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Characterization of α_2-adrenoceptors mediating contraction of dog saphenous vein: identity with the human α_{2A} subtype

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1 In the dog saphenous vein α_1- and α_2-adrenoceptors mediate noradrenaline-induced contractions *in vitro*. In order to study the α_2-adrenoceptor in isolation, α_1-adrenoceptors were inactivated by treatment of tissues with the alkylating agent phenoxybenzamine (3.0 μM for 30 min) in the presence of rauwolscine (1 μM) to protect α_2-adrenoceptors.

2 Noradrenaline-induced contractions of tissues treated with phenoxybenzamine were antagonized competitively by the selective α_2-adrenoceptor antagonist rauwolscine, $pK_B = 8.63 \pm 0.07$ (means ± s.e.-mean; $n = 3$), consistent with an interaction at α_2-adrenoceptors.

3 Noradrenaline was a full agonist at α_2-adrenoceptors in dog saphenous vein. By use of the method of partial receptor alkylation and analysis of concentration-effect curve data by direct, operational model fitting methods, the affinity (pK_A) and efficacy (τ) were 5.74 ± 0.07 and 7.50 ± 1.05, respectively ($n = 6$). Nine other agonists which were examined each had affinities higher than noradrenaline, but with the exception of the imidazoline, A-54741 (5,6-dihydroxy-1,2,3,4-tetrahydro-1-naphthyl-imidazoline) had relatively lower efficacies.

4 To compare the α_2-adrenoceptor in dog saphenous vein to the human recombinant subtypes, the affinities of twenty-one compounds were estimated in functional studies in the dog saphenous vein and in radioligand binding studies for the human α_{2A}, α_{2B} and α_{2C} receptor subtypes expressed in Chinese hamster lung (CHL) cells.

5 Of twenty-one compounds examined in ligand binding studies, only nine had greater than ten fold selectivity for one human receptor subtype over either of the other two. These compounds were A-54741, oxymetazoline, guanfacine, guanabenz, prazosin, spiroxatrine, tolazoline, WB 4101 and idazoxan. In dog saphenous vein, their affinities (pK_A and pK_B for agonists and antagonists respectively) were: A-54741 ($pK_A = 8.03 \pm 0.05$), oxymetazoline ($pK_A = 7.67 \pm 0.09$), guanfacine ($pK_A = 6.79 \pm 0.03$); guanabenz ($pK_A = 7.02 \pm 0.13$); prazosin ($pK_B = 5.19 \pm 0.08$), spiroxatrine ($pK_B = 6.59 \pm 0.04$), tolazoline ($pK_B = 6.21 \pm 0.07$), WB 4101 ($pK_B = 7.42 \pm 0.09$) and idazoxan ($pK_B = 7.11 \pm 0.08$).

6 Comparisons of affinity estimates for these nine compounds at the receptor in dog saphenous vein and at the human recombinant subtypes suggest that the vascular receptor is most similar to the h α_{2A} subtype; correlation coefficients (r) were 0.82 (h α_{2A}), 0.24 (h α_{2B}) and 0.04 (h α_{2C}).

Keywords: Dog saphenous vein; α_2-adrenoceptors; vascular smooth muscle; human recombinant receptors; operational-model

Introduction

α_2-Adrenoceptor agonists are used therapeutically as antihypertensives, as analgesic agents and as sedation-inducing adjuncts in veterinary anaesthesia (see Ruffolo *et al*., 1995). Clonidine, dexmedetomidine and guanfacine are agonists with selectivity for α_2-over α_1-adrenoceptors (McGrath *et al*., 1989), but little is known about their affinity and efficacy at the subtypes of α_2-adrenoceptors (α_{2A}, α_{2B}, α_{2C}) which have been identified by molecular biological and pharmacological techniques. Thus, the affinities of both clonidine ($K_i = 123$ and 150 nM) and dexmedetomidine ($K_i = 8.1$ and 5.9 nM) are similar at human recombinant α_{2A} and α_{2B} receptors respectively, when expressed in sf9 cells (Jansson *et al*., 1995). In the latter study agonist relative efficacies were determined by measuring the inhibition of forskolin-stimulated adenosine 3′:5′-cyclic monophosphate (cyclic AMP) production. Dexmedetomidine had the same intrinsic activity as noradrenaline at both the h α_{2A} and h α_{2B} adrenoceptors, while clonidine was a partial agonist at each subtype. Guanfacine has a selectivity of 10–30 fold for the human α_{2A} receptor over other subtypes (Uhlen *et al*., 1994). However, these limited data on the affinity and efficacy of α_2-adrenoceptor agonists are insufficient to draw any conclusions on whether the multiple effects of these agonists are due to interactions with one or several of the α_2 subtypes.

As part of a programme concerned with defining the roles of the different of α_2-adrenoceptor subtypes, it was necessary to establish robust, functional assays for the different subtypes in order to estimate agonist efficacies as well as agonist and antagonist affinities. Many α_2-adrenoceptors in the CNS and peripheral tissues are located pre-junctionally and have been characterized by measuring the overflow of radiolabelled neurotransmitter (Smith *et al*., 1992; Smith & Docherty, 1992; Limberger *et al*., 1995a, b; Funk *et al*., 1995; Molderings & Gothert, 1995; Trendelenburg *et al*., 1995). However, these assays are not amenable to agonist affinity and efficacy estimation. Another commonly used assay is the field-stimulated rat vas deferens in which α_2-agonists inhibit an electrically-evoked twitch response (see Smith *et al*., 1992 for references).

Pharmacological characterization of this receptor has shown that it is similar to the human α_{2A} subtype (Smith *et al*., 1992), but that it has a low affinity for rauwolscine and yohimbine suggesting it is a species homologue, and classified as an α_{2D}-adrenoceptor (see Bylund *et al*., 1994). However, this assay is complex, since two other groups (Akers *et al*., 1991; Oriowo *et al*., 1991) have obtained affinity estimates for the antagonist SK&F 104078 that are agonist-dependent, thus suggesting heterogeneity of receptors.

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Collectively, the lack of well-defined, robust assays for α_2-adrenoceptor subtypes has led us to characterize other tissues used previously as assays for α_2-adrenoceptors. In this respect, contraction of dog isolated saphenous vein has been used for many years to study α_2-adrenoceptor function, although α_1-adrenoceptors contribute to the constictor effects of non-selective agonists such as noradrenaline in this tissue (Sullivan & Drew, 1980; Constantine *et al*., 1982; Flavahan *et al*., 1984; Alabaster *et al*., 1985). The objectives of this study were, therefore, to isolate the α_2-adrenoceptor population in dog saphenous vein in order to study this receptor exclusively and to define the pharmacological characteristics in relation to recombinant receptor subtypes. Direct, operational model-fitting of concentration-effect curve data (Black & Leff, 1983; Leff *et al*., 1990) was used to estimate agonist affinities and efficacies in dog saphenous vein. In the absence of data from recombinant canine α_2-adrenoceptors, we compared ligand affinities for the receptor in dog saphenous vein with their affinities for the human cloned subtypes, obtained by ligand binding techniques. Some of these data have been published in abstract form (MacLennan *et al*., 1996).

Methods

Isolated tissues

Saphenous veins were removed from beagle or mongrel dogs of either sex (10–15 kg) which had been killed by an overdose of pentobarbitone sodium (200 mg kg^{-1}, i.v.). The veins were cleared of adhering connective tissue and used either that day or stored overnight at 4°C in normal Krebs solution. The reactivity (potency or maximum response) of these tissues to noradrenaline was not different.

Ring preparations (3–5 mm) were suspended between tungsten wire hooks (500 μm diameter) within 20 ml organ baths filled with Krebs solution maintained at 37°C and aerated with 95%O_2/5%CO_2. Isometric tension changes were measured by force transducers (Grass FTO3c) and recorded on a polygraph (Grass Model 7D). The Krebs solution also contained cocaine and corticosterone (each 30 μM), to prevent neuronal and extraneuronal uptake of catecholamines respectively, EDTA (23 μM) to prevent oxidative degradation of catecholamines, (±)-propranolol (1 μM) to block β_1-adrenoceptors and nitrendipine (0.1 μM) to block L-type calcium channels (see Oriowo *et al*., 1991). The tissues were allowed to stabilize for 20 min before being placed under a tension of 3 g which was re-applied twice at 15 min intervals.

Pharmacological isolation of α_2-receptors

A critical part of the experiment was to eliminate α_1-adrenoceptor-mediated effects and thus 'isolate' the α_2-receptor population. This was undertaken as follows: during the time the tissues were placed under passive force, they were exposed to the α_2-adrenoceptor antagonist rauwolscine (1 μM; 470 fold greater than its K_B at this receptor) and phenoxybenzamine (3 μM) for 30 min. Rauwolscine was added 5 min before phenoxybenzamine. The antagonists were washed out by 4 exchanges of the Krebs solution at 5 min intervals. The concentration of phenoxybenzamine used was the minimum concentration required to abolish responses to the α_1-adrenoceptor agonist phenylephrine (results not shown). Tissues were then challenged with KCl (100 mM) to assess tissue viability and to act as reference contracture with which to scale other responses. This protocol, by which α_1-adrenoceptors were inactivated, was followed in all experiments with the exception of those shown in Figure 1a.

Experimental protocols

Antagonist studies Concentration-effect (E/[A]) curves to noradrenaline were reproducible within a particular tissue (see results) which therefore allowed a paired-curve design to be employed throughout. In all experiments a first cumulative E/[A] curve to noradrenaline was constructed by increasing the bath concentration in 0.5 $\log_{10}$ M increments. Noradrenaline was removed from the bath by several exchanges of the Krebs solution. Following complete relaxation of the tissue a second E/[A] curve was constructed to noradrenaline, or other agonists, in the absence or presence of antagonist which had been added 60 min previously. The antagonist concentrations were chosen based on their affinities at the human recombinant subtypes (if known), or their correlates in other tissues such as the HT29 cell line, rat lung, opossum kidney cell line, from either published data (Bylund *et al*., 1992; Uhlen *et al*., 1994) or our own initial studies (functional and ligand binding studies were being conducted in parallel). The concentrations chosen were 10–30 fold higher than their lowest affinity for any of the α_2 subtypes to ensure a quantifiable shift of noradrenaline E/[A] curves in dog saphenous vein.

Agonist studies In order to estimate agonist affinities and relative efficacies, the technique of partial, irreversible receptor alkylation was adopted (Furchgott, 1966). Two E/[A] curves to noradrenaline were constructed, the second curve to noradrenaline after treatment of the tissues with phenoxybenzamine (0.5 μM) for 30 min followed by 4 exchanges of the Krebs. Analysis of these data by direct, operational model fitting showed that noradrenaline was a full agonist (see results) and therefore allowed the estimation of partial agonist affinities by a comparative analysis with noradrenaline (Barlow *et al*., 1967). The first curve was to noradrenaline and the second to a partial agonist. To estimate efficacies of partial agonists relative to noradrenaline in the same tissue, the affinity of noradrenaline ($pK_A = 5.74$) has to be assumed in the model fitting procedure. This allows the efficacy of noradrenaline to be estimated. The affinity estimate for noradrenaline used in these analyses had been determined in the experiment described above.

The imidazolines, A-54741 and UK-14,304 had similar maximum responses as noradrenaline. However, first and second E/[A] curves to these agonists were not reproducible and in experiments with these agonists the first E/[A] curve was to noradrenaline and the second E/[A] curve was to A-54741 or UK-14,304 with or without treatment of the tissue with phenoxybenzamine (0.5 μM for 30 min). In this experimental design a set of four E/[A] curves from two tissues were generated for subsequent analysis.

Analysis of E/[A] curve data

Logistic curve fitting and analysis of antagonism The Hill equation was fitted to individual E/[A] curves:

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

in which E, α, $[A_{50}]$ and n_H are effect, upper-asymptote, midpoint location and slope parameters respectively. Location parameters were actually estimated as logarithms ($-\log_{10}[A_{50}]$). If antagonists produced parallel, surmountable displacements of agonist concentration-effect curves, Schild analysis of the data was conducted by use of least squares linear regression of $\log(r-1)$ vs $\log[B]$ where r is the concentration-ratio between control and antagonist (B) treated tissues (Arunlakshana & Schild, 1959). If the Schild plot slope was not significantly different from unity it was constrained to this value to estimate K_B, the antagonist dissociation equilibrium constant. In experiments in which only a single concentration of antagonist was used, affinity estimates (apparent pK_Bs) were estimated from the equation

$$pK_B = \log[r-1] - n\log[B] \quad (2)$$

provided that the shift produced by the antagonist was parallel

and making the assumption of simple competitive antagonism, i.e. that n was unity.

Operational model-fitting The operational model of agonism (Black & Leff, 1983; Leff *et al*., 1990) was fitted directly to the E/[A] curve data. This model describes agonist action in a one-receptor: one-transducer system by the equation:

$$E = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n} \quad (3)$$

where E is the pharmacological effect, of which E_m is the maximum effect possible, [A] is the agonist concentration, K_A is the dissociation equilibrium constant of the agonist, τ is the efficacy of the agonist in the system and n is the slope of the transducer relation linking [AR] to E. The solver function of Microsoft Excel was used to fit the model equation directly to E/[A] curve data.

Cell culture

Chinese Hamster Lung (CHL) cells were stably transfected with the human α_{2A} or α_{2B} genes by use of the pBC expression vector (Cullen, 1987). To increase expression levels, the α_{2C} gene was spliced into the pCDNA3 expression vector (Invitrogen, CA, U.S.A.). Cells were maintained in Dulbecco's modified Eagle medium (DMEM, 4.5 g l^{-1} glucose, without sodium pyruvate) with 10% foetal bovine serum and 250 μg ml^{-1} G-418 and grown in a 5% CO_2 environment at 37°C. Cells were harvested with 2 mM EDTA in phosphate buffered saline and centrifuged at 500 *g* for 5 min and the pellets stored at −70°C.

Membrane preparation

Membranes were prepared from frozen cell pellets with a Polytron P10 disrupter (setting 5, 5 s bursts) and centrifuged at 30,000 *g* for 15 min at 4°C. The pellets were resuspended in a small volume of homogenization buffer (50 mM Tris-base, 1 mM EDTA (free acid), 150 mM NaCl, 2 mM $MgCl_2$, pH 7.4 at 4°C) by use of a Polytron and frozen in aliquots at −70°C.

Radioligand binding assays

Cell membranes were thawed and briefly homogenized in assay buffer (50 mM Tris-base, 1 mM EDTA (free acid), 150 mM NaCl, 2 mM $MgCl_2$, 100 μM GppNHp, pH 7.4 at 25°C) with a Polytron. To each assay tube was added membranes from 80,000–250,000 cells (5–10 μg protein), 0.3–0.5 nM [^{3}H]-MK 912 and 100 μM GppNHp. Nonspecific binding was determined with 1 μM RX 821002. For competition binding studies, drugs were tested over a range of concentrations in duplicate, in a minimum of three separate experiments. Following a 90 min incubation at 25°C, the tubes were filtered over GF/B glass fibre filtermats (Whatman, NJ, U.S.A.) with a Brandel 48 well harvestor or a Packard Top Count 96 well cell harvester. The tubes were then rinsed three times with ice-cold 50 mM tris-base, pH = 7.4 (3 × 1 ml/sample). Radioactivity was determined by liquid scintillation counting (Topcount, Packard Instrumentation Co., CT, U.S.A.).

Analysis of radioligand binding data

The K_D and B_{max} values of [^{3}H]-MK 912 for each receptor were determined from binding isotherms by non-linear regression (Prism; GraphPad Software, CA, U.S.A.). For each test compound, the concentration producing 50% inhibition of binding (IC_{50}) and Hill slope were determined (Prism) and from the radioligand K_D value, the inhibition dissociation constant, K_i, of each drug was calculated from the Cheng-Prusoff (1973) relationship:

$$K_i = \frac{IC_{50}}{1 + [L]/K_D} \quad (4)$$

where L is the concentration of [^{3}H]-MK 912.

Statistics

Statistical analysis of data were undertaken by use of either *t* tests or analysis of variance where appropriate, with $P < 0.05$ regarded as statistically significant. Comparisons between the affinities of compounds from functional and ligand binding experiments were made by linear regression (Microsoft Excel) and Pearson's correlation coefficient, *r*, given for each analysis.

Materials

The Krebs solution (Sigma, MO, U.S.A.) had the following composition (mM); NaCl 118.41, $NaHCO_3$ 25.00, KCl 4.75, KH_2PO_4 1.19, $MgSO_4$ 1.19, glucose 11.10 and $CaCl_2$ 2.50. DMEM, phosphate-buffered saline, G-418 and foetal bovine serum were purchased from Gibco (NY, U.S.A.). The cDNAs (in pBC vector) for the human α_{2A}, α_{2B} or α_{2C} receptors were obtained from Duke University (NC, U.S.A.).

The following drugs were purchased: (−)-noradrenaline bitartrate, guanabenz, oxymetazoline hydrochloride, prazosin hydrochloride, phentolamine hydrochloride, yohimbine hydrochloride, (±)-propranolol hydrochloride, (−)-phenylephrine hydrochloride (all Sigma, MO, U.S.A.); clonidine hydrochloride (Calbiochem-Novabiochem Corp., CA, U.S.A.); tolazoline hydrochloride (Aldrich, WI, U.S.A.); rauwolscine hydrochloride; RX821002 (2-(2,3-dihydro-2-methoxy-1,4-benzodioxin-2-yl)-4,5-dihydro-1H-imidazole) hydrochloride, WB 4101 (2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane) hydrochloride, spiroxatrine (all RBI; MA, U.S.A.); phenoxybenzamine hydrochloride (ICN Pharmaceuticals, CA, U.S.A.); [^{3}H]-MK 912 ((2S,12bS)1′,3′-dimethylspiro (1, 3, 4,5′,6′,7,12b) octahydro - 2H - benzo [b] furo [2,3-a] quinazoline-2,4′-pyrimidin-2′-one, New England Nuclear; MA, U.S.A.), UK-14,304 (5-bromo-6-[2-imidazoline-2-glamino]-quinoxaline) hydrochloride, dexmedetomidinne hydrochloride, A-54741 (5,6-dihydroxy-1,2,3,4-tetrahydro-1-naphthyl-imidazoline) hydrobromide, ST-91 (N-2,6-diethylphenyl)-4,5-dihydro-1H-imidazol-2(3H)-imine), idazoxan hydrochloride and nitrendipine were synthesized at Roche Bioscience for which Dr R. Clark, H. Cai, J.P. Dunn and J.M. Caroon are thanked. The following drugs were generous gifts for which the companies are thanked: B-HT 920 (2-amino-6-allyl-5,6,7,8-tetrahydro-4H-thiazolo[4,5-d]-azepine, Boehringer Ingelheim); SK&F 104078 6-chloro-9-[(3-methyl-2-butenyl)oxy]-3-methyl-1H- 2,3,4,5 - tetrahydro- 3- benzazepine, and SK&F 104856 2-vinyl-7-chloro-3,4,5,6-tetrahydro-4-methylthieno[4,3,2ef][3]benzazepine, (Dr Paul Hieble, SmithKline Beecham Pharmaceuticals); guanfacine hydrochloride (Sandoz).

All drugs were dissolved in water with the exception of spiroxatrine (2 mM), dexmedetomidine (20 mM), tolazoline (20 mM), guanfacine (20 mM), guanabenz (20 mM), prazosin (20 mM), SK&F 104078 (2 mM), ST-91 (20 mM) and B-HT 920 (20 mM) which were dissolved in dimethylsulphoxide (DMSO) at the stated concentrations and diluted in water (purified and de-ionised). Phenoxybenzamine (2 mM) and nitrendipine (1 mM) were dissolved in absolute ethanol and diluted in water. At the final concentrations in the organ bath none of the drug vehicles affected tissue responsiveness.

Results

Antagonist studies

In rings of dog saphenous vein E/[A] curves to noradrenaline were monophasic with a Hill slope (n_H) of 1.04 ± 0.02 ($n = 28$). The mid-point location (p[A_{50}]) was 6.70 ± 0.07 and maximum

response of 22.86 ± 1.06 g (28 tissues from 4 animals; Figure 1a). E/[A] curves to noradrenaline were antagonized by the selective α_2-adrenoceptor antagonist rauwolscine in a non-competitive manner. Rauwolscine (10 nM – 1.0 μM) caused rightward displacements of noradrenaline E/[A] curves without affecting the maximum response but the slope of the Schild plot was significantly lower than unity (0.56, mean; 95% confidence limits 0.45 – 0.67; 20 degrees of freedom (d.f.), tissue from 4 animals) suggesting the involvement of other receptors (probably α_1) in addition to the α_2 subtype, in noradrenaline-induced contraction.

In order to isolate α_2-adrenoceptors it was necessary to abolish α_1-adrenoceptor-mediated effects. Contractions induced by phenylephrine, an α_1-adrenoceptor agonist, were abolished by phenoxybenzamine (3.0 μM, $n = 4$; data not shown) therefore all subsequent experiments were conducted after a 30 min treatment of tissues with phenoxybenzamine (3.0 μM) in the presence of rauwolscine (1.0 μM) to protect the α_2-adrenoceptors. Following this treatment, noradrenaline E/[A] curves were significantly steeper ($n_H = 1.92 \pm 0.36$), but the potency was not changed ($p[A_{50}] = 6.67 \pm 0.21$). The maximum response (9.63 ± 0.43 g wt; 18 tissues from 3 animals) was also significantly lower than in tissues not exposed to phenoxybenzamine. Noradrenaline-induced contractions were now competitively antagonized by rauwolscine (10 nM – 0.3 μM; Figure 1b): E/[A] curves to noradrenaline were displaced in a parallel fashion with no significant change in the maximum and the Schild plot slope was not significantly different from unity (0.98; 0.83 – 1.14; 10 d.f., tissue from 3 animals). With the Schild plot constrained to unity the pK_B was 8.63 (8.39 – 8.87). These data show that phenoxybenzamine (in the presence of rauwolscine) effectively inactivated non-α_2-adrenoceptors involved in noradrenaline-induced contraction, therefore this treatment regimen was followed in all subsequent experiments.

Noradrenaline-induced contractions of dog saphenous vein were reproducible as E/[A] curve parameters were not different between first and second cumulative curves. Differences between first and second curves were ($n = 4$): $\Delta p[A_{50}]$ 0.12 ± 0.04; Δn_H 0.02 ± 0.11; Δmaximum 0.01 ± 0.04. This allowed a paired curve experimental design to be adopted. Thus, antagonist affinities were assessed by the extent of

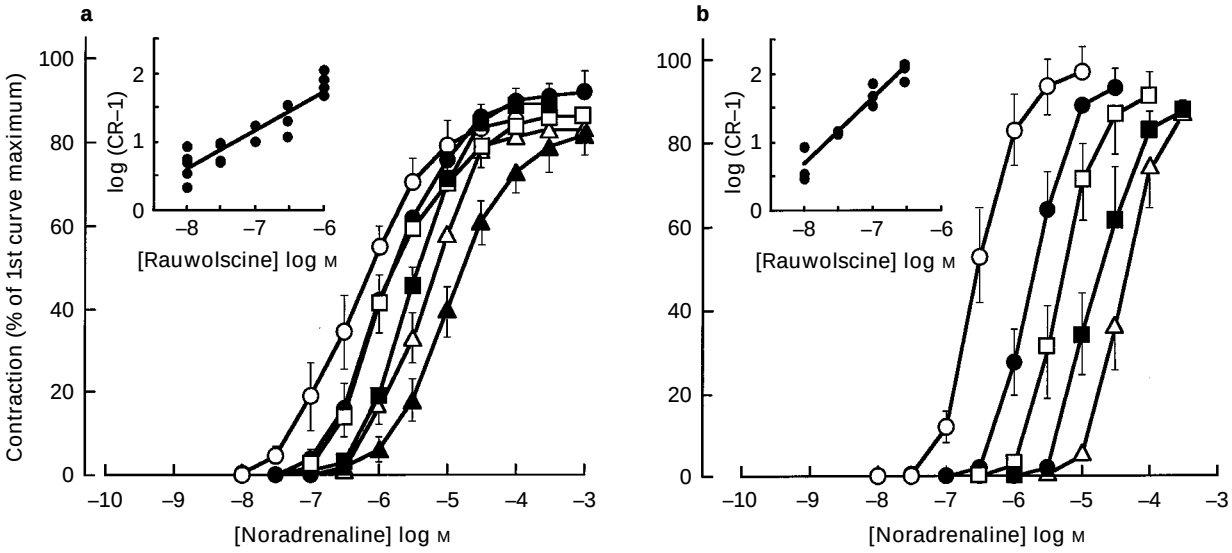

Figure 1 Antagonism of noradrenaline-induced contraction of dog saphenous vein by rauwolscine. (a) Two concentration-effect curves to noradrenaline were constructed, the second curve constructed in the absence (○) or presence of rauwolscine (●, 0.01 μM); (□, 0.03 μM); (■, 0.1 μM); (△, 0.3 μM); (▲, 1.0 μM). (b) Tissues were first exposed to phenoxybenzamine (3.0 μM) in the presence of rauwolscine (1.0 μM) for 30 min which was subsequently washed out before construction of concentration-effect curves to noradrenaline as described for (a). Data shown are for the second curves only and are expressed as a % of the first curve maximum. In (a) and (b), the inserts show the Schild plot for which the slopes (mean) were (a) 0.56, (b) 0.98. Vertical lines show s.e.mean of $n = 4$ (a) or $n = 3$ (b); these are shown when they were larger than the symbols.

Table 1 Antagonist affinity estimates at the α_2-adrenoceptor in dog saphenous vein and at the human recombinant α_2-adrenoceptor subtypes

	Dog saphenous vein		*Human α_2 (pK_i)*		
Antagonist	pK_B	*[Antagonist]* (μM)	*h α_{2A}*	*h α_{2B}*	*h α_{2C}*
Rauwolscine	8.63 ± 0.07	0.01 – 0.3	9.02 ± 0.20	8.78 ± 0.10	9.24 ± 0.08
Yohimbine	8.66 ± 0.05	0.1	8.67 ± 0.06	8.35 ± 0.14	7.97 ± 0.05
RX 821002	8.55 ± 0.04	0.03	9.36 ± 0.11	8.69 ± 0.15	8.84 ± 0.02
Phentolamine	7.46 ± 0.06	0.1	7.49 ± 0.17	7.24 ± 0.11	6.74 ± 0.09
WB 4101	7.42 ± 0.09	0.1	7.99 ± 0.02	7.49 ± 0.01	8.90 ± 0.02
Idazoxan	7.11 ± 0.08	1.0	7.89 ± 0.02	7.41 ± 0.03	6.60 ± 0.02
SKF 104856	6.88 ± 0.25	3.0	7.35 ± 0.02	8.10 ± 0.02	7.15 ± 0.05
SKF 104078	6.50 ± 0.08	3.0	7.13 ± 0.13	6.78 ± 0.08	6.37 ± 0.04
Spiroxatrine	6.59 ± 0.04	10.0	7.43 ± 0.06	9.32 ± 0.13	9.00 ± 0.13
Tolazoline	6.21 ± 0.07	30.0	6.90 ± 0.03	5.96 ± 0.07	5.54 ± 0.11
Prazosin	5.19 ± 0.08	30.0	6.12 ± 0.06	7.38 ± 0.10	7.23 ± 0.09

pK_B estimates in dog saphenous vein were estimated functionally from the antagonism of noradrenaline-induced contraction. pK_i values for the human α_2 subtypes are from ligand binding studies. Values are the mean and s.e.mean of 3 – 4 estimates, each estimate obtained in tissue from a different animal, or number of replicates in ligand binding studies.

shift of noradrenaline E/[A] curves in the same tissue. Agonist affinity and efficacy data were determined by comparison of E/[A] curve data for noradrenaline and a test agonist in the same tissue.

Each of the antagonists (with the exception of rauwolscine) was examined at a single concentration only (see Table 1). Each antagonist caused parallel displacements of noradrenaline E/[A] curves without a significant effect on the maximum response. The estimated affinities (apparent pK_Bs) of prazosin and spiroxatrine, which in ligand binding studies showed some selectivity between the human α_2 subtypes, were 5.19 ± 0.08 ($n=3$) and 6.59 ± 0.04 ($n=3$), respectively, in dog saphenous vein. Lower concentrations of prazosin (3 μM) had no effect, as would be anticipated if it behaved as a simple competitive antagonist with a pK_B of 5.19.

Agonist studies

Treatment of tissues with phenoxybenzamine (0.5 μM for 30 min) caused a righward shift and depressed the maximum response of noradrenaline E/[A] curves (Figure 2 and Table 2). Operational model fitting of the E/[A] curve data suggested that noradrenaline was a full agonist, achieving the same maximum response as the estimated value of E_m, the maximum possible effect in the system, which was $102 \pm 1\%$ ($n=5$). The slope parameter describing the occupancy-effect relation was 1.80 ± 0.15 and the estimated pK_A was 5.74 ± 0.07. This affinity estimate was used in all other operational model fitting procedures in order to estimate efficacy (τ) for noradrenaline in the same tissue as τ was estimated for a test agonist and thus provide within-tissue relative efficacy estimates. The ratio of the efficacies for noradrenaline after and before alkyation (τ_2/τ_1) was 0.11 ± 0.03.

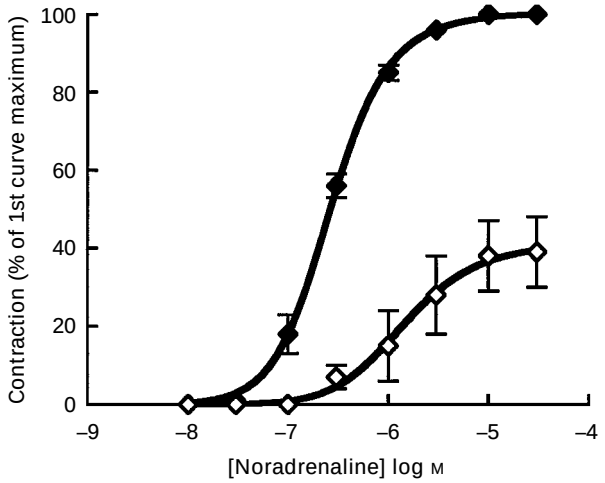

Figure 2 Effect of phenoxybenzamine on noradrenaline-induced contraction of dog saphenous vein. α_1-Adrenoceptors were first inactivated by phenoxybenzamine (3.0 μM) in the presence of rauwolscine (1.0 μM). Following washout of antagonists, concentration-effect curves to noradrenaline were constructed before (◆) and after (◇) exposure of tissues to phenoxybenzamine (0.5 μM for 30 min) which was washed out before responses to noradrenaline were obtained. Vertical lines show s.e.mean ($n=5$) when larger than the symbols. The lines through the data are the result of fitting simultaneously the operational model of agonism (equation 3) to the experimental data.

The effects of phenoxybenzamine were shown to be caused by a reduction of receptor number, and not due to post-receptor actions, as its effects could be prevented by concommitant exposure of the tissues to rauwolscine (Figure 3). Higher concentrations of phenoxybenzamine (5.0 μM for 30 min) abolished noradrenaline-induced contractions.

Each of the other agonists examined here produced monophasic E/[A] curves. Operational modelling of E/[A] curve data found that each agonist was of higher affinity than noradrenaline but with the exception of A-54741 behaved as partial agonists (Table 2). The average estimates of E_m and n from the analysis of A-54741 were 104 ± 2 and 1.86 ± 0.08, respectively ($n=5$), and from the analysis of UK-14,304 were 104 ± 1 and 1.67 ± 0.07 ($n=3$). These estimates of E_m and n were not significantly different from the estimates obtained with noradrenaline, as would be expected since they are parameters which describe the receptor system. Examples of data sets of A-54741 and dexmedetomidine which provided single estimates of operational-model parameters, are shown in Figures 4 and 5, respectively.

Agonist-independent estimates of the affinity of rauwolscine were obtained, showing that the contractile effects of each agonist were mediated by the same receptor (Table 3).

Ligand binding studies

Saturation binding isotherms for the binding of [^{3}H]-MK 912 to each of the α_2 subtypes expressed in CHL cells were described by a single-site fit. From the isotherms the K_D values (nM) and B_{max} (pmol mg^{-1} protein) were calculated: h α_{2A} $K_D = 0.58 \pm 0.13$, B_{max} 1.5 ± 0.10; h α_{2B} $K_D = 0.64 \pm 0.06$, $B_{max} = 2.80 \pm 0.20$; h α_{2C} $K_D = 0.04 \pm 0.01$, $B_{max} = 1.3 \pm 0.16$ ($n = 3-4$ estimates in each case). At each of the human recombinant α_2-adrenoceptor subtypes all of the compounds displaced [^{3}H]-MK-912 with Hill slopes not significantly different from unity thus confirming the presence of a single class of binding site in each assay (Tables 1 and 2).

Table 2 Affinity and efficacy data for agonists acting at α_2-adrenoceptors in dog saphenous vein and affinity estimates at the human recombinant α_2-adrenoceptor subtypes

	Dog saphenous vein				*Human α_2 (pK_i)*		
Agonist	*pA_{50}*	*Intrinsic activity* (NA = 1.0)	*pK_A*	*Relative efficacy* (NA = 1.0)	*h α_{2A}*	*h α_{2B}*	*h α_{2C}*
Noradrenaline (NA)	6.54 ± 0.06	1.0	5.74 ± 0.07	1.0	5.12 ± 0.10	5.33 ± 0.06	5.70 ± 0.20
A-54741	8.77 ± 0.13	1.01 ± 0.02	8.03 ± 0.05	0.98 ± 0.09	7.38 ± 0.03	6.47 ± 0.05	6.18 ± 0.06
UK-14304	7.64 ± 0.09	0.95 ± 0.06	7.02 ± 0.08	0.65 ± 0.18	6.71 ± 0.05	6.28 ± 0.05	5.97 ± 0.05
ST-91	6.82 ± 0.11	0.71 ± 0.08	6.47 ± 0.07	0.36 ± 0.07	6.48 ± 0.03	6.37 ± 0.04	6.02 ± 0.09
B-HT 920	6.56 ± 0.05	0.63 ± 0.05	6.36 ± 0.05	0.26 ± 0.05	6.36 ± 0.08	6.04 ± 0.12	6.02 ± 0.01
Dexmedetomidine	8.12 ± 0.08	0.67 ± 0.04	7.93 ± 0.07	0.24 ± 0.03	7.99 ± 0.04	7.53 ± 0.04	7.30 ± 0.04
Clonidine	7.36 ± 0.13	0.56 ± 0.04	7.24 ± 0.13	0.20 ± 0.05	7.15 ± 0.03	7.22 ± 0.09	6.62 ± 0.06
Oxymetazoline	7.66 ± 0.13	0.44 ± 0.07	7.67 ± 0.09	0.13 ± 0.02	8.19 ± 0.03	5.73 ± 0.03	7.42 ± 0.04
Guanabenz	7.04 ± 0.15	0.39 ± 0.07	7.02 ± 0.13	0.13 ± 0.04	7.77 ± 0.05	6.56 ± 0.02	6.58 ± 0.08
Guanfacine	6.82 ± 0.06	0.41 ± 0.06	6.79 ± 0.03	0.13 ± 0.02	7.12 ± 0.03	5.86 ± 0.07	5.74 ± 0.11

pA_{50} and intrinsic activity data in dog saphenous vein are from fitting equation (1) to E/[A] curve data. Affinity (pK_A) and relative efficacies were derived from operational model fitting procedures. Affinities (pK_i) at the human recombinant α_2 subtypes (α_{2A}, α_{2B} and α_{2C}) expressed in CHL cells are ligand binding estimates. Values are the mean ± s.e.mean of 3–5 estimates.

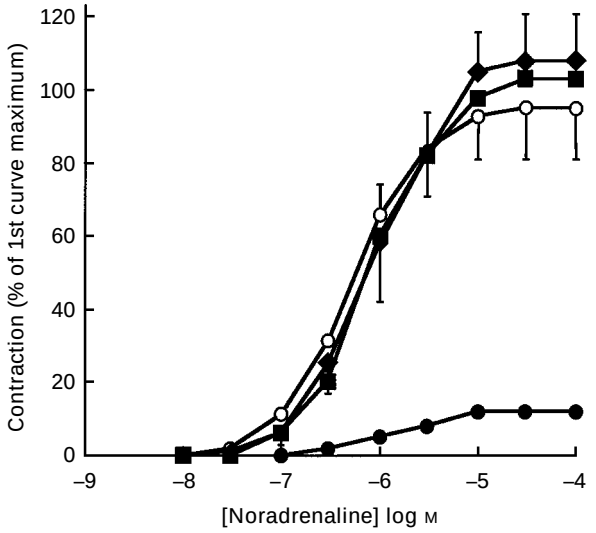

Figure 3 Noradrenaline-induced contraction of dog saphenous vein. α_1-Adrenoceptors were first inactivated by phenoxybenzamine (3.0 μM for 30 min) in the presence of rauwolscine (1.0 μM) followed by washout. Following washout of these antagonists, two concentration-effect curves to noradrenaline were constructed, the second after exposure of tissues to phenoxybenzamine (0.5 μM; ●), rauwolscine (1.0 μM; ◆), rauwolscine and phenoxybenzamine (■), or control (○, no treatment). Each of these treatments was washed out before constructing the second curve to noradrenaline. Data shown are for the second curves only and are expressed as a % of the first curve maximum. Vertical lines show s.e.mean ($n=4$) when larger than the symbols.

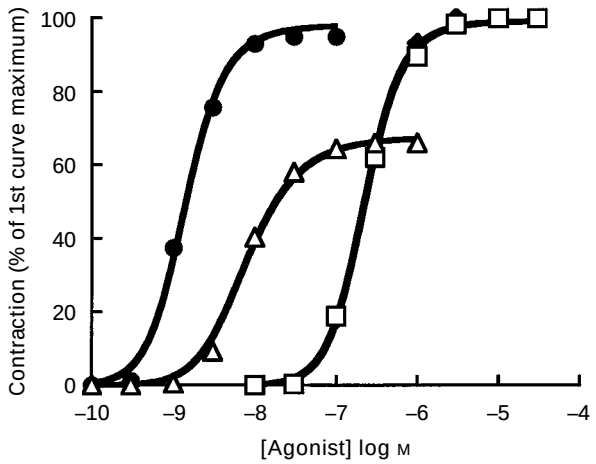

Figure 4 α_2-Adrenoceptor-mediated contraction of dog saphenous vein. α_1-Adrenoceptors were first inactivated by phenoxybenzamine (3.0 μM for 30 min) in the presence of rauwolscine (1.0 μM) followed by washout. The figure shows a representative experiment which provided single estimates of operational model parameters for the agonist A-54741. Concentration-effect curves to noradrenaline were constructed in two tissues (◆, □). After washout, one tissue was exposed to phenoxybenzamine (0.5 μM for 30 min) which was washed out before construction of concentration-effect curves to A-54741 in both tissues (●, △). Open and solid symbols refer to the two different tissues. The lines through the data are the result of fitting simultaneously the operational model of agonism (equation 3) to all of the experimental data which gave an estimate of E_m (100), n (2.07), τ (A-54741, 6.3) and pK_A (8.12). The operational model of agonism was fitted to the E/[A] curve data for noradrenaline, assuming a pK_A of 5.74 obtained from a prior analysis, in order to generate efficacy (τ) estimates of 9.1 and 8.9 for the two tissues. The relative efficacy of A-54741 was therefore 0.70. Note that the noradrenaline curves are almost superimposed.

Comparison of all the ligand affinities at the α_2-adrenoceptor mediating contraction of dog saphenous vein and the cloned human receptors showed the vascular receptor correlated best with the h α_{2A} subtype ($r=0.88$, Figure 6a). Correlations with data obtained for the h α_{2B} (Figure 6b) and h α_{2C} (Figure 6c) subtypes were much lower ($r=0.46$ and 0.54, respectively). A total of 21 compounds were analysed of which only nine had a selectivity greater than 10 fold for any one of the human subtypes over either of the other two. Of these nine compounds, four were agonists in the dog saphenous vein (A-54741, guanfacine, guanabenz and oxymetazoline), the others antagonists (prazosin, spiroxatrine, WB 4101, idazoxan and tolazoline). Comparisons of ligand affinities for the vascular receptor and the human subtypes, by use of the affinities of these nine selective compounds only, gave the following correlation coefficients: h α_{2A} $r=0.82$; h α_{2B} $r=0.24$, h α_{2C} $r=0.04$.

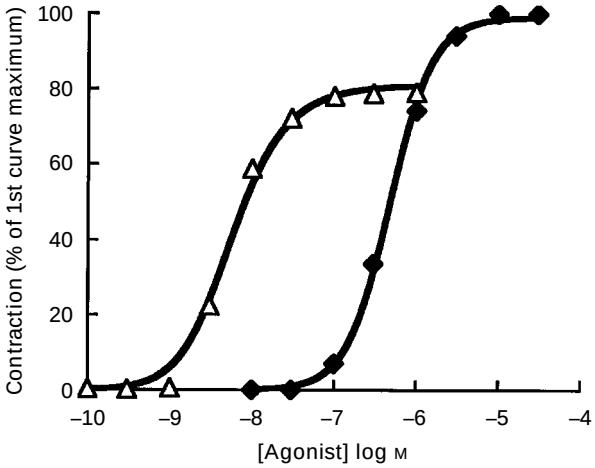

Figure 5 α_2-Adrenoceptor-mediated contraction of dog saphenous vein. α_1-Adrenoceptors were first inactivated by phenoxybenzamine (3.0 μM for 30 min) in the presence of rauwolscine (1.0 μM) followed by washout. The figure shows a representative experiment which provided single estimates of operational model parameters for the agonist dexmedetomide. Concentration-effect curves were constructed to noradrenaline (◆) followed by dexmedetomide (◇) in the same tissue. The lines through the data are the result of fitting simultaneously the operational model of agonism (equation 3) to the dexmedetomidine data and a logistic function (equation 1) to the noradrenaline data. This gave an estimate of E_m (99), n (1.67), τ for dexmedetomidine (2.4) and pK_A for dexmedetomidine (7.93). The operational model of agonism was fitted separately to the concentration-effect curve data for noradrenaline, assuming a pK_A of 5.74 obtained from a prior analysis, in order to generate an efficacy estimate of 7.9. The relative efficacy of dexmedetomidine was therefore 0.30.

Table 3 Antagonist affinity estimates for rauwolscine against various agonists at the α_2-adrenoceptor mediating contraction of the dog saphenous vein

Agonist	*Rauwolscine* pK_B
Noradrenaline	8.66±0.06
A-54741	8.57±0.17
UK-14,304	8.77±0.09
ST-91	8.74±0.13
B-HT 920	8.53±0.11
Dexmedetomidine	8.80±0.18
Clonidine	8.71±0.08
Oxymetazoline	8.24±0.14
Guanabenz	8.54±0.22
Guanfacine	8.53±0.10

pK_B estimates for rauwolscine were calculated from equation (2) where the concentration of rauwolscine was 30 nM. Values are the mean±s.e.mean of 3–4 estimates.

The compounds with greatest discriminatory power were prazosin, spiroxatrine, A-54741 and oxymetazoline which were approximately 100 fold different in affinity at the receptor in dog saphenous vein than at the human α_{2B} receptor. Prazosin, spiroxatrine and A-54741 were approximately 100 fold different in affinity at the receptor in dog saphenous vein than at the human α_{2C} receptor.

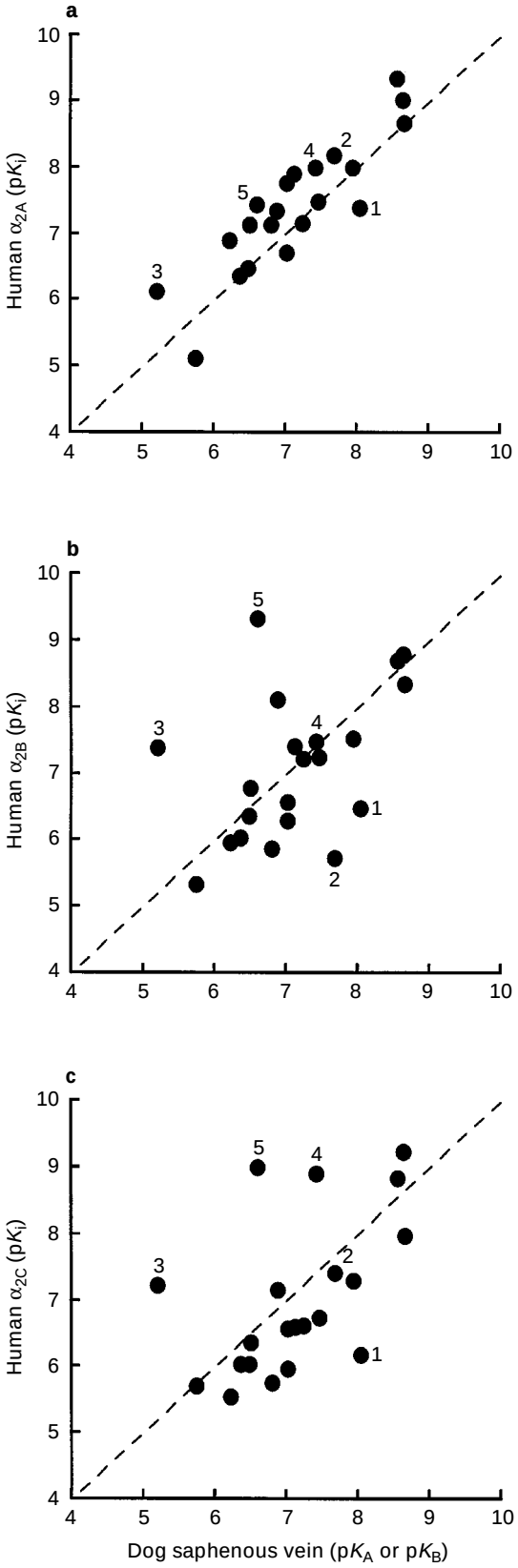

Figure 6 Comparisons of the affinities of compounds for the α_2-adrenoceptor in dog saphenous vein (pK_A or pK_B) and (a) the human α_{2A}, (b) the human α_{2B} and (c) the human α_{2C} receptors expressed in CHL cells (pK_i). Key to figures: (1) A-54741; (2) oxymetazoline; (3) prazosin; (4) WB 4101; (5) spiroxatrine. The broken line is the line of identity between the axes.

Discussion

This study confirms previous findings that α_1- in addition to α_2-adrenoceptors mediate noradrenaline-induced contraction of dog saphenous vein (see Introduction for references), as the effects of this non-selective agonist were antagonized non-competitively by rauwolscine. However, we have also shown that is is possible to eliminate α_1-adrenoceptors and thus isolate the α_2-adrenoceptor population for pharmacological examination. Previous authors have also used phenoxybenzamine to alkylate irreversibly α_1-adrenoceptors in this tissue. Thus Flavahan *et al.* (1984) used 0.5 μM phenoxybenzamine but still found that a significant component of the response to phenylephrine remained. It is not known if this remaining effect was mediated by α_1- or α_2-receptors. We have used a higher concentration (3 μM) as this was the minimum concentration required to abolish phenylephrine responses. To protect α_2-adrenoceptors the tissues were also exposed to rauwolscine. This approach was successful as subsequent responses to noradrenaline were competitively antagonized by rauwolscine and were insensitive to prazosin (3 μM). This protocol was sufficient to protect α_2-receptors completely, as the p[A_{50}] for UK-14,304 (7.64), a selective α_2 agonist, was similar to that found previously in tissues not exposed to phenoxybenzamine (7.77; Alabaster *et al.*, 1985).

Pharmacological characterization of the α_2-adrenoceptor in dog saphenous vein showed that, with respect to the human recombinant α_2-adrenoceptors, it is most similar to the α_{2A} subtype. This conclusion was reached on the basis of a few key ligands which are able to discriminate between the subtypes. Of twenty-one compounds which have been examined, only nine were found to have greater than 10 fold selectivity for one human subtype over the others. Nevertheless, a comparison of ligand affinities of these nine compounds shows clearly the vascular receptor is most similar to the h α_{2A} subtype as the correlation coefficient, r, was 0.82, while the correlations were much weaker at h α_{2B} or h α_{2C} receptors. With respect to agonists, oxymetazoline is the only compound advocated thus far for use in α_2-adrenoceptor characterization studies (Bylund *et al.*, 1994). However, A-54741 may have an advantage in functional studies since it has considerably higher efficacy than oxymetazoline, indeed equal to noradrenaline, and therefore may be useful in systems having a low receptor density or poor coupling efficiency. Other studies in our group have shown that A-54741 is a highly efficacious agonist at each of the human α_2 subtypes when expressed in HEK-293 cells, where agonist effects were measured by the incorporation of GTPγ^{35}S (Jasper *et al.*, 1996).

The population of α_2-adrenoceptors in dog saphenous vein appeared to be homogeneous. This conclusion was based on the effects of the partial agonists oxymetazoline, guanabenz and guanfacine, each of which had simple monophasic concentration-effect curves. These ligands have an affinity 10 fold higher at the human α_{2A} receptor than at the other receptor subtypes while they have similar efficacy at each subtype expressed in HEK-293 cells (Jasper *et al.*, 1996). Thus, if more than one α_2-adrenoceptor was present in dog saphenous vein, we might have observed biphasic curves to these partial agonists as their effects mediated by two receptor subtypes might be expected to be additive. This was never observed.

With respect to antagonist affinities for the human α_2 clones, we have confirmed the ability of WB 4101 and prazosin to discriminate between the subtypes. WB 4101 is useful for distinguishing α_{2C} from α_{2A} or α_{2B}, where it has similar affinity, though lower. Prazosin can distinguish α_{2A} from α_{2B} or α_{2C}, where it has similar affinity, though higher. When examined in the dog saphenous vein the low affinity estimates for each

compound clearly reinforce the likeness of this receptor to the α_{2A} subtype, as suggested by the agonist affinity estimates. Spiroxatrine is another key ligand in this study having an affinity 300 fold lower in dog saphenous vein than either the human α_{2B} or α_{2C} receptors. In binding studies it also has appreciably lower affinity (30 – 100 fold) for the α_{2A} over the other subtypes. This compound should be useful in other α-adrenoceptor characterization studies and perhaps should be added to the list of pharmacological tools recommended for classification studies (Bylund *et al*., 1994). However, this compound needs to be used with caution as it has little selectivity over some other monoamine receptors, notably the 5-HT_{1A} receptor (Hoyer, 1988).

Precise affinity estimates for prazosin at the α_2-adrenoceptor in dog saphenous vein have not been made previously, although one study indicated an affinity of less than 6 μM (Alabaster *et al*., 1985). Our estimate for prazosin (pK_B 5.19) was lower (almost 10 fold) in the dog saphenous vein than our estimate for the human α_{2A} subtype (6.12), but is consistent with the observation that a lower concentration (3 μM) was without effect on noradrenaline responses. The binding affinity value for prazosin of 6.12 is higher than that found previously, 5.69 (Link *et al*., 1992). With respect to this latter value, the low affinity in dog saphenous vein (5.19) does not appear to be so markedly different. Furthermore, the affinity of prazosin at the human α_{2A} adrenoceptor expressed in CHL cells, i.e. the same cell as used in our binding studies, is less than 10 μM when examined with the cytosensor microphysiometer, which measures extracellular acidification (unpublished observations). The medium in this assay was DMEM at 37°C which is quite different from the binding assay medium which was Tris at 25°C. These data suggest that antagonist affinities in the dog saphenous vein are generally lower than in the binding studies due to methodological differences which include assay buffer and temperature. Such differences are known to influence receptor binding affinity estimates, including those for the α_2-receptor (Bylund & Ray-Prenger, 1989). In this latter study affinity estimates for prazosin (and several other antagonists) at the α_{2A} receptor in HT29 cells were considerably lower in functional experiments (pK_B = 5.77 with DMEM at 37°C) than in binding studies (pK_i = 6.47 with Tris at 23°C).

Isolation of the α_2-adrenoceptors mediating contraction of the rabbit saphenous vein has been achieved by use of the same procedures as described in the present study by Daly *et al*. (1988). As with many studies, pharmacological examination of α_2-adrenoceptors in rabbit saphenous vein pre-dates the recognition of several subtypes of this receptor. On the basis that prazosin (pK_B = 6.58) and spiroxatrine (pK_B = 7.18) have low affinities for the α_2-adrenoceptor, our provisional experiments in this tissue suggest it is an α_{2A}-adrenoceptor (Luong & MacLennan, unpublished observations). It will be of interest to determine which α_2-adrenoceptor subtypes mediate contraction of other vascular tissues. Preliminary data suggest α_{2A}-adrenoceptors mediate contraction of various porcine tissues (Blaylock & Wilson, 1995), while α_{2C}-adrenoceptors mediate contraction of the rat caudal artery (Craig *et al*., 1995) and human saphenous vein (Gavin & Docherty, 1996).

Emerging evidence suggests that many of the physiological actions of α_2-agonists are mediated by the α_{2A} subtype. Thus, agents such as clonidine, dexmedetomide and UK-14,304 are effective antinociceptive agents (Yaksh, 1985; Eisenach & Tong, 1991; Eisanach, 1994) although their clinical use is limited by hypotension and sedation (Brown, 1989; Eisenach & Tong, 1991; Ruffolo *et al*., 1993; Eisanach, 1994). The conclusion that α_{2A}-adrenoceptors are involved in each response is based on both traditional pharmacological and molecular biological approaches. Thus, Millan *et al*. (1994) used both agonists and antagonists having some subtype selectivity to show that the α_{2A}-adrenoceptor mediated the antinociceptive and motor-sedative effects of α_2 agonists. By use of genetically altered mice in which the α_{2A}-adrenoceptor was mutated (MacMillan *et al*., 1996) or α_{2B} and α_{2C}-adrenoceptor 'knocked-out' (Link *et al*., 1996), it has been shown that the prolonged hypotensive response following intra-arterial injection of α_2 agonists is mediated by the α_{2A} subtype. From these studies it appears there is little potential to develop α_2 agonists as analgesic agents which lack sedative and haemodynamic effects.

However, from a theoretical standpoint it may still be possible to develop α_{2A} agonists as analgesic agents without significant side-effects if their efficacy is lower at the receptors mediating sedation and hypotension. There is tentative evidence to support this notion. Oxymetazoline is an effective analgesic agent following intrathecal administration in rats (Sherman *et al*., 1987), but has less effect on blood pressure than clonidine when given either systemically or centrally (Strucyker Boudier *et al*., 1974). In our studies in dog saphenous vein, clonidine had a higher efficacy than oxymetazoline at α_{2A} receptors supporting the notion that low efficacy α_{2A} agonists may be analgesic but without unwanted side-effects. A caveat to this proposal is that oxymetazoline has little selectivity over α_1-adrenoceptors which also mediate spinal analgesia (Yaksh, 1985).

In conclusion, this study has shown that it is possible to isolate the α_2-adrenoceptor in dog saphenous vein by irreversible alkylation of the α_1-adrenoceptor population with phenoxybenzamine in the presence of rauwolscine to protect α_2-adrenoceptors. This finding mirrors similar studies in rabbit saphenous vein (Daly *et al*., 1988) and thus allows the use of non-selective agonists, e.g. noradrenaline, without the inclusion of 'selective' α_1 antagonists. Affinity estimates for agonists and antagonists suggests the α_2-adrenoceptor in dog saphenous vein belongs to the α_{2A}-adrenoceptor family.

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