Alpha-adrenoceptors in equine digital veins: Evidence for the presence of both alpha$_1$ and alpha$_2$-receptors mediating vasoconstriction

J. ELLIOTT

Department of Veterinary Basic Sciences, Royal Veterinary College, Royal College Street, London NW1 0TU, UK

Elliott, J. Alpha-adrenoceptors in equine digital veins: Evidence for the presence of both alpha$_1$ and alpha$_2$-receptors mediating vasoconstriction. *J. vet. Pharmacol. Therap.* **20,** 308–317.

Rings of equine digital vein examined under conditions of isometric tension recording constricted to alpha-adrenoceptor agonists with an order of potency of 5-bromo-6-[2-imidazolin-2-yl-amino]-quinoxaline bitartrate (UK 14304) = noradrenaline > 6-Allyl-2-amino-5,6,7,8-tetrahydro-4H-thiazolo-(4,5-d) azepine (BHT-920) > phenylephrine > dopamine > methoxamine. The maximum force generated was greatest for the non-selective agonist noradrenaline and lowest for the alpha$_2$-selective agonist BHT-920 with the other agonists between these two extremes. Selective inactivation of alpha$_1$-adrenoceptors (achieved by treating yohimbine-protected tissues with phenoxybenzamine) reduced the maximum responses of all agonists, the EC_{50} values of UK 14304, BHT-920 and noradrenaline and increased the EC_{50} values of phenylephrine and methoxamine. Prazosin (30 nM) had no inhibitory effect on responses to low concentrations of BHT-920 and UK 14304 and caused competitive inhibition of responses to phenylephrine and noradrenaline giving pK_b values of 8.49 ± 0.18 and 8.23 ± 0.14, respectively. Yohimbine (0.1 μM) caused significant competitive inhibition of responses to BHT-920 and noradrenaline with calculated pK_b values of 8.43 ± 0.11 for BHT-920 and 7.43 ± 0.31 for noradrenaline and non-competitive inhibition of responses to UK 14304. 2-[2-methoxy-1,4-benzodioxan-2-yl]-2-imidazoline (RX 821002; 10 nM) caused competitive inhibition of responses to BHT-920 (pK_b 9.04 ± 0.27) and dopamine (pK_b 8.2 ± 0.2). These data indicate that equine digital veins possess both post-synaptic alpha$_1$ and alpha$_2$-adrenoceptors.

(Paper received 4 November 1996; accepted for publication 11 April 1997)

Jonathan Elliott, Department of Veterinary Basic Sciences, Royal Veterinary College, Royal College Street, London NW1 0TU, UK.

INTRODUCTION

Alpha-adrenoceptors were originally classified into alpha$_1$ and alpha$_2$ sub-types on the basis of their anatomical location (Langer, 1974) with alpha$_1$-adrenoceptors being found post-junctionally and alpha$_2$-adrenoceptors located on nerve terminals. It soon became clear that this simple anatomical categorization did not hold true and that post-junctional receptors with the pharmacological characteristics of alpha$_2$-adrenoceptors could be found on the effector organ (Timmermans & Van Zwieten, 1981). Post-synaptic adrenoceptors mediating vasoconstriction of blood vessels which were resistant to inhibition by prazosin were first reported by Bentley *et al.* (1977) in whole animal studies and by Jauering *et al.* (1978) in human isolated digital arteries. Canine saphenous vein was one of the first isolated vascular preparations where convincing pharmacological evidence was presented for the presence of post-synaptic alpha$_2$-adrenoceptors (Sullivan & Drew, 1980; De Mey & Vanhoutte, 1981; Flavahan *et al.*, 1984).

The convincing demonstration of both types of alpha-adrenoceptor in functional studies using isolated tissues is not simple. It has been complicated by the discovery of sub-types for both alpha$_1$ and alpha$_2$-adrenoceptors (Bylund *et al.*, 1994). If one relies only on selective agonists to characterize the receptors mediating a response, differences in receptor reserve may lead, for example, to agonists which are apparently selective for alpha$_2$-receptors in one tissue having substantial efficacy at alpha$_1$-receptors in another tissue, which has high alpha$_1$-receptor reserve (Minneman, 1988). Add to this the possibility of one or more subtypes of alpha$_1$ and alpha$_2$-adrenoceptors co-existing and interacting within a given tissue and the potential for confusion is great. Finally, a number of drugs used to characterize alpha-adrenoceptors have recently been found to have activity at receptors which are not activated by catecholamines, so-called imidazoline preferring receptors (Lehmann, 1989). Thus, pharmacological characterization of post-synaptic vascular adrenoceptors mediating vasoconstriction requires careful investigation.

Isolated equine digital blood vessels have been shown to contract in response to noradrenaline (Baxter *et al.*, 1989) and dopamine (Baxter *et al.*, 1991) but no detailed investigation of the type(s) of adrenoceptor mediating these responses has been undertaken. Baxter *et al.* (1991) examined the effect of prazosin on the responses of equine digital vessels to dopamine and noradrenaline and found evidence for prazosin-resistant responses to dopamine, although the effect of prazosin was difficult to interpret in these studies, being apparently non-competitive. Recently, Bryant & Clarke (1996) presented evidence for presynaptic alpha$_2$-adrenoceptors modulating responses to electrical stimulation of isolated spiral strips of equine saphenous vein. In addition, the data generated by Bryant & Clarke (1996) supported the existence of post-synaptic alpha$_2$-adrenoceptors mediating vasoconstriction in this tissue but extensive antagonist studies were not undertaken to verify this finding. Knowledge of the post-synaptic adrenoceptors present in the digital circulation is of fundamental importance in understanding the control of blood flow through this vascular bed, particularly in relation to the pathophysiology of the ischaemic disease laminitis. Moreover, a number of drugs with alpha$_2$-adrenoceptor agonist properties are currently commonly used in equine practice for their sedative and analgesic properties, so a knowledge of the distribution of post-synaptic vascular alpha$_2$-adrenoceptors is important.

The aim of the present study was to characterize pharmacologically the post-synaptic alpha-adrenoceptor subtype(s) mediating vasoconstriction to noradrenaline in isolated equine digital veins. Some of these results have been presented in abstract form elsewhere (Elliott & Soydan, 1994).

MATERIALS AND METHODS

Animals

Equine digital veins were collected from West Sussex Abattoir, Crawley, UK. Mixed breed adult horses were killed and the hind limb was removed within 10 min of death. The digital artery was cannulated at the level of the fetlock joint and 40 mL of ice-cold modified Krebs Henseleit solution (Krebs solution) was infused through the catheter. The skin was then reflected from above the coronary band to reveal the coronary venous plexus. This was dissected, placed in ice cold Krebs solution and transported to the laboratory. The veins were cleared of connective tissue and cut into rings of 4 mm in width. Those which were not used on the day of collection were stored overnight in oxygenated Krebs solution at 4°C until required.

Isometric tension recording in isolated digital veins

The intimal surface of the blood vessel segments was gently rubbed using a wooden cocktail stick to remove the endothelial layer. We have previously shown that this procedure abolishes the relaxant effects of carbachol in this tissue (Elliott *et al.*, 1994). Rings of digital vein were suspended between two parallel stainless steel wires contained within a jacketed organ bath and bathed in Krebs Henseleit solution which was aerated with 95% O_2 and 5% CO_2 and maintained at 30°C. One wire was fixed and the other connected to an isometric force transducer (HSE force transducer, Type K30; Linton Instrumentation Ltd, Diss, Norfolk, UK). The output from the force transducer was fed via an amplifier (HSE type 301, Linton) to a two channel pen recorder (Linseis L6512, Linton). Each vessel segment was stretched to 2 *g* tension and an equilibration period of 1 h was allowed prior to the administration of vasoconstrictor agents. Preliminary experiments showed that this passive stretch gave optimal vasoconstrictor responses in vessel segments of this size.

The viability of each vessel segment was tested initially by exchanging the Krebs solution for one in which the sodium chloride had been replaced with potassium chloride to produce a depolarising Krebs solution (DKS; 118 mM KCl). At the peak tension obtained, the tissues were washed with Krebs solution and the tension returned to baseline. Vessel segments which produced less than 1 *g* tension in response to DKS were considered to be non-viable and were discarded. Concentration response curves (CRCs) were then obtained by cumulative addition of noradrenaline (1 nM to 0.1 mM), dopamine (10 nM to 0.1 mM), BHT-920 (1 nM to 10 μM), UK 14304 (1 nM to 10 μM), phenylephrine (1 nM to 0.1 mM) and methoxamine (10 nM to 0.1 mM). Only one CRC was obtained to one agonist in any given vessel segment. For some vessel segments the following pretreatment protocol was used prior to obtaining CRC to the agonists. Yohimbine (0.1 μM) was added to the bathing solution and 15 min later phenoxybenzamine (1 μM) was also added. The two drugs remained in contact with the tissues for a further 30 min and then the bathing solution was exchanged 10 times at 5 min intervals using drug-free Krebs solution. This produced tissues which were 'yohimbine-protected phenoxybenzamine treated'. CRCs to all agonists were then produced in these tissues and compared with tissues which had had no pre-treatment.

The effects of antagonists were studied using weight matched vessel segments from the same horse in the same experiment and adding the antagonist (either prazosin, 30 nM; yohimbine, 0.1 μM; or RX 821002, 10 nM) 30 min prior to the addition of agonists. All the agonists except methoxamine were examined in the presence and absence of these antagonists. In addition, the effect of prazosin and yohimbine were tested against noradrenaline and phenylephrine in tissues which had been pre-treated with yohimbine and phenoxybenzamine as described above. The results obtained in the presence of antagonists were then compared with those obtained in a vessel segment from the same horse, where no antagonist was added to the Krebs solution prior to the construction of the CRC.

Drugs and reagents

L-Noradrenaline bitartrate, L-phenylephrine hydrochloride, yohimbine hydrochloride, methoxamine hydrochloride, dopamine hydrochloride, DL-propranolol hydrochloride, ethylene diaminetetraacetic acid (EDTA), L-ascorbic acid and prazosin hydrochloride were all obtained from Sigma Chemical Company Ltd, Poole, Dorset, U.K. UK 14304 (5-bromo-6-[2-imidazolin-2-yl-

amino]-quinoxaline bitartrate, RX 821002 (2-[2-methoxy-1,4-benzodioxan-2-yl]-2-imidazoline), phenoxybenzamine hydrochloride were purchased from Research Biochemicals Inc., Natik, MA, USA and BHT-920 (6-Allyl-2-amino-5,6,7,8-tetrahydro-4H-thiazolo-(4,5-d) azepine) was a generous gift from Boehringer Ingelheim, Ingelheim, Germany. Cocaine hydrochloride BP was purchased from Macarthys Medical Ltd, Romford, Essex, UK. All other reagents were of analytical grade where possible. The modified Krebs-Henseleit solution had the following composition (mM):- NaCl 118, KCl 4.57, $CaCl_2$ 1.27, KH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 25, glucose 5.55, EDTA 0.07 and L-ascorbic acid 0.11. Cocaine (3 μM) and propranolol (1 μM) were added to the Krebs solution to block neuronal uptake and beta-adrenoceptors, respectively, and were present throughout all the experiments described above. All drugs were freshly prepared on the day of the experiment. Unless otherwise stated, drugs were dissolved in distilled water or 0.01 M hydrochloric acid and further dilutions were made in normal saline. Phenoxybenzamine was dissolved in ethanol and prazosin in methanol. These vehicles alone did not affect the responses of the vessels to the alpha agonists.

STATISTICAL ANALYSIS OF RESULTS

Vasoconstrictor responses were measured as the increase in tension expressed as a percentage of the peak rise in tension caused by DKS and plotted against log agonist concentration. Cumulative CRCs were fitted to a modified Marquart equation:

$$\text{Increase in tension} = \text{Resp}_{max} \times D^n/(D^n + EC_{50}{}^n)$$

where Resp_{max} represents the maximum response, D is the concentration of drug used and *n* is the Hill slope of the response curve. A non-linear curve fitting procedure (using Multifit®, Day Computing Cambridge, UK, running on a Macintosh SE30) was employed to derive the three CRC parameters for each vessel segment tested with each agonist. The best fit values for EC_{50}, maximum response and Hill slope were then used to calculate the mean ± SEM value of these parameters for each agonist derived from vessel segments from four to 24 horses.

Where appropriate, the effects of antagonist on the responses obtained to a given agonist were tested for statistical significance using a Student's *t*-test or one way ANOVA, followed by Dunnett's comparison and $P < 0.05$ was taken to indicate statistical significance. Concentration ratios (CR) were calculated for the antagonists used by dividing the EC_{50} value obtained in the presence of the antagonist by the EC_{50} value obtained in the absence of the antagonist. Where an antagonist gave a parallel shift in the CRC such that there was no significant change in the slope parameter or the maximum response, an apparent pK_b value for the antagonist was derived using the following formula (Furchgott, 1972):

$$pK_b = -\log \times (\text{CR-1}/[\text{Antagonist}]).$$

RESULTS

The CRCs to the alpha-adrenoceptor agonists are presented in Fig. 1a and b and the best fit parameters derived from these curves are shown in Table 1. The order of potency for the six agonists examined was found to be UK 14304 = noradrenaline > BHT-920 > phenylephrine > dopamine > methoxamine. The maximum responses varied considerably between agonists and, when normalized for the tissue responses to DKS, noradrenaline produced the largest response, followed by phenylephrine, dopamine, methoxamine, UK 14304 and BHT-920.

The responses of tissues treated with phenoxybenzamine are shown in Fig. 2a and b. The best fit parameters of these CRCs are presented in Table 2. This treatment reduced the maximum response of the six agonists such that all produced similar

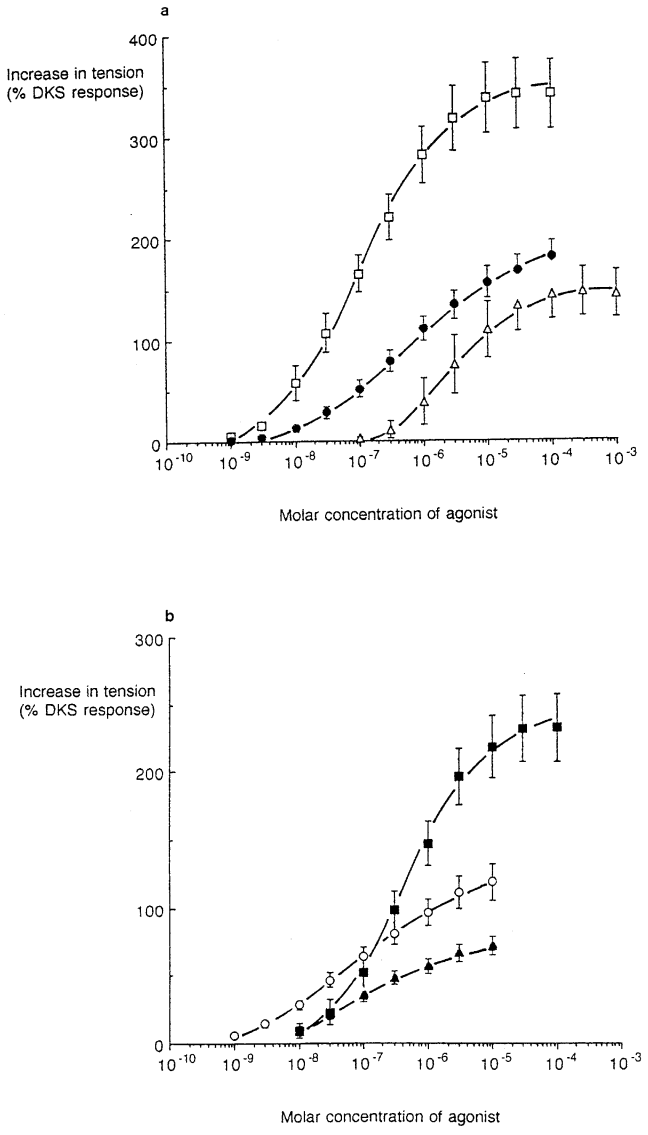

Fig. 1. Responses of equine digital veins to alpha-adrenoceptor agonists. Cumulative concentration response curves were obtained to each agonist in rings of EDV from 5 to 24 different horses. Each point represents the mean ± SEM value. The best fit parameters derived from non-linear curve fitting of these data are presented in Table 1. (a) Noradrenaline (open squares); dopamine (closed circles); methoxamine (open triangles) and (b) UK 14304 (open circles); BHT-920 (close triangles) and phenylephrine (closed squares).

Table 1. Order of potency of alpha-adrenoceptor agonists in equine digital vein.

Agonist	*n*	EC_{50} (μM)	Maximum response (% DKS response)	Hill slope
UK 14304	17	0.138 ± 0.034	129.8 ± 15.7	0.64 ± 0.05
Noradrenaline	14	0.138 ± 0.030	345.2 ± 35.5	0.76 ± 0.04
BHT-920	18	0.415 ± 0.135	79.6 ± 7.72	0.85 ± 0.13
Phenylephrine	14	0.567 ± 0.104	237.4 ± 26.7	0.91 ± 0.04
Dopamine	24	4.306 ± 1.598	192.4 ± 16.1	0.65 ± 0.03
Methoxamine	5	4.901 ± 1.561	145.3 ± 23.0	1.29 ± 0.07

Concentration response curve parameters were derived by non-linear curve fitting for each CRC and the mean value ± sem for each agonist is quoted.

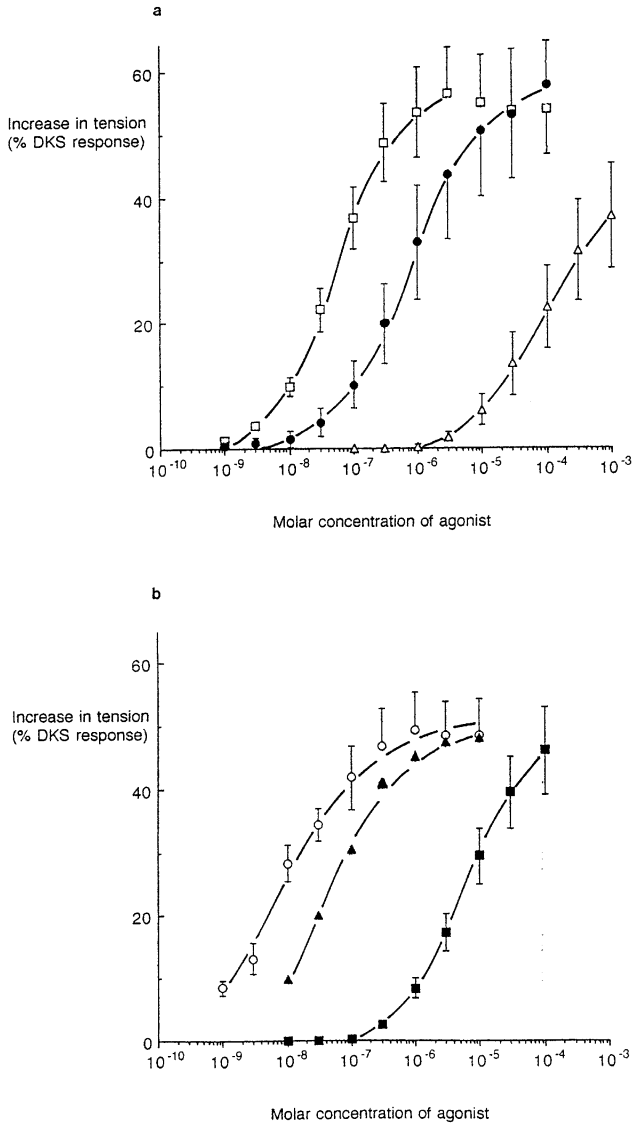

Fig. 2. Responses of equine digital blood vessels to alpha agonists following pre-treatment with yohimbine and phenoxybenzamine. Cumulative concentration response curves were obtained to each agonist in rings of EDV from 5 to 12 different horses following pre-treatment with yohimbine (0.1 μM) and phenoxybenzamine (1 μM). Each point represents the mean ± SEM value. The best fit parameters derived from non-linear curve fitting of these data are presented in Table 2. (a) Noradrenaline (open squares); dopamine (closed circles); methoxamine (open triangles) and (b) UK 14304 (open circles); BHT-920 (close triangles) and phenylephrine (closed squares).

maximum responses. The EC_{50} values for noradrenaline, UK 14304 and BHT-920 were significantly reduced by phenoxybenzamine treatment, whereas the change in the EC_{50} value for dopamine did not reach statistical significance. The EC_{50} values for phenylephrine and methoxamine were significantly increased. Three of the agonists demonstrated a significant increase in the slope factor following treatment with phenoxybenzamine. These were noradrenaline, UK 14304 and dopamine.

The effects of the antagonists tested in these studies are presented in Table 3. Concentration ratios are quoted for each antagonist tested against noradrenaline, phenylephrine, BHT-920, UK 14304 and dopamine. Statistical significance of the change in EC_{50} value is also indicated. An apparent pK_b value has been calculated provided the antagonist did not cause a significant change in the slope parameter or maximum response of the CRC. These are quoted even if the change in EC_{50} value did not reach statistical significance, provided all tissues examined in the presence of the antagonist showed an increase in the EC_{50} value when compared to the appropriate control tissue.

Prazosin (30 nM) caused significant increases in the EC_{50} values for noradrenaline and phenylephrine (see Fig. 3a). The effect of prazosin on the responses of phenoxybenzamine treated tissues to noradrenaline and phenylephrine was substantially less than that seen in untreated tissues, such that prazosin no longer caused significant inhibition of the responses to these two agonists (see Fig. 3b). The responses of equine digital vein (EDV) to low concentrations of UK 14304 and BHT-920 were resistant to inhibition by prazosin (Figs 4a and 5). In the case of UK 14304, the CRC was clearly biphasic in the presence of prazosin (Fig. 4a). The maximum response of EDV to BHT-920 tended to be reduced by prazosin treatment (from 69.5 ± 5.43 to 58.9 ± 11.4% DKS response) although this reduction did not reach statistical significance. A similar pattern of inhibitory effect was seen when dopamine was used as the agonist with a small prazosin-resistant component of the response being evident (Fig. 6).

Yohimbine (0.1 μM) produced potent competitive inhibition of the responses of EDV to BHT-920 whereas its action against UK 14304 was apparently non-competitive causing an increase in the slope of the CRC (0.59 ± 0.03 vs. 0.87 ± 0.10; $P < 0.001$; see Fig. 4a). Competitive antagonism was noted for yohimbine against noradrenaline but the calculated apparent pK_b value was almost an order of magnitude lower than that calculated with BHT-920 as the agonist (Table 3). The inhibitory action of yohimbine against noradrenaline was much greater in tissues which had been pretreated with phenoxybenzamine.

Table 2. Order of potency of alpha-adrenoceptor agonists in yohimbine (0.1 μM) protected phenoxybenzamine (1 μM) treated equine digital vein

Agonist	*n*	EC_{50} (μM)	Maximum response (% DKS response)	Hill slope
UK 14304	4	0.010 ± 0.004**	49.8 ± 5.84**	0.78 ± 0.05*
Noradrenaline	11	0.041 ± 0.005**	55.6 ± 7.52***	1.01 ± 0.05**
BHT-920	5	0.055 ± 0.012*	48.8 ± 11.7*	0.94 ± 0.04
Dopamine	7	3.692 ± 1.980	61.3 ± 10.3***	0.93 ± 0.12*
Phenylephrine	13	7.502 ± 1.206***	49.6 ± 7.56***	0.98 ± 0.07
Methoxamine	5	100.385 ± 20.99***	41.2 ± 8.84***	1.09 ± 0.23

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. concentration response curve parameters derived for the same agonist in untreated tissue. Comparisons were made using a Student's *t*-test.

Table 3. Effects of alpha antagonists on the responses of equine digital veins to alpha agonists

Agonist	Antagonist: Prazosin (30 nM)			Yohimbine (0.1 μM)			RX 821002 (10 nM)		
(*n* = 4–7)	CR	Type of inhibition	pK_b	CR	Type of inhibition	pK_b	CR	Type of inhibition	pK_b
NA	7.80 ± 2.46***	competitive	8.23 ± 0.14	6.48 ± 2.8*	competitive	7.43 ± 0.31	1.97 ± 0.51	no effect	NA
NA (PBZ)	1.37 ± 0.25	no effect	NA	18.2 ± 2.4***	competitive	8.22 ± 0.06	ND	ND	ND
PE	13.3 ± 5.38*	competitive	8.49 ± 0.18	3.36 ± 1.93	competitive	7.37 ± 0.25	1.07 ± 0.09	no effect	NA
PE (PBZ)	2.04 ± 0.48	competitive	NA	18.9 ± 6.00**	competitive	8.11 ± 0.14	ND	ND	ND
BHT-920	0.98 ± 0.50	no effect	NA	26.0 ± 4.95*	competitive	8.34 ± 0.11	25.2 ± 11.3**	competitive	9.04 ± 0.27
UK 14304	0.23 ± 0.07[#]	biphasic CRC	NA	8.36 ± 3.23	non-competitive	NA	1.92 ± 0.50	no effect	NA
Dopamine	ND[#]	biphasic CRC	NA	10.9 ± 5.67	competitive	7.70 ± 0.31	3.73 ± 1.04*	competitive	8.24 ± 0.20

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ for the EC_{50} values obtained in the presence of antagonist when compared with the EC_{50} value obtained in the absence of antagonist. Comparisons have been made by paired Student's *t*-tests or one way analysis of variance followed by Dunnett's comparison as appropriate. CR – concentration ratio; NA – not applicable; ND – not determined; PBZ -phenoxybenzamine. [#]In the case of UK 14304, a concentration ratio for prazosin has been calculated on the basis of the EC_{50} value for the first phase of the biphasic CRC divided by the EC_{50} value obtained in the absence of prazosin. No CR is quoted for dopamine as there was no distinct plateau in the CRC in the presence of prazosin.

Responses to phenylephrine were relatively insensitive to inhibition by yohimbine with the increase in the EC_{50} value failing to reach statistical significance at the concentration used (Fig. 3a and Table 3). However, in phenoxybenzamine treated tissues, the same concentration of yohimbine caused marked competitive antagonism of the responses to phenylephrine (Fig. 3b).

RX 821002 (10 nM) had a significant inhibitory effect on the responses of EDV to BHT-920 (Fig. 5) and to dopamine (Fig. 6). This antagonist failed to produce a significant inhibitory effect at the concentration tested against UK 14304 (Fig. 4b) or either of the other agonists examined (Table 3).

DISCUSSION

The results of the present study clearly demonstrated the presence of both $alpha_1$ and $alpha_2$-adrenoceptors mediating contraction of EDV. The combination of agonist and antagonist data provides evidence which supports this statement and allows us to estimate the relative contribution of each receptor to the response to each agonist. In addition, the data seem to suggest that there was an interaction between $alpha_1$ and $alpha_2$-receptors in mediating vasoconstriction of EDV, a phenomenon which has been reported for other blood vessels possessing both types of alpha-adrenoceptors (Daly *et al.*, 1988).

The order of potency data showed that the $alpha_2$-selective agonists UK 14304 (Cambridge, 1981) and BHT-920 (Motram, 1983) and the non-selective naturally occurring agonist, noradrenaline, were more potent than the $alpha_1$-selective agonists, phenylephrine and methoxamine. This finding suggested a contribution of $alpha_2$-adrenoceptors to the responses recorded. These data are similar to those reported by Bryant & Clarke (1996) who found an order of potency of noradrenaline > BHT-920 > UK 14304 > phenylephrine in spiral segments of equine saphenous vein. The reason for the discrepancy in potency of UK 14304 between the present study and that reported by Bryant & Clarke (1996) is not clear, although the relative densities of $alpha_1$ and $alpha_2$-adrenoceptors has been shown to alter when moving from proximal to distal portions of limb blood vessels from other species (Guimaraes & Nunes, 1990).

In the present study, the maximal responses obtained to noradrenaline were much larger than those obtained to any of the other agonists. The next most efficacious agonist was phenylephrine, the $alpha_1$-selective agonist. From the antagonist data obtained in the present study, it would seem that noradrenaline activates both $alpha_1$ and $alpha_2$-receptors over the same concentration range which could lead to synergism in

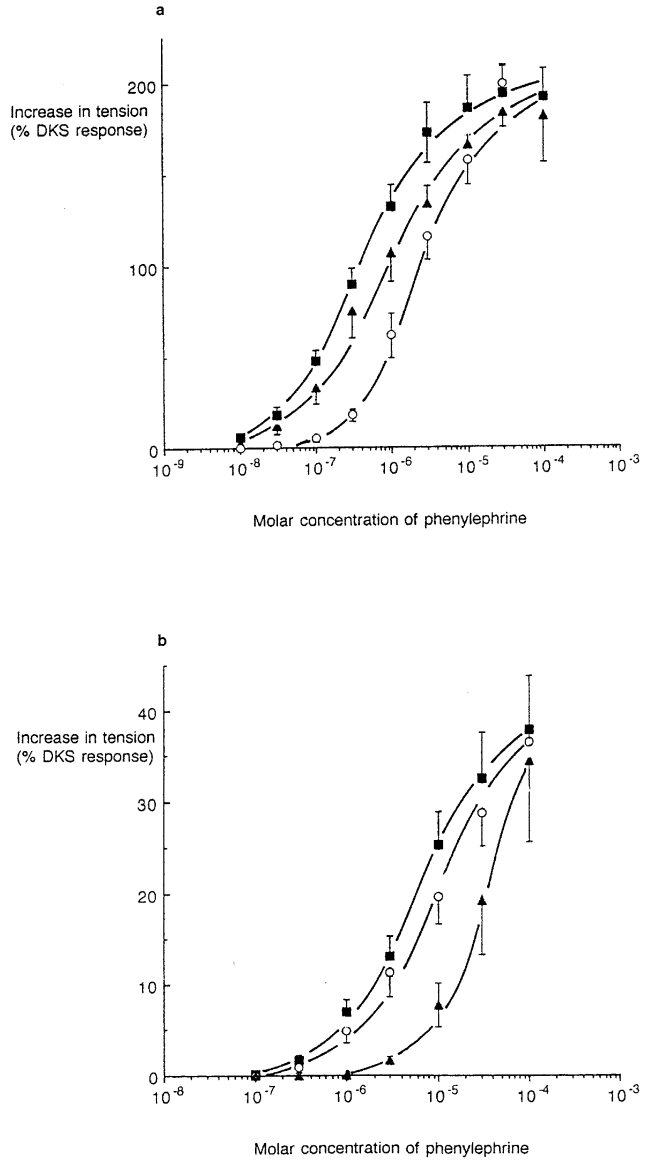

Fig. 3. Effects of yohimbine and prazosin on responses of EDV to phenylephrine. Responses have been obtained in the absence of antagonist (closed squares), in the presence of 0.1 μM yohimbine (closed triangles) and 30 nM prazosin (open circles). Antagonists were added 30 min prior to the cumulative addition of phenylephrine. Each point represents the mean ± SEM value from 5 to 7 horses. (a) Vessel segments which had not been pre-treated; (b) vessel segments pre-treated with yohimbine (0.1 μM) and phenoxybenzamine (1 μM).

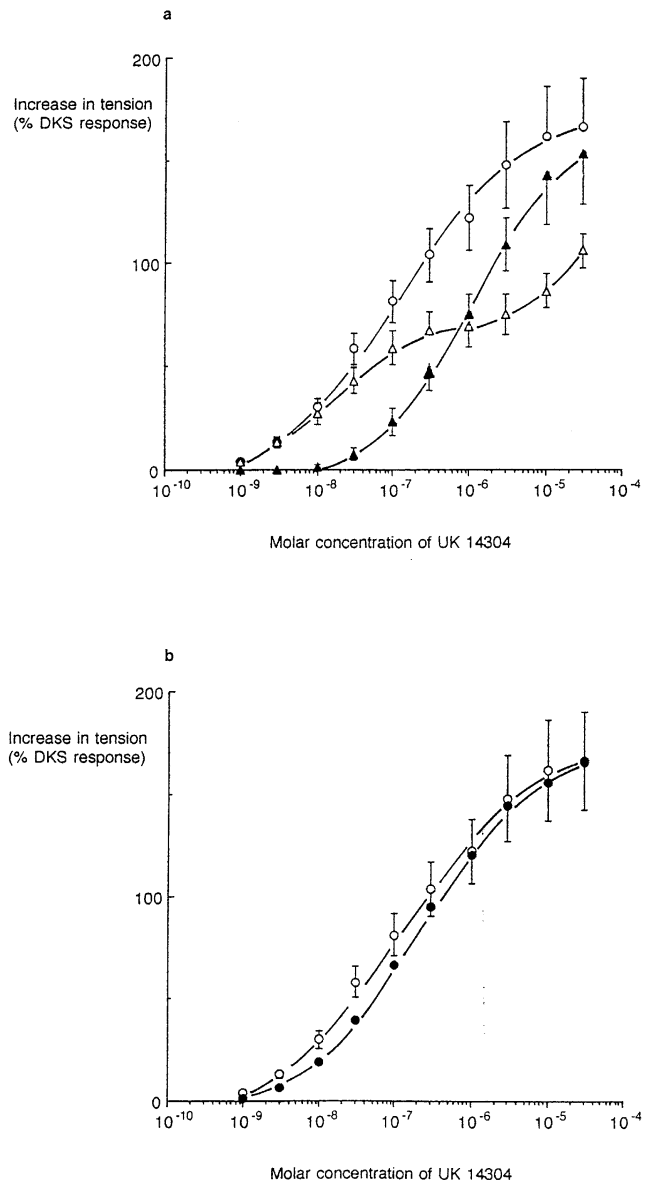

Fig. 4. Effects of yohimbine, prazosin and RX 821002 on responses of EDV to UK 14304. Responses have been obtained in the absence of antagonist (open circles), in the presence of 0.1 μM yohimbine (closed triangles), 30 nM prazosin (open triangles) and 10 nM RX 821002 (closed circles). Antagonists were added 30 min prior to the cumulative addition of UK 14304. Each point represents the mean ± SEM value from 5 or 6 horses.

the contractile response. It is hard to explain on this basis, however, why other agonists such as dopamine and UK 14304, which also stimulate both receptor types, do not produce the same maximum response as noradrenaline. An additional explanation for this finding may be that noradrenaline was a full agonist at both receptor types whereas the other agonists tested were partial agonists at one or both of the receptor types in EDV.

The Hill slopes for the agonists also varied considerably, the lowest values being found for UK 14304, dopamine and noradrenaline followed by BHT-920, phenylephrine and methoxamine. UK 14304 in particular, had a shallow Hill slope compared to the other agonists, a fact which might indicate that this agonist stimulates another pathway, over the same range of concentrations tested here, which inhibits smooth muscle contraction (or stimulates smooth muscle relaxation) and so reduces the slope of the CRC. Alpha$_2$-adrenoceptor-mediated vasoconstriction has been shown to be quite markedly influenced by the presence of an intact endothelium in some blood vessels, such as rat aorta (Egleme *et al.*, 1984). This can not be the explanation for the shallow CRC observed here because the

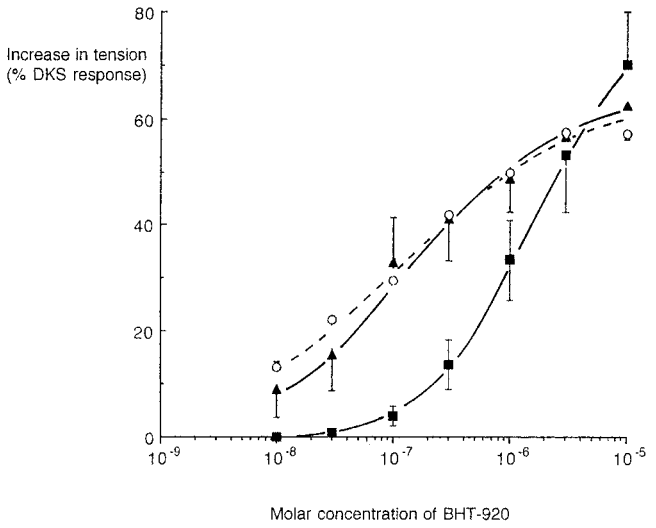

Fig. 5. Effects of prazosin and RX 821002 on responses of EDV to BHT-920. Responses have been obtained in the absence of antagonist (closed triangles), in the presence of 30 nM prazosin (open circles) and 10 nM RX 821002 (closed squares). Antagonists were added 30 min prior to the cumulative addition of BHT-920. Each point represents the mean ± SEM value from 5 horses.

tested. In addition, inclusion of a selective antagonist which separated the two components of the responses, increased the Hill slopes of UK 14304, noradrenaline and dopamine but not BHT-920, phenylephrine and methoxamine.

The irreversible antagonist, phenoxybenzamine was used in the present study to inhibit the alpha$_1$-adrenoceptor component of the responses to the alpha agonists tested. Phenoxybenzamine has been shown to have selectivity for alpha$_1$-adrenoceptors (Doxey *et al.*, 1977) and in some studies has been used alone at concentrations of 0.05–0.5 μM to produce selective and irreversible inhibition of alpha$_1$-adrenoceptors (Flavahan *et al.*, 1984). Given the fact that our preliminary data suggested that alpha$_1$-adrenoceptors predominated in this tissue, we decided to protect the alpha$_2$-adrenoceptors with yohimbine (alpha$_2$-selective antagonist) in an attempt to inactivate as many alpha$_1$-adrenoceptors as possible, leaving alpha$_2$-receptors intact. It might be argued that a more selective alpha$_2$-adrenoceptor antagonist should have been used to provide protection against alpha$_2$-receptor inactivation by phenoxybenzamine. Nevertheless, yohimbine was used at a concentration which was some 20 times higher than its pK_a for alpha$_2$-receptors and below the reported pK_a of yohimbine for alpha$_1$-adrenoceptors (Ruffolo *et al.*, 1981). This, coupled with the fact

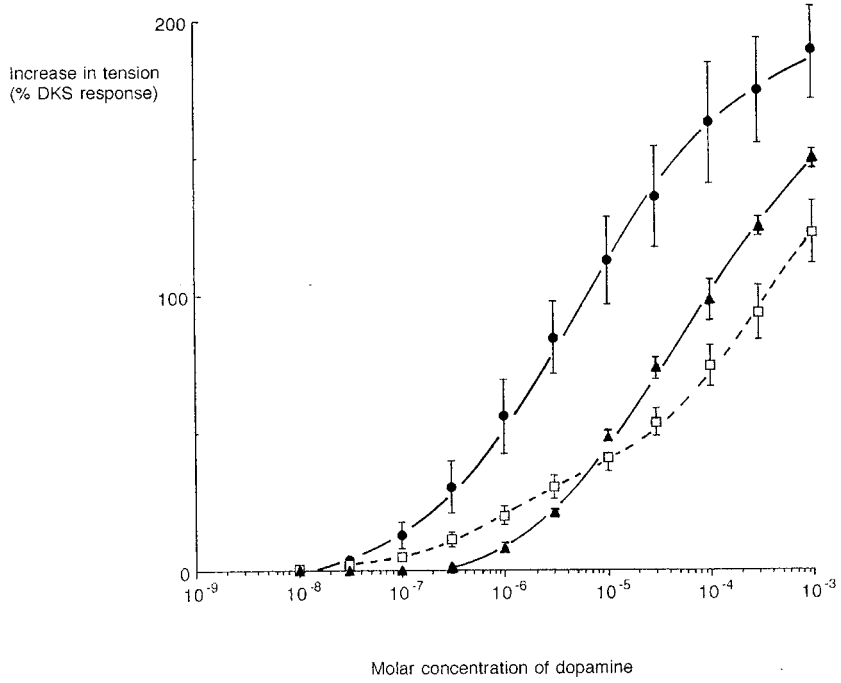

Fig. 6. Effects of prazosin and RX 821002 on responses of EDV to dopamine. Responses have been obtained in the absence of antagonist (closed circles), in the presence of 30 nM prazosin (open squares) and 10 nM RX 821002 (closed triangles). Antagonists were added 30 min prior to the cumulative addition of dopamine. Each point represents the mean ± SEM value from 6 horses.

vessels which we used had been denuded of their endothelium. In addition, BHT-920, the other alpha$_2$-selective agonist used in the present study, had a Hill slope of close to unity. An alternative explanation for this finding of a shallow Hill slope is that it comes about by the interaction at the second messenger level when two receptor pathways are stimulated simultaneously. In support of this explanation is the fact the agonists with shallow Hill slopes appear to be those which stimulate both alpha-adrenoceptors types within the concentration ranges that phenoxybenzamine has been shown to have a much lower affinity for alpha$_2$-receptors than it has for alpha$_1$-receptors (Doxey *et al.*, 1977), justifies the regime used in the present study to inactivate alpha$_1$-receptors leaving alpha$_2$-receptors intact. At 0.1 μM, yohimbine has little or no selectivity for the different alpha$_2$-receptor subtypes currently characterized (Murphy & Bylund, 1988) and the alpha$_2$-receptor population which is left on EDV following this treatment protocol could consist of more than one sub-type of alpha$_2$-receptor.

The results of the studies on yohimbine-protected phenoxybenzamine-treated tissues further support the contention that EDVs have both alpha$_1$ and alpha$_2$-adrenoceptors mediating vasoconstriction. A reduction in the maximum response was seen for all agonists following phenoxybenzamine treatment, such that all agonists gave similar maximum responses in treated tissues and suggested that each had an action at receptors other than alpha$_2$-adrenoceptors. Those agonists which are reported to have selectivity for alpha$_2$-adrenoceptors (BHT-920 and UK 14304) or which are non-selective naturally occurring agonists (noradrenaline and dopamine) showed no change or a decrease in their EC_{50} values following this treatment. The two agonists which are reported to be alpha$_1$-selective (phenylephrine and methoxamine) had increases of 13 and 23 fold in their EC_{50} values, respectively. These data show that all agonists were capable of stimulating alpha$_2$-adrenoceptors which were protected from inactivation by 1 μM phenoxybenzamine by concomitant incubation with yohimbine (0.1 μM). The order of potency of the agonists following phenoxybenzamine treatment supports the view that the alpha-adrenoceptors remaining are predominantly of the alpha$_2$ type. The Hill slopes for all the agonist CRCs moved towards unity following phenoxybenzamine with significant changes for noradrenaline, UK 14304 and dopamine. This finding supports the hypothesis discussed above that shallow Hill slopes were the result of an interaction occurring when both receptor subtypes were activated simultaneously for a large part of the CRC. An alternative explanation for this phenomenon could be blockade of extraneuronal uptake by phenoxybenzamine (Burgen & Iversen, 1965) which potentiates the action of high concentrations of catecholamines. This explanation seems less likely, as preliminary experiments using corticosterone (30 μM) as an inhibitor of extraneuronal uptake failed to demonstrate any effect on CRC to noradrenaline (data not shown).

Further studies were undertaken using reversible adrenoceptor antagonists, prazosin, yohimbine and RX 821002 to characterize the alpha-adrenoceptors in EDV. Prazosin proved useful in this respect because at the concentration chosen for the present experiments, the responses of EDV to low concentrations of UK 14304 and BHT-920 proved resistant to prazosin. This finding indicated that the type of alpha$_2$-adrenoceptor present on the smooth muscle of EDV did not have a high affinity for prazosin. Recently, alpha$_2$-adrenoceptors have been classified into four subtypes, two of which have an affinity for prazosin in the nanomolar concentration range (alpha$_{2B}$ and alpha$_{2C}$) (see Bylund *et al.*, 1994). The alpha$_2$-adrenoceptors on the smooth muscle of EDV are more likely to be of the alpha$_{2A}$ or alpha$_{2D}$ sub-types as prazosin (30 nM) did not cause any inhibition of the responses to low concentrations of UK 14304 or BHT-920. The biphasic nature of the CRC to UK 14304 in the presence of prazosin is consistent with the finding that phenoxybenzamine treatment reduced the maximum responses to this agonist. It would appear that a substantial component of the response to UK 14304 in EDV was mediated by alpha$_1$-adrenoceptors. An alternative but less likely explanation for this finding would be that an additional alpha$_2$-adrenoceptor subtype is also present on EDV which was sensitive to inhibition by prazosin and which was not protected by yohimbine against inactivation by phenoxybenzamine.

Prazosin caused competitive inhibition of the responses to noradrenaline and phenylephrine with pK_b values in line with the reported affinity of prazosin for alpha$_1$-adrenoceptors. In most species, the pA_2 value of prazosin for alpha$_1$-adrenoceptors lies between 8 and 9, except for the rat where it lies between 9 and 10 (Agrawal *et al.*, 1984). Prazosin inhibited responses to dopamine in EDV revealing a small prazosin-resistant component to dopamine. This is in agreement with the findings of Baxter *et al.* (1991). The reason that such a prazosin-resistant component was evident for dopamine but not for noradrenaline might reflect the larger contribution of alpha$_2$-adrenoceptors to the contractile effect of dopamine.

In phenoxybenzamine treated tissues, the responses to noradrenaline and phenylephrine were not significantly inhibited by prazosin at 30 nM, confirming that this treatment has removed the alpha$_1$-adrenoceptors in EDV and that the remaining alpha$_2$-adrenoceptors of EDV have a low affinity for prazosin.

Yohimbine caused competitive inhibition of responses of EDV to BHT-920. The calculated apparent pK_b value of yohimbine for the alpha$_2$-receptors in the present study was very close to that reported for yohimbine for alpha$_2$-receptors in blood vessels from other species (Ruffolo *et al.*, 1981; Stevens & Moulds, 1981) and in rat brain (Brown *et al.*, 1990). Yohimbine caused competitive inhibition of the responses of EDV to noradrenaline but the calculated apparent pK_b value was 0.9 log units lower than that calculated with BHT-920 as the agonist. There are at least two possible explanations for this finding. Firstly, the apparently competitive effect might be the result of shifting the concentration at which alpha$_2$-receptor activation occurred and the influence this had on the alpha$_1$-receptor mediated response. This explanation seems unlikely because no similar effect was measured for the selective alpha$_2$-adrenoceptor antagonist, RX 821002 against noradrenaline even though this antagonist was just as effective as yohimbine against BHT-920. An alternative explanation is that the apparent pK_b value calculated vs. noradrenaline could, in part, reflect the affinity yohimbine has for alpha$_1$-adrenoceptors in EDV. This seems more likely even though in most other blood vessels examined, the affinity of yohimbine for alpha$_1$-adrenoceptors has been found to be $\approx$ 10 times lower than that reported here (Ruffolo *et al.*, 1981). Nevertheless, alpha$_1$-adrenoceptors in some blood vessels, such as canine saphenous vein, have been reported to have an affinity for yohimbine of the same order of magnitude as reported in the present study (Sullivan & Drew, 1980; see Flavahan & Vanhoutte, 1986a). Although yohimbine (0.1 μM) did not produce significant inhibition of responses to phenylephrine, all the EC_{50} values calculated in the presence of yohimbine were higher than those found in vessel segments taken from the same horse where yohimbine was not included in the bathing fluid. The apparent pK_b calculated for yohimbine vs. phenylephrine was in close agreement with that calculated for yohimbine vs. noradrenaline.

Phenoxybenzamine treatment produced vessels with a population of alpha-adrenoceptors stimulated by noradrenaline and phenylephrine, which were much more sensitive to inhibition by yohimbine. Indeed, the pK_b values calculated for yohimbine in phenoxybenzamine-treated tissues, with noradrenaline and phenylephrine as agonists, were in much closer agreement with the pK_b value calculated for yohimbine vs. BHT-920 in untreated tissue. These findings also support the contention that the method used in the present study of treating tissues with phenoxybenzamine whilst protected with yohimbine, did produce tissue which had predominantly $alpha_2$-adrenoceptors.

The effect of yohimbine on responses to UK 14304 and dopamine was complicated by the fact that a substantial proportion of the responses to both these agonists was mediated by $alpha_1$-receptors. Against UK 14304, yohimbine inhibited responses to low concentrations of the agonist to a greater extent than higher concentrations, leading to a change in the Hill slope of the CRC and apparently non-competitive inhibition. These data complement those discussed above for prazosin against UK 14304, where responses to low concentrations of UK 14304 were resistant to inhibition by prazosin, resulting in a biphasic CRC. Dopamine appeared to be inhibited in a competitive fashion by yohimbine. The change in EC_{50} value produced by yohimbine, did not, however, reach statistical significance because of the small number of vessels tested ($n = 4$) and the degree of variability in the effect of yohimbine (which caused an increase in EC_{50} value for all animals tested). The apparent pK_b value calculated for yohimbine vs. dopamine was between that calculated for yohimbine vs. BHT-920 and noradrenaline, possibly reflecting the intermediate proportion of $alpha_2$-receptors contributing to the overall response of EDV to dopamine, when compared with noradrenaline and BHT-920.

RX 821002 is a highly selective $alpha_2$-adrenoceptor antagonist (Wallace *et al.*, 1994). A significant competitive inhibitory effect of this antagonist was found against the $alpha_2$-selective agonist, BHT-920 and the non-selective agonist, dopamine. The apparent pK_b value calculated for RX 821002 was about 0.75 of a log unit higher when BHT-920 was the agonist than when dopamine was the agonist. This may well be the result of a stimulatory effect of dopamine on $alpha_1$-receptors which tended to mask the inhibitory action of RX 821002. This was likely the reason why no significant inhibitory effect of RX 821002 could be identified when noradrenaline was used as the agonist. The proportion of the total response to noradrenaline which was mediated by $alpha_2$-receptors in EDV was $\approx$ 16% whereas for dopamine this figure was about 32%, perhaps explaining why a significant inhibitory effect of RX 821002 was discernible vs. dopamine but not noradrenaline. It was surprising therefore to find that RX 821002 (10 nM) had no significant effect against the $alpha_2$-selective agonist, UK 14304. This finding is not readily explained by the fact that UK 14304 also stimulated $alpha_1$-adrenoceptors in EDV. The proportion of the response to UK 14304 which was resistant to phenoxybenzamine was 35%, so one would expect to see an effect of RX 821002 on the responses to UK 14304, particularly at low concentrations of this agonist. This might indicate that there is more than one type of $alpha_2$-adrenoceptor present in EDV mediating contraction, or that UK 14304 activates a different sort of receptor to produce the prazosin-resistant contraction found in this vessel. Given the imidazoline-containing structure of this agonist, an action at imidazoline preferring receptors (Lehmann, 1989) has to be a possible explanation for this finding but more work is necessary to investigate this further. RX 821002 has minimal affinity for non-adrenoceptor imidazoline preferring sites (Wallace *et al.*, 1994), despite being an imidazoline derivative of idazoxan. Bryant and Clarke (1996) postulated that the pre-synaptic actions of medetomidine, another imidazoline $alpha_2$-adrenoceptor agonist, were mediated at an imidazoline receptor. The present study would suggest that the post-synaptic contractile effects of UK 14304 which were resistant to inhibition by RX 821002, were inhibited by yohimbine at a concentration which was well below the reported affinity of yohimbine for central imidazoline receptors (pK_i 5.28; Brown *et al.*, 1990), perhaps favouring the view that more than one type of $alpha_2$-adrenoceptor may be present in the EDV post-synaptically.

In conclusion, the present study has provided conclusive evidence that EDV contain post-synaptic alpha-adrenoceptors of both $alpha_1$ and $alpha_2$ types which mediate vasoconstriction. The evidence presented suggests that the $alpha_2$-adrenoceptor was resistant to inhibition by 30 nM prazosin and so is most likely to be of the $alpha_{2A}$ or $alpha_{2D}$ sub-type. Further studies are necessary to characterize this fully and to determine whether more than one $alpha_2$-adrenoceptor subtype is present in this blood vessel. The sub-type of $alpha_1$-adrenoceptor which mediates the major part of the vasoconstrictor response to noradrenaline, remains to be characterized. The results presented here suggest that simultaneous activation of these two receptor types by the endogenous full agonist, noradrenaline, may lead to synergistic interactions in terms of the maximum contractile force developed but also seems to reduce the slope of the concentration response curve. In other superficial blood vessels, temperature has been shown to influence the operation of these two receptor types such that cooling favours constriction of vessels from superficial tissues (Flavahan & Vanhoutte, 1986b). This may be physiologically important for the diversion of blood from the superficial toward the deeper tissues and hence in the conservation of heat. It is worthy of note that all the experiments conducted for the present study were undertaken in the summer months (June to August). Preliminary observations suggest the sensitivity of EDV to $alpha_1$-selective agonists is lower in winter than in summer (unpublished observations). Seasonal changes have been noted in vasoconstrictor action in digital vessels of the fallow deer (Callingham *et al.*, 1996) and it is interesting to speculate that if such changes occur in the horse they might in some way be linked to the seasonal occurrence of laminitis.

ACKNOWLEDGMENTS

This work was funded by an Equine Welfare Grant from the Home of Rest for Horses. The excellent technical assistance of Mrs Jale Soydan and the help and co-operation of the owners

and staff of West Sussex Abattoir, Crawley, UK are gratefully acknowledged.

REFERENCES

Agrawal, D.K., Tringle, C.R. & Daniel, E.E. (1984) Pharmacological characterization of the postsynaptic *alpha* adrenoceptors in vascular smooth muscle from canine and rat mesenteric vascular beds. *Journal of Pharmacology and Experimental Therapeutics*, **229**, 831–838.

Baxter, G.M., Laskey, R., Tackett, R.L., Moore, J.N. & Allen, D. (1989) *In vitro* reactivity of digital arteries and veins to vasoconstrictive mediators in healthy horses and horses with early laminitis. *American Journal of Veterinary Research*, **50**, 508–517.

Baxter, G.M., Moore, J.N. & Tackett, R.L. (1991) *In vitro* responses of equine digital vessels to dopamine and fenoldopam. *Equine Veterinary Journal*, **23**, 48–52.

Bentley, S.M., Drew, G.M. & Whiting, S.B. (1977) Evidence for two distinct types of post-synaptic α-adrenoceptor. *British Journal of Pharmacology*, **61**, 116–117P.

Brown, C.M., MacKinnon, A.C., McGrath, J.C., Spedding, M. & Kilpatrick, A.T. (1990) α_2-adrenoceptor subtypes and imidazoline-like binding sites in the rat brain. *British Journal of Pharmacology*, **99**, 803–809.

Bryant, C.E. & Clarke, K.W. (1996) The actions of medetomidine may not be mediated exclusively by α_2-adrenoceptors in the equine saphenous vein. *Journal of Veterinary Pharmacology and Therapeutics*, **19**, 124–129.

Burgen, A.S.V. & Iversen, L.L. (1965) The inhibition of noradrenaline uptake by sympathomimetic amines in the rat isolated heart. *British Journal of Pharmacology*, **25**, 34–49.

Bylund, D.B., Eikenberg, D.C., Hieble, J.P., Langer, S.Z., Lefkowitz, R.J., Minneman, K.P., Molinoff, P.B., Ruffolo, R.R. Jr. & Trendelenburg, U. (1994) International Union of Pharmacology Nomenclature of Adrenoceptors. *Pharmacological Reviews*, **46**, 121–137.

Callingham, B.A., White, R., Scarlett, J.A. & Brown, G. (1996) Seasonal differences in the actions of vasoactive agents on segments of digital arteries of the fallow deer. *British Journal of Pharmacology*, **119**, 22P.

Cambridge, D. (1981) UK-14304, a potent and selective alpha$_2$-adrenoceptor agonist for the characterisation of alpha-adrenoceptor subtypes. *European Journal of Pharmacology*, **72**, 413–415.

Daly, C.J., McGrath, J.C. & Wilson, V.G. (1988) Pharmacological analysis of postjunctional α-adrenoceptors mediating contractions to (-)-noradrenaline in the rabbit isolated lateral saphenous vein can be explained by interacting responses to simultaneous activation of α_1- and α_2-adrenoceptors. *British Journal of Pharmacology*, **95**, 485–500.

De Mey, J. & Vanhoutte, P.M. (1981) Uneven distribution of postjunctional alpha$_1$ and alpha$_2$-like adrenoceptors in canine arterial and venous smooth muscle. *Circulation Research*, **48**, 875–884.

Doxey, J.R., Smith, C.F. & Walker, J.M. (1977) Selectivity of blocking agents for pre- and post-synaptic α-adrenoceptors. *British Journal of Pharmacology*, **60**, 91–96.

Egleme, T., Godfraind, T. & Miller, R.C. (1984) Enhanced responsiveness of rat isolated aorta to clonidine after removal of endothelial cells. *British Journal of Pharmacology*, **81**, 16–18.

Elliott, J. & Soydan, J. (1994) Dopamine activates both alpha$_1$ and alpha$_2$ adrenoceptors to cause vasoconstriction in equine digital veins. *Journal of Autonomic Pharmacology*, **14**, 17.

Elliott, J., Bryant, C.E. & Soydan, J. (1994) The role of nitric oxide in the responses of equine digital veins to vasodilator and vasoconstrictor agents. *Equine Veterinary Journal*, **26**, 378–384.

Flavahan, N.A. & Vanhoutte, P.M. (1986a) α_1-adrenoceptor subclassification in vascular smooth muscle. *Trends in Pharmacological Science*, **6**, 347–349.

Flavahan, N.A. & Vanhoutte, P.M. (1986b) Effect of cooling on alpha$_1$ and alpha$_2$ adrenergic responses in canine saphenous and femoral veins. *Journal of Pharmacology and Experimental Therapeutics*, **238**, 139–147.

Flavahan, N.A., Rimele, T.J., Cooke, J.P. & Vanhoutte, P.M. (1984) Characterization of postjunctional *alpha$_1$* and *alpha$_2$* adrenoceptors activated by exogenous or nerve-released norepinephrine in the canine saphenous vein. *Journal of Pharmacology and Experimental Therapeutics*, **230**, 699–705.

Furchgott, R.F. (1972) The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In *Catecholamines – Handbook of Experimental Pharmacology*, Vol. 33. Eds Blashko, H. & Muscoll, E. pp. 283–335. Springer-Verlag, Berlin.

Guimaraes, S. & Nunes, J.P. (1990) The effectiveness of α_2-adrenoceptor activation increases from the distal to the proximal part of the veins of canine limbs. *British Journal of Pharmacology*, **101**, 387–393.

Jauering, R.A, Moulds, R.F.W. & Shaw, J. (1978) The action of prazosin in human vascular preparations. *Archives Internationales de Pharmacodynamie et de Therapie* **231**, 81–89.

Langer, S.Z. (1974) Presynaptic regulation of catecholamine release. *Biochemical Pharmacology*, **23**, 1793–1800.

Lehmann, J. (1989) The immidazoline preferring receptor. *Life Sciences*, **45**, 1609–1615.

Minneman, K.P. (1988) α_1-adrenergic receptor subtypes, inositol phosphates and sources of cell Ca^{2+}. *Pharmacological Reviews*, **40**, 87–119.

Motram, D.R. (1983) Pre-junctional α_2-adrenoceptor activity of BHT-92. *Journal of Pharmacy and Pharmacology*, **35**, 652–655.

Murphy, T.J. & Bylund, D.B. (1988) Characterisation of alpha-2-adrenergic receptors in the OK Cell, an opossum cell line. *Journal of Pharmacology and Experimental Therapeutics*, **244**, 571–578.

Ruffolo, R.R. Jr., Waddell, J.E. & Yaden, E.L. (1981) Postsynaptic alpha adrenergic receptor subtypes differentiated by yohimbine in tissues from the rat. Existence of alpha-2 adrenergic receptors in rat aorta. *Journal of Pharmacology and Experimental Therapeutics*, **217**, 235–240.

Stevens, M.J. & Moulds, R.F.W. (1981) Heterogeneity of post-junctional α-adrenoceptors in human vascular smooth muscle. *Archives Internationales de Pharmacodynamie et de Therapie*, **254**, 43–57.

Sullivan, A.T. & Drew, G.M. (1980) Pharmacological characterisation of pre- and postsynaptic α-adrenoceptors in dog saphenous vein. *Naunyn Schmiedeberg's Archives of Pharmacol.*, **314**, 249–258.

Timmermans, P.B.M.W.M. & Van Zwieten, P.A. (1981) The postsynaptic α_2-adrenoceptor. *Journal of Autonomic Pharmacology*, **1**, 171–183.

Wallace, D.R., Muskardin, D.T., Zahniser, N.R. (1994) Pharmacological characterisation of [^{3}H]idazoxan, [^{3}H]RX821002 and *p*-[^{125}I]iodoclonidine binding to α_2-adrenoceptors in rat cerebral cortical membranes. *European Journal of Pharmacology*, **258**, 67–76.