

RESEARCH PAPER

Investigation of the distribution and function of α -adrenoceptors in the sheep isolated internal anal sphincter

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BACKGROUND AND PURPOSE

We have investigated the distribution of α -adrenoceptors in sheep internal anal sphincter (IAS), as a model for the human tissue, and evaluated various imidazoline derivatives for potential treatment of faecal incontinence.

EXPERIMENTAL APPROACH

Saturation and competition binding with ³H-prazosin and ³H-RX821002 were used to confirm the presence and density of α -adrenoceptors in sheep IAS, and the affinity of imidazoline compounds at these receptors. A combination of *in vitro* receptor autoradiography and immunohistochemistry was used to investigate the regional distribution of binding sites. Contractile activity of imidazoline-based compounds on sheep IAS was assessed by isometric tension recording.

KEY RESULTS

Saturation binding confirmed the presence of both α_1 - and α_2 -adrenoceptors, and subsequent characterization with sub-type-selective agents, identified them as α_{1A} - and α_{2D} -adrenoceptor sub-types. Autoradiographic studies with ³H-prazosin showed a positive association of α_1 -adrenoceptors with immunohistochemically identified smooth muscle fibres. Anti- α_1 -adrenoceptor immunohistochemistry revealed similar distributions of the receptor in sheep and human IAS. The imidazoline compounds caused concentration-dependent contractions of the anal sphincter, but the maximum responses were less than those elicited by L-erythro-methoxamine, a standard non-imidazoline α_1 -adrenoceptor agonist. Prazosin (selective α_1 -adrenoceptor antagonist) significantly reduced the magnitude of contraction to L-erythro-methoxamine at the highest concentration used. Both prazosin and RX811059 (a selective α_2 -adrenoceptor antagonist) reduced the potency (pEC₅₀) of clonidine.

CONCLUSIONS AND IMPLICATIONS

This study shows that both α_1 - and α_2 -adrenoceptors are expressed in the sheep IAS, and contribute (perhaps synergistically) to contractions elicited by various imidazoline derivatives. These agents may prove useful in the treatment of faecal incontinence.

Abbreviation

IAS, internal anal sphincter

Introduction

Faecal incontinence is a common condition which, depending on criteria applied, affects up to 2% of the population (Perry *et al.*, 2002) and can have a devastating effect on quality of life. Clinical management of the condition has historically relied on non-pharmacological approaches, although there is increasing interest in modulating the contractile state of the anal sphincter smooth muscle to alleviate symptoms (Tuteja and Rao, 2004).

It has long been recognized that sympathetic activation of α -adrenoceptors contributes to continence in man (Parks *et al.*, 1969; Frenckner and Ihre, 1976). In 1975, Gutierrez and Shah reported that intravenous infusion or oral administration of α -adrenoceptor antagonists caused a reduction in anal sphincter pressure (Gutierrez and Shah, 1975). Similarly, the selective α_1 -adrenoceptor antagonist indoramin caused a reduction in anal sphincter pressure in patients suffering from anal fissures (Pitt *et al.*, 2001). These observations are consistent with reports on isolated segments of the human internal sphincter that α_1 -adrenoceptors mediate constriction (Speakman *et al.*, 1990; Glavind *et al.*, 1997). Several recent clinical trials have been conducted to examine the effect of α -adrenoceptor agonists on anal sphincter pressure in both healthy volunteers and patients (Carapeti *et al.*, 1999; 2000; Cheetham *et al.*, 2001; Badvie and Andreyev, 2005; Park *et al.*, 2007). Anal (topical) application of the selective α_1 -adrenoceptor agonist phenylephrine was found to cause a significant increase in resting anal pressure in healthy volunteers (Carapeti *et al.*, 1999). Subsequent use of this agonist in patients with faecal incontinence noted a significant increase in maximum resting pressures, but only when phenylephrine was employed at higher concentrations (10% w/w) than in volunteers and which may account for the incidence of local side effects (Carapeti *et al.*, 2000). Later trials with patients suffering with passive faecal incontinence (Cheetham *et al.*, 2001), radiation-induced faecal incontinence (Badvie and Andreyev, 2005) and incontinence resulting from low anterior resection (Park *et al.*, 2007) produced mixed results. Based on incontinence scoring, radiation-induced faecal incontinence was improved by 10–20% (w/w) phenylephrine (Badvie and Andreyev, 2005), while a 30% (w/w) gel was insufficient to improve the continence scores or resting pressure of low anterior resection patients (Park *et al.*, 2007). Conversely, in patients suffering with passive faecal incontinence, 30% (w/w) phenylephrine gel was sufficient to increase resting pressure (Cheetham *et al.*, 2001).

Mortensen and colleagues reported that an isomer of methoxamine, L-erythro-methoxamine, was four times more potent than phenylephrine as a constrictor agent in the porcine isolated internal anal sphincter (IAS) *in vitro* (Jones *et al.*, 2003). Based on these findings, trials have been conducted in human volunteers and patients. Intra-anal and rectal application of L-erythro-methoxamine gel in healthy volunteers demonstrated rapid and sustained increase in resting pressure from a concentration as low as 1% w/w (Nisar *et al.*, 2005). Furthermore, when 1% gel preparations were applied to patients suffering with faecal incontinence, resting pressures similarly increased rapidly and gave a sustained response (Nisar *et al.*, 2007). However, application of 3% L-erythro-methoxamine in an immediate release formulation was associated with an increased systemic absorption and side effects of hypertension and bradycardia in some patients.

The potential role of α_2 -adrenoceptors in faecal continence has not been explored in man, but work in the anaesthetized opossum suggests that the imidazoline derivative clonidine can activate the myenteric neurones responsible for inhibition of the recto-anal inhibitory reflex (Yamato and Rattan, 1990). Although there are no comparable studies in man, α_2 -adrenoceptors are known to be present in the duodenum and proximal colon (Zhang *et al.*, 1992; Valet *et al.*, 1993), and administration of selective α_2 -adrenoceptor agonists (e.g. clonidine) is associated with an increase in colonic and rectal compliance (Viramontes *et al.*, 2001; Camilleri *et al.*, 2003). Interestingly, α_2 -adrenoceptors have been reported to play a role in noradrenaline-mediated constriction of the pig isolated IAS, but only at high concentrations, suggesting that α_1 -adrenoceptors are predominant in contractile responses to α -adrenoceptor agonists (Mills *et al.*, 2008).

As changes in modern surgical practice have led to a significant increase in sphincter-preserving surgery which allows the post-operative patient to maintain voluntary continence, access to human tissue is limited. With this in mind, the current study has utilized a sheep model of the IAS to characterize the α -adrenoceptors in sheep IAS, and used this model to investigate potential pharmacological tools for the therapeutic treatment of faecal incontinence. The aim of the current study was twofold. First, to establish the presence and where possible the distribution of α -adrenoceptors in sheep and human IAS; and second, to investigate whether a variety of imidazoline compounds compared to known α_1 -adrenoceptor agonists exhibit functional effects at α -adrenoceptors expressed in sheep IAS.

Methods

Human IAS tissue was collected in accordance with ethical approval obtained from the University of Nottingham Ethics committee (05/Q2403/171) from patients undergoing a proctectomy either as part of an abdominoperineal resection or completion procedure as a consequence of suffering from ulcerative colitis. Care was taken to ensure that none of the patients had active disease affecting the anal canal at the time of operation.

Sheep anus, containing IAS and rectum, were obtained from a local abattoir and transported to the lab on ice within 120 min and stored at 4°C. Within a further 2 h, the anal canal was divided along its ventral longitudinal axis and pinned to a corkboard with the mucosa uppermost. The mucosa was reflected at the dentate line to expose the underlying white circular muscle fibres of the IAS. Further dissection from the underlying external sphincter enabled a thin strip of circular internal sphincter to be prepared. The IAS was dissected and used for the following experiments.

Radioligand binding experiments

Sheep IAS was dissected from anal tissue on the day of collection and frozen at -20°C until required. When required, the defrosted tissue was crudely chopped and homogenized using an Omni macro homogenizer (Omni International, Kennesaw, GA, USA) in a minimal volume of ice-cold buffer (50 mM, Tris-HCl pH 7.6). Tissue typically required three 30 s homogenizations at maximum speed to achieve complete tissue disruption. Ice-cold Tris buffer was used to increase the homogenate volume to 10 times original tissue wet weight before centrifugation at 1500× *g* for 10 min at 4°C. The supernatant was passed through a gauze filter then centrifuged at 30 000× *g* for 30 min at 4°C. The resulting pellet was then washed and resuspended in 2 volumes tissue wet weight of ice-cold Tris buffer. Following radioligand binding, fractions of membrane preparations were retained and stored at -20°C for estimation of protein concentration by Lowry assay (Lowry *et al.*, 1951). In all cases, membrane preparations were made from frozen tissue and used for experiments on the same day, because preliminary experiments had previously revealed that if frozen membrane preparations were used, they failed to reveal significant binding of the ligands.

Saturation binding with [³H]-prazosin (selective antagonist for α_1 -adrenoceptors) was performed in a 500 μ L reaction volume consisting of 200 μ L membrane preparation; 200 μ L 50 mM Tris-HCl, pH 7.6; 50 μ L of 50 mM TE buffer (50 mM Tris, 1 mM EDTA,

pH 7.4) or 100 μ M noradrenaline (used to define non-specific binding) and 50 μ L [³H]-prazosin using eight doubling concentrations in a range of 0.04–5 nM (final concentration). Reactions were initiated by the addition of membrane preparation, and after thorough mixing, were incubated at 25°C for 30 min. Reactions were terminated by filtration under vacuum using a Brandel cell harvester (Semat, Herts, UK). Filters were washed three times with ice-cold TE buffer to remove unbound radioligand, and remaining bound ligand was evaluated by scintillation counting. All experiments were performed in triplicate. Saturation studies with [³H]-RX821002 (a selective antagonist for α_2 -adrenoceptors) were performed as described above, but rauwolscine (100 μ M final concentration) was used instead of noradrenaline, to define non-specific binding. Binding reactions were incubated for 60 min rather than 30 min, and the concentration range for [³H]-RX821002 was 0.08–10 nM. These conditions have been used in previous studies using these radioligands (Wright *et al.*, 1995). Competition studies using [³H]-prazosin and [³H]-RX821002 were performed in a 500 μ L volume using 200 μ L membrane preparation, 200 μ L 50 mM Tris-HCl, 50 μ L [³H]-antagonist at a fixed concentration and 50 μ L of competitor at increasing concentrations. The relative affinities of seven different imidazoline derivatives at α_1 - and α_2 -adrenoceptors were compared to L-erythro-methoxamine and noradrenaline using competition binding.

Immunohistochemistry

Strips of sheep and human circular IAS were embedded in OCT (BDH, Poole, Dorset, UK), and sections (10 μ m thick) were cut in a cryostat at approximately -20°C and thaw-mounted on poly-lysine-coated slides. Immunohistochemical staining of sections proceeded according to instructions for Vectastain ABC-AP kit (Vector laboratories Inc., Burlingame, CA, USA). Briefly, sections were allowed to equilibrate to room temperature prior to fixation in cold acetone at -20°C for 20 min. After residual fixative had evaporated, slides were incubated in phosphate-buffered saline (PBS) containing 10% normal horse serum and incubated at room temperature for 10 min in humid conditions to block non-specific binding. All subsequent incubations were also carried out at room temperature in humid conditions. After removing the blocking solution, the slides were incubated in PBS (negative control) or PBS containing one of the following antibodies for 30 min: anti- α -smooth muscle actin (1:200 dilution; to identify smooth muscle cells), anti-CD31 (1:100; endothelial cell marker), anti-NF200 (1:200; for identification of neurones), anti-tyrosine

hydroxylase (1:250; adrenergic neurone marker) or anti- α_{1A} -adrenoceptor (1:400; Genway Biotech Inc., San Diego, CA, USA). The slides were washed three times in PBS before incubating with biotinylated universal antibody in blocking solution (as per manufacturer's instructions) for 10 min before washing three times in PBS again and incubating with the ABC-AP reagent for 5 min. The slides were once again washed three times in PBS before development with alkaline phosphatase substrate solution (in 200 mM Tris buffer, pH 8.2). Reactions were terminated by immersion and rinsing in water.

The slides were subsequently counterstained with haematoxylin for 15 s, rinsed in running tap water, dehydrated in increasing concentrations of methanol, clearing in Histoclear (National Diagnostics, Hull, UK) and coverslipped using DPX mountant (BDH). A minimum of three tissues (two non-consecutive sections from each tissue) were evaluated for each antibody used.

Autoradiography

Sections of sheep IAS (10 μ m) were thaw-mounted on poly-lysine-coated slides as described for the immunohistochemistry, and pre-incubated in 50 mM Tris-HCl, pH 7.6, for 10 min at 4°C to remove endogenous ligand. The sections were then transferred to 50 mM Tris-HCl, pH 7.6, containing 5 nM 3 H-prazosin, then incubated for 1 h at 4°C. Non-specific binding was evaluated by incubating adjacent sections in the presence of 100 μ M noradrenaline. The binding reaction was terminated by washing the slides twice in TE buffer (4°C) before rinsing in distilled water (4°C). The sections were dried in a continuous stream of cold air, followed by air-drying overnight at room temperature (~22°C). A parallel series of experiments were conducted using [3 H]-RX821002 (2–25 nM) where non-specific binding was evaluated in the presence of rauwolscine (100–250 μ M).

For low-resolution autoradiography, slide-mounted sections were exposed to 3 H Hyperfilm (Amersham Biosciences, Little Chalfont, UK) in X-ray cassettes for up to 9 weeks at 4°C. Films were developed using Kodak D19 and fixed using Hypam (Ilford, Moberly, Cheshire, UK). For high-resolution autoradiography, sections were dipped in molten nuclear emulsion (K2, Ilford) following air-drying. The slides were stored in light-proof boxes containing dessicant at 4°C and processed once low-resolution (film) autoradiography provided evidence of binding to tissue sections. Following development, the sections were counterstained with haematoxylin and eosin, visualized using an Olympus BX50 microscope (Olympus Corporation, Tokyo, Japan) and photographed where appropriate

on an AxioCam digital camera (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) with images stored on a KS300 imaging system (Imaging Associates, Bicester, UK).

Contractile studies

Following collections of the sheep anal tissue (as described above), a thin strip of circular internal sphincter measuring approximately 50 mM long, 5 mM wide and 2 mM thick was removed and stored overnight in Krebs–Henseleit solution at 4°C. The following day, tissue strips were allowed to warm up to room temperature. Additional connective tissue was removed by sharp dissection from the specimen, and up to a maximum of four strips of internal sphincter tissue (approximately 10 \times 2 \times 2 mM) containing visible parallel muscle fibres were prepared and used for experiments. Silk ligatures were tied to each end of the strips, and the lower end was attached to a perspex holder between two parallel platinum wire electrodes (although electrical field stimulation was not performed in this series of experiments). The upper end was secured to a Grass FT-03 isometric force transducer (Grass Technologies, West Warwick, RI, USA) ensuring some laxity in the suspended tissue which was bathed for approximately 30 min in 20 mL organ baths containing Krebs–Henseleit solution (pH 7.4), gassed (95% O₂/5% CO₂) at 37°C. The transducer was connected to a MacLab Bridge amplifier (AD Instruments, Chalgrove, UK) and linked via a four-channel MacLab unit to a Macintosh LC II computer running Chart 3.5.

The strips were initially placed under 19.62 mN tension, which has previously been identified as an optimal stretch tension necessary for the development of spontaneous myogenic tone in sheep isolated IAS (Munday *et al.*, 2000). Once stable myogenic tone had been reached, cumulative concentration curves were constructed by half-logarithm increments using a total of six imidazoline compounds, which were compared to responses to the α -adrenoceptor agonists L-erythro-methoxamine and noradrenaline. Only one agonist was used per strip. A minimum of 5 min was allowed between the addition of individual concentrations, and subsequent additions were made only after the previous response had reached equilibrium. In addition, the responses of L-erythro-methoxamine and clonidine were examined in the presence or absence of 100 nM prazosin and 100 nM RX-811059, antagonists selective for α_1 - and α_2 -adrenoceptors, respectively (McGrath *et al.*, 1989; Mallard *et al.*, 1992). The antagonists were allowed to equilibrate for 30 min prior to construction of the concentration curve.

Data analysis

For saturation binding experiments, K_d and B_{max} values were calculated using GraphPad Prism (GraphPad, San Diego, CA, USA) and represented as the mean \pm SEM for triplicate experiments. Competition binding curves were similarly constructed, and the mean pK_i values \pm SEM for triplicate experiments calculated. In contractile studies, changes in tension with the addition of drugs were determined as the maximal increase or decrease in tone, and are expressed as a percentage of the initial stable myogenic tone. Individual concentration–response curves were constructed for each tissue, and the negative logarithm of the concentration required to achieve 50% of the maximum response (pEC_{50}) and the maximum response relative to myogenic tone (R_{max}) were calculated using GraphPad Prism. Differences between mean pEC_{50} and R_{max} values for L-erythromethoxamine and clonidine in the presence or absence of antagonists were compared using a one-way ANOVA with Dunnett's *post hoc* test and considered significant if $P < 0.05$.

Materials

The composition of the modified Krebs–Henseleit saline was (in mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2, glucose 11.1. TE buffer was (in mM): Tris 50, EDTA 1, pH 7.4. The drugs used were obtained from Sigma-Aldrich (Poole, UK) with the exception of: prazosin hydrochloride (Pfizer, Sandwich, UK); 2-(2-ethoxy-1,4-benzodioxan-2-yl)-2-imidazoline (RX-811059, Reckitts and Coleman, Hull, UK), L-erythromethoxamine (Norgine, Hengoed, UK), rauwolscine (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), moxonidine (Tocris Bioscience, Bristol, UK), xylometazoline (Chemos GmbH, Regenstauf, Germany), 5-methyl-3-[3-[3-[4-[2-(2,2,2-trifluoroethoxy)phenyl]-1-piperazinyl]propyl]-2,4-(1H,3H)-pyrimidinedione hydrochloride (RS100329, Tocris Bioscience), 8-[2-[4-(methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride (BMY7378, Tocris Bioscience), 2-[(4,5-dihydro-1H-imidazol-2-yl)methyl]-2,3-dihydro-1-methyl-1H-isoindole malate (BRL44408, Tocris Bioscience).

[3H]-RX821002 (2-methoxyidazoxan) and [3H]-prazosin were obtained from GE Healthcare Ltd (Little Chalfont, UK). Prazosin (1 mM) was dissolved in 0.1 M lactic acid. All further dilutions were made in distilled water. The concentration of the vehicle never exceeded 0.3% v/v. All drug and molecular target nomenclature follows Alexander *et al.* (2009).

Results

Radioligand binding

Saturation binding. Figure 1 shows that [3H]-prazosin and [3H]-RX-821002 were able to bind to membranes of the sheep IAS in a concentration-dependent manner that was inhibited by 100 μ M noradrenaline and 100 μ M rauwolscine respectively. At the half-maximally effective concentration (K_d), approximately 87 and 90% of the binding of [3H]-prazosin and [3H]-RX-821002, respectively, was inhibited. [3H]-prazosin was calculated to have a K_d value of 0.58 ± 0.07 nM ($n = 3$) and identified sites at density of 222 ± 4 fmol·mg $^{-1}$ protein ($n = 3$), while [3H]-RX821002 was slightly less potent ($K_d = 0.7 \pm 0.14$ nM, $n = 3$) and labelled approximately one-third the number of sites (72 ± 7 fmol·mg $^{-1}$ protein, $n = 3$). These findings indicate that the sheep isolated IAS possesses both α_1 - and α_2 -adrenoceptor binding sites.

Discrimination of α -adrenoceptor sub-type using radioligand binding. Competition binding analysis was subsequently used to investigate which sub-type of α -adrenoceptor was identified by each ligand in this tissue. The pK_i values for the competing agents generated from these experiments are summarized in Table 1. With the exception of RS100329, all of the Hill slope values were close to unity (>0.8). In each instance, the competing agents displaced $>90\%$ of the specific binding of the ligands (data not shown). RS100329 (an α_{1A} -adrenoceptor-selective antagonist; Williams *et al.*, 1999), 5-methyl-urapidil (discriminates α_{1A} from α_{1B}/α_{1D} -adrenoceptor sub-types; Ford *et al.*, 1997) and BMY7378 (α_{1D} -adrenoceptor sub-type-selective antagonist; Piascik *et al.*, 1995) were used to investigate the sub-type of α_1 -adrenoceptor present in sheep IAS. The data summarized in Table 1 indicate that the rank order of affinity for these agents is 5-methylurapidil \geq RS100329 \gg BMY7378, suggesting the presence of the α_{1A} -adrenoceptor sub-type.

Four agents were used to provide information on the α_2 -adrenoceptor sub-type present. BRL44408 was used as a selective α_{2A}/α_{2D} -adrenoceptor antagonist (Wikberg-Matsson *et al.*, 1995); prazosin, although non-selective at α_1 -adrenoceptor sub-types, can be employed to discriminate between α_{2C} -adrenoceptors (for which it has high affinity) and α_{2A}/α_{2D} -adrenoceptors (McGrath *et al.*, 1989); phentolamine and rauwolscine were also used, as the relative affinities of these two agents can be used to discriminate between α_{2A} - and α_{2D} -adrenoceptor sub-types. If the affinity of rauwolscine is greater than phentolamine, it is indicative of the presence

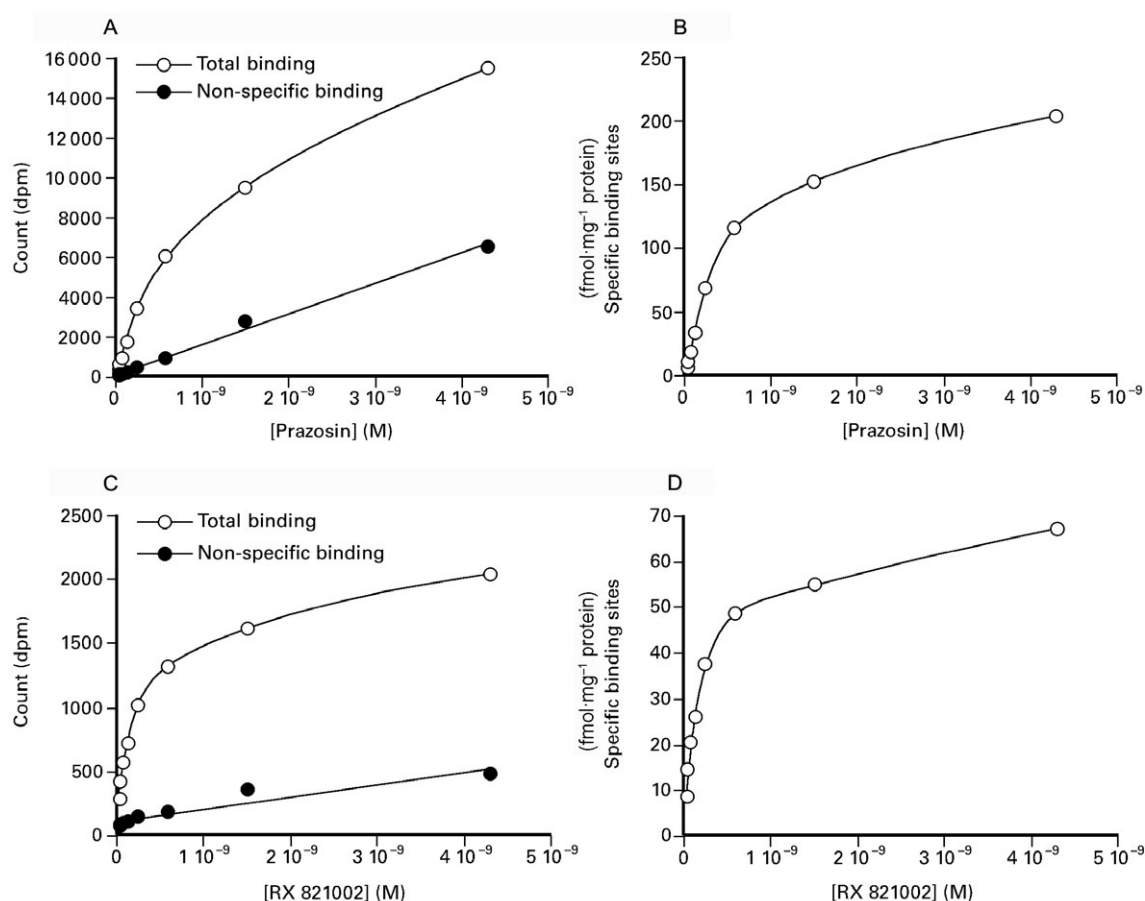


Figure 1

Saturation binding data for [³H]-prazosin (A,B) and [³H]-RX-821002 (C,D). Representative data showing total and non-specific binding from a single experiment are shown in (A) for [³H]-prazosin and (C) for [³H]-RX821002. (B) and (D) show representative specific binding curves from a single experiment using [³H]-prazosin and [³H]-RX821002 respectively.

Table 1

Mean pK_i values (±SEM of three experiments) for sub-type-selective inhibitors at α₁- ([³H]-prazosin) or α₂-adrenoceptors ([³H]-RX821002) on sheep IAS

	pK _i (Hill slope) [³ H]-RX-821002	pK _i (Hill slope) [³ H]-prazosin
RS100329	n/d	9.14 ± 0.11 (−0.68 ± 0.06)
5-Methyl-urapidil	n/d	9.52 ± 0.03 (−1.27 ± 0.08)
BM7378	n/d	6.61 ± 0.04 (−0.98 ± 0.09)
BRL44408	8.85 ± 0.13 (−0.81 ± 0.06)	n/d
Prazosin	5.32 ± 0.13 (−1.07 ± 0.09)	n/d
Rauwolscline	7.91 ± 0.12 (−0.83 ± 0.08)	n/d
Phentolamine	8.66 ± 0.08 (−0.84 ± 0.07)	n/d

n/d, the experiment was not done.

of an α_{2A}-adrenoceptor; if the affinity of phentolamine is greater than rauwolscline, then an α_{2D}-adrenoceptor is implied (Trendelenburg *et al.*, 1996; Naselsky *et al.*, 2001). The data summarized in

Table 1 indicate that the rank order of affinity for these agents is BRL44408 > phentolamine > rauwolscline > prazosin, which is consistent with the presence of α_{2D}-adrenoceptors.

Table 2

Mean pK_i values (\pm SEM of three experiments) for imidazoline compounds at both α_1 - ($[^3H]$ -prazosin) and α_2 -adrenoceptor ($[^3H]$ -RX-821002) on the sheep IAS

	pK_i (Hill slope) $[^3H]$ -RX-821002	pK_i (Hill Slope) $[^3H]$ -prazosin	α_2/α_1 Ratio
Naphazoline	7.99 ± 0.01 (-0.79 ± 0.01)	6.84 ± 0.09 (-1.04 ± 0.08)	14
Clonidine	7.55 ± 0.09 (-0.91 ± 0.07)	6.39 ± 0.10 (-0.95 ± 0.02)	14
Oxymetazoline	7.23 ± 0.09 (-0.94 ± 0.02)	7.67 ± 0.13 (-0.87 ± 0.05)	0.36
Xylometazoline	6.92 ± 0.05 (-1.07 ± 0.10)	7.10 ± 0.15 (-0.82 ± 0.04)	0.6
Tizanidine	6.75 ± 0.03 (-0.82 ± 0.04)	5.57 ± 0.05 (-0.76 ± 0.03)	15
Rilmenidine	6.45 ± 0.04 (-0.87 ± 0.05)	5.33 ± 0.04 (-0.89 ± 0.05)	13
Moxonidine	5.98 ± 0.06 (-0.75 ± 0.05)	4.62 ± 0.02 (-0.88 ± 0.02)	23
Noradrenaline	5.62 ± 0.08 (-0.90 ± 0.14)	5.47 ± 0.06 (-0.83 ± 0.02)	1.4
L-Erythro-methoxamine	4.00 ± 0.03 (-0.99 ± 0.08)	5.40 ± 0.07 (0.86 ± 0.04)	0.04

Competition binding with agonists. All of the putative agonists examined using competition binding showed concentration-dependent displacement of both radioligands and, with the exception of tizanidine ($[^3H]$ -prazosin) and moxonidine ($[^3H]$ -RX-821002), the Hill slope values were close to unity (>0.8). The pK_i values for these compounds are included in Table 2. The affinity of the imidazoline compounds against $[^3H]$ -prazosin varied a 1000-fold, with oxymetazoline and moxonidine the most and least potent respectively. Against $[^3H]$ -RX821002 binding sites, a 100-fold range in potency was observed between the imidazoline derivatives (Table 2).

The ratio of mean $[^3H]$ -RX821002 and $[^3H]$ -prazosin pK_i values (α_2/α_1 adrenoceptor binding) was used to establish receptor selectivity of agonists. Based on this analysis, a ratio with a value higher than 1 indicates an α_2 -selective agonist, whereas a value of less than 1 suggests an α_1 -adrenoceptor selective agent. The majority of the agonists exhibited 3- to 30-fold selectivity for α_2 -adrenoceptor binding sites over α_1 -adrenoceptor binding sites, with moxonidine exhibiting the greatest selectivity. In contrast, L-erythro-methoxamine possessed 25-fold greater selectivity for α_1 -adrenoceptor binding sites (Table 2). Noradrenaline, xylometazoline and oxymetazoline did not distinguish between the α_1 - and α_2 -adrenoceptor binding sites.

Immunohistochemistry

Sections of sheep IAS that had been exposed to the anti- α -smooth muscle actin antibody and counterstained with haematoxylin showed a pronounced positive staining (red colouration) throughout the tissue (Figure 2A,B). The staining was most intense in bundles surrounded by a loose network of pale pink-stained tissue.

Anti-CD31 antibodies were used as a marker for the presence of endothelial tissue, and therefore indicative of the vasculature associated with the tissue sections of sheep IAS (Figure 2C,D). Unlike the smooth muscle actin, this antibody stained discrete areas of the tissue sections that appear to correspond to the areas of pale pink immunostaining surrounding the bundles of α -smooth muscle actin-rich tissue.

Two antibodies were used to identify neuronal-derived tissue. As shown in Figure 2E,F, anti-NF200 was found to have a distribution of staining largely similar to anti-CD31 antibodies in the areas of weak α -smooth muscle actin staining. However, closer examination of the tissue also revealed a weaker distribution of staining with anti-NF200 that also corresponds to the α -smooth muscle actin-rich bundles of tissue. In addition to NF200, an anti-tyrosine hydroxylase antibody was also used to identify adrenergic nerves. The profile of intense staining was similar to that observed using the NF200 antibody, where positive staining was observed in both the α -smooth muscle actin-rich tissue and surrounding tissue which was weakly stained by the α -smooth muscle actin antibody (Figure 2G,H). In the absence of primary antibody, the slides revealed a low level of staining (pale pink) that corresponded to the α -smooth muscle actin-poor regions of the tissue (Figure 2I,J).

Based on the results of α_1 -adrenoceptor sub-type competition binding, sections of sheep and human IAS were also incubated with an α_{1A} -adrenoceptor antibody. Results using this antibody can be seen in Figure 3 with their corresponding negative controls. Overall, both tissues showed a similar distribution of positive staining: with a diffuse pink positive staining over the smooth muscle bundles, as well as discrete areas of dense positive staining which could

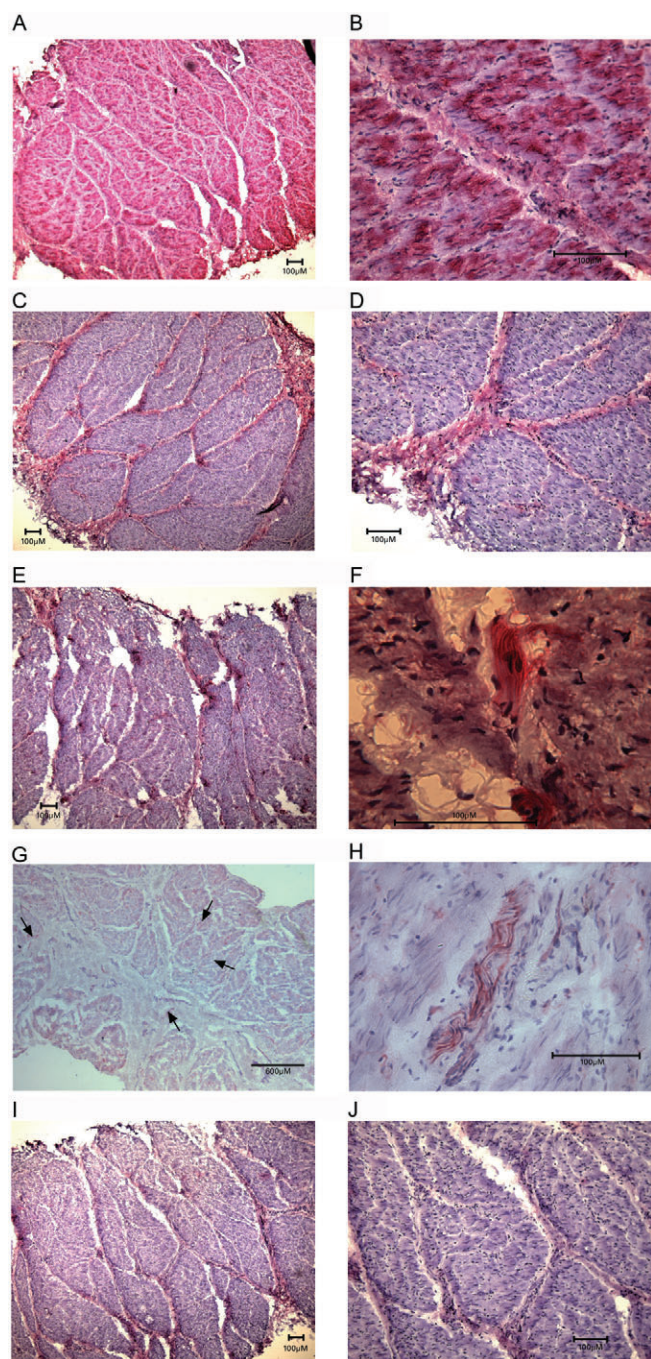


Figure 2

Anti- α -smooth muscle actin antibody at low (A) and high magnification (B); CD31 antibody at low (C) and higher magnification (D); NF200 antibody at low (E) and high magnification (F); tyrosine hydroxylase antibody at low (G) and high magnification (H); negative control (no primary antibody) at low (I) and high magnification (J). Arrows are used to indicate dense areas of positive staining in Figure 2 (G). Positive immunostaining is red and sections counterstained with haematoxylin (blue).

be found either in or between the muscle bundles. This discrete dense staining was particularly apparent in the human specimens.

Autoradiography

Using 5 nM [3 H]-prazosin, significant binding was observed in sheep IAS after 8 weeks exposure (a typical example is shown in Figure 4). Specific binding occurs in discrete areas of smooth muscle bundles, with no specific binding observed in connective tissue between smooth muscle bundles or adventitia (Figure 4A,B). Attempts to generate comparable images with 2–25 nM [3 H]-RX-821002 were unsuccessful. Even after 12 weeks exposure, no evidence of 25 μ M [3 H]-RX821002 rauwolscine-sensitive 'hotspots' were observed ($n = 3$), although specific binding could be observed using sections of rat brain as positive controls incubated under the same conditions (data not shown).

Contractile experiments

The sheep IAS responded to the initial stretch with the generation of sustained increase tension above baseline (35.61 ± 2.84 mN, $n = 28$). All of the compounds examined elicited slow concentration-dependent contractions that took 5–10 min to attain a peak response that declined slowly thereafter. Rilmenidine was not investigated in detail because initial experiments revealed that the maximum response was not appreciably different from the other imidazoline derivatives (data not shown). Moreover, the sub-type selectively displayed in the radioligand binding experiments was comparable to clonidine and tizanidine (see Table 2). Based on the pEC₅₀ values (Table 3), there was less than a 10-fold difference in potency between all six of the imidazoline compounds tested; in the case of moxonidine, many preparations failed to produce a true maximum response so the pEC₅₀ values shown is an estimate. Xylometazoline was the most potent imidazoline agonist, which was between 10- and 100-fold more potent than L-erythro-methoxamine and noradrenaline, respectively (Table 3). Although L-erythro-methoxamine and noradrenaline were less potent than the imidazoline derivatives, the maximum responses were significantly larger (see Table 3); based on the increase in tone calculated relative to the baseline tone (% of resting tone), the response to L-erythro-methoxamine was approximately twofold greater than that elicited by clonidine (Student's unpaired *t*-test, $P < 0.01$).

Figure 5A shows cumulative concentration curves for L-erythro-methoxamine in the presence or absence of 100 nM prazosin (a selective α_1 -adrenoceptor antagonist) or 100 nM RX-811059

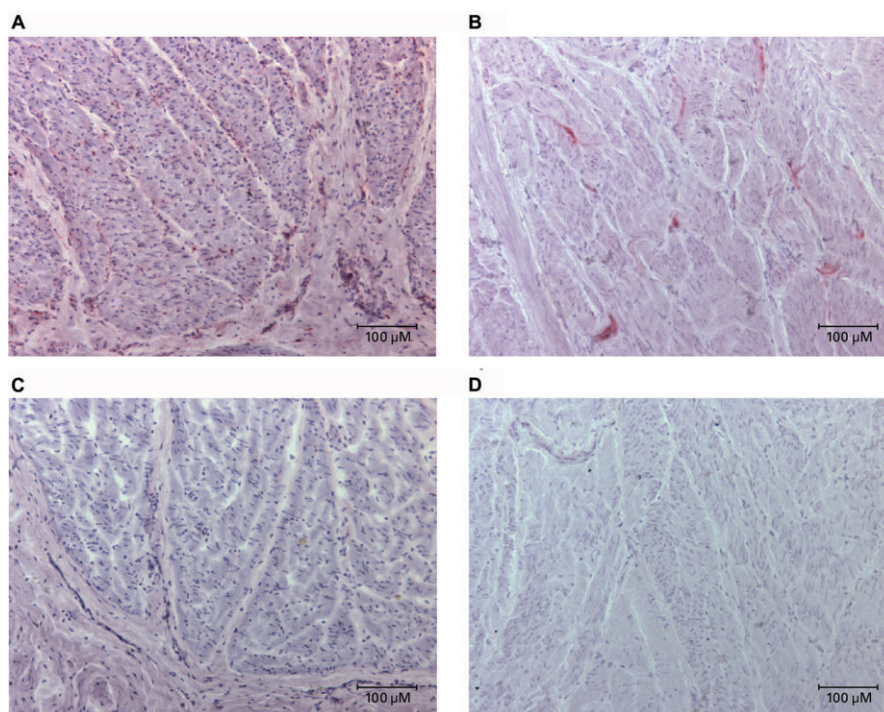


Figure 3

Comparison of staining using anti- α_{1A} -adrenoceptor antibody in sheep (A) and human (B) IAS at high magnification. Negative controls (no primary antibody) are also shown for sheep (C) and human (D) tissues. Positive immunostaining is pink or red, with haematoxylin counterstaining (blue).

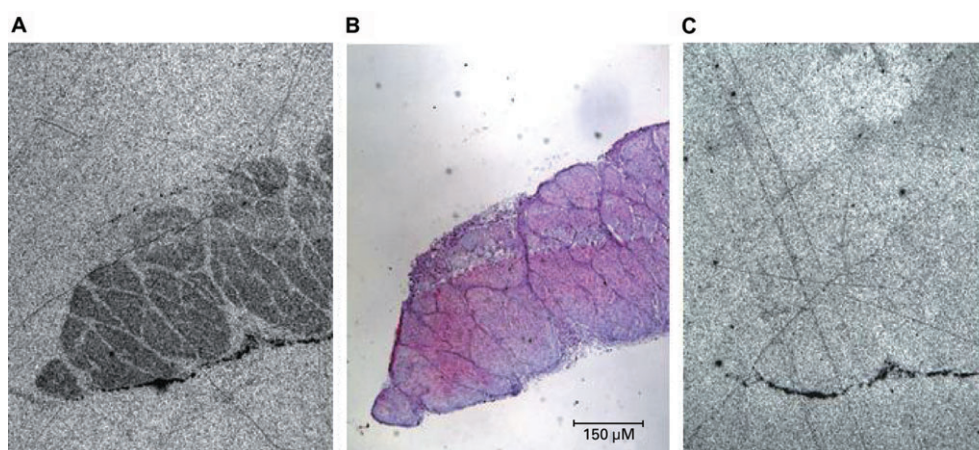


Figure 4

Film images showing the localization of 3 H-prazosin binding in sections of sheep IAS: (A) Total binding of 5 nM 3 H-prazosin to sheep IAS with some areas of dense binding. (B) Tissue underlying total binding, counterstained with haematoxylin and eosin. (C) Non-specific binding of 5 nM 3 H-prazosin in the presence of 100 μ M noradrenaline. These images are representative of sections taken from three individual sheep IASs.

(a selective α_2 -adrenoceptor antagonist). Contractile responses observed in the presence of 100 nM RX-811059 did not differ significantly in terms of pEC_{50} (control 5.63 ± 0.09 , $n = 14$; RX-811059 5.43 ± 0.10 , $n = 9$) or maximum contraction (control 50.72 ± 5.59 mN, $n = 14$; RX811059 56.31 ± 3.83 mN, $n = 9$) from those observed in control

experiments. In contrast, 100 nM prazosin caused a 30-fold rightward displacement of the concentration response curve to L-erythro-methoxamine; at the highest concentration of the agonist employed (100 μ M), there was a significant reduction in the response (control $136 \pm 18\%$, $n = 14$; prazosin $59 \pm 15\%$, $n = 12$; Student's paired t -test $P < 0.005$).

Table 3

The potency of imidazoline agonists, noradrenaline and L-erythro-methoxamine as contractile agents in sheep IAS

	pEC ₅₀	R _{max} (mN)	Maximal responses (% of resting tone)	n
Xylometazoline	6.89 ± 0.06	21.88 ± 5.49	63 ± 23	7
Naphazoline	6.78 ± 0.36	19.13 ± 2.84	58 ± 13	10
Oxymetazoline	6.46 ± 0.20	28.15 ± 7.26	57 ± 13	9
Clonidine	6.44 ± 0.07	30.51 ± 4.22	62 ± 7	14
Tizanidine	6.01 ± 0.17	27.37 ± 5.79	102 ± 37	7
Moxonidine	5.22 ± 0.11	19.91 ± 4.02	67 ± 10	7
L-Erythro-methoxamine	5.60 ± 0.08	42.58 ± 4.91	133 ± 17	20
Noradrenaline	4.68 ± 0.29	34.14 ± 5.20	214 ± 57	5

Values shown are the mean ± SEM.

Cumulative concentration curves for clonidine in the presence or absence of 100 nM prazosin and 100 nM RX811059 are shown in Figure 5B. Both antagonists significantly affected the pEC₅₀ values calculated compared to control (control 6.55 ± 0.10, *n* = 8; prazosin 4.52 ± 0.52, *n* = 9; RX811059 6.03 ± 0.16, *n* = 8) based on analysis by one-way ANOVA with Dunnett's *post hoc* test (prazosin *P* < 0.01; RX811059 *P* < 0.05). Neither antagonist affected the R_{max} values observed (control 37.57 ± 5.40 mN, *n* = 8; prazosin 43.65 ± 9.03 mN, *n* = 9; RX811059 34.43 ± 4.91 mN, *n* = 8).

Discussion

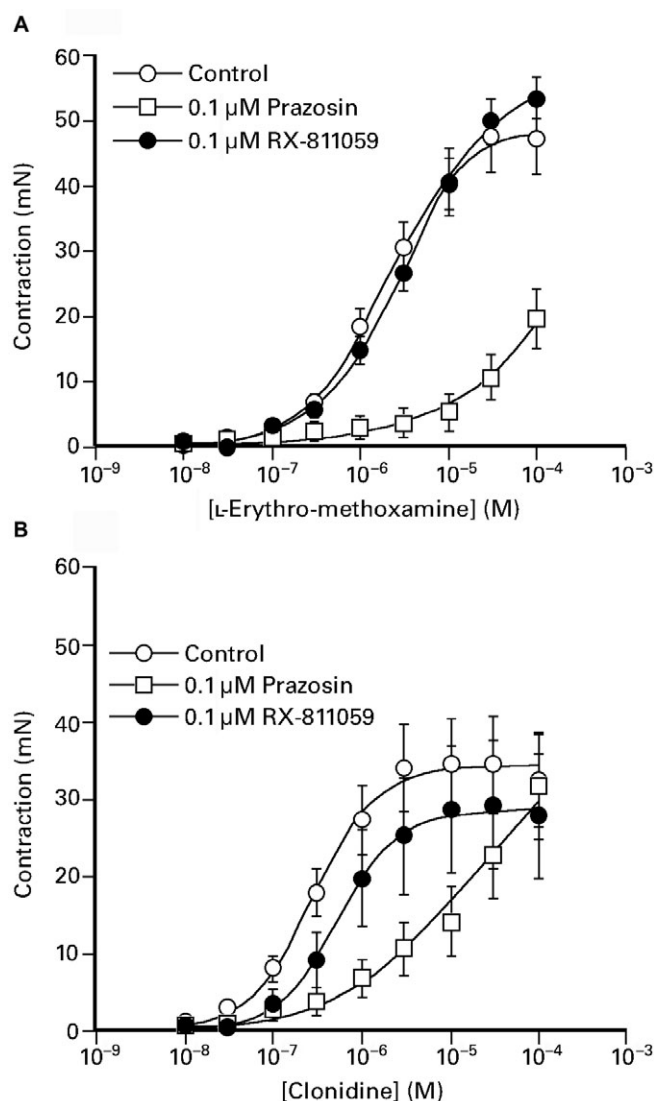
Presence and distribution of α-adrenoceptors in the sheep IAS

Saturation binding analysis of membrane preparations of the sheep IAS with [³H]-prazosin and [³H]-RX821002 revealed the presence of two discrete populations of α-adrenoceptor binding sites. The density of α₁-adrenoceptor binding sites was approximately threefold greater than that of α₂-adrenoceptor binding sites.

Using sub-type-selective α₁-adrenoceptor antagonists, the rank order of affinity against [³H]-prazosin binding in the sheep IAS was RS100329 ≥ 5-methylurapidil >> BMY7378. Based on the published data for recombinant human receptors (McGrath *et al.*, 1989; Mallard *et al.*, 1992), the rank order of affinity is consistent with that described for the α_{1A}-adrenoceptor sub-type. This conclusion is in agreement with recent observations in the pig isolated IAS where RS100329 was reported to competitively inhibit phenylephrine-induced contractions with a pA₂ value of 9.0 (Mills *et al.*, 2008).

For the population of α₂-adrenoceptor binding sites, sub-type-selective antagonists suggested the presence of α_{2D}-adrenoceptors. This conclusion is based on the finding that BRL4408 showed high affinity (pK_i 8.85) against [³H]-RX821002 binding, while prazosin was only weakly active (see Table 1), which suggests the presence of the α_{2A/D}-adrenoceptor. These species orthologues of α₂-adrenoceptors can be distinguished on the basis of the relative potency of phentolamine and rauwolscine (Trendelenburg *et al.*, 1996; Naselsky *et al.*, 2001). As phentolamine was approximately fivefold more potent than rauwolscine, the sub-type present in sheep is similar to that found in the rat and cow (α_{2D}-adrenoceptor) rather than that previously described in man and pig (α_{2A}-adrenoceptor). As far as we are aware, the available data suggest that the two species orthologues respond in a similar manner to known α₂-adrenoceptor agonists, so the functional significance of this difference is unclear.

Immunohistochemical analysis revealed that the sheep IAS is composed of bundles of smooth muscle fibres. Interestingly, the smooth muscle-poor regions of the tissue were highly stained by antibodies to CD31 and NF200, which suggests that vascular endothelium and enteric nerves are closely associated with connective tissue that links the bundles of smooth muscles. The finding that positive staining for adrenergic nerves (using a tyrosine hydroxylase antibody) can be identified in close association with the smooth muscle bundles is consistent with adrenergic innervation of the IAS as observed in sheep (Acheson *et al.*, 2009). Autoradiographic studies with [³H]-prazosin revealed the presence of α₁-adrenoceptor binding sites on the smooth muscle bundles that were largely absent from the smooth muscle-poor regions of the tissue. Positive staining

**Figure 5**

Comparison of the effect of (A) L-erythro-methoxamine and (B) clonidine on the sheep isolated IAS in the absence (control) and the presence of 0.1 μ M prazosin and 1 μ M RX-811059. The responses shown are the mean \pm SEM of 9–14 observations.

in smooth muscle bundles of human and sheep IAS was further confirmed using an antibody specifically targeting the α_1 -adrenoceptor sub-type indicated by radioligand binding experiments (α_{1A} -adrenoceptors). In light of the pattern of adrenergic neurones revealed by the immunohistochemistry, confirmation of the presence of α_1 -adrenoceptors in sheep and human tissue lends further weight to the interpretation of adrenergic innervation.

Surprisingly, the distribution of α_2 -adrenoceptor binding sites could not be revealed by this method. Even with several modifications to the protocol, including elevation of the concentration of [3 H]-RX821002 to 10-fold greater than the K_d value,

elevation of the concentration of rauwolscine (the displacing agent) to 100 μ M and increased duration of exposure of the tissue segments to the film, no specific binding was detected. In contrast, 'hot spots' of [3 H]-RX821002 binding sites was observed in slices of the rat cerebral cortex (approx' 250 fmol·mg $^{-1}$ protein) when run in parallel experiments (unpublished observations). Thus, it seems likely that the low density of α_2 -adrenoceptor binding sites in the sheep IAS precludes autoradiographic detection.

The activity of imidazoline derivatives compounds at α -adrenoceptors

Three observations prompted a detailed study of biological activity of imidazoline derivatives in the sheep IAS. First, clinical evidence that the greater potency of L-erythro-methoxamine at α_1 -adrenoceptors, compared to phenylephrine, allowed for low concentrations to be used to raise anal sphincter pressure in volunteers and patients (Carapeti *et al.*, 1999; 2000; Cheetham *et al.*, 2001; Badvie and Andreyev, 2005; Nisar *et al.*, 2007; Park *et al.*, 2007). Second, imidazoline derivatives (e.g. xylometazoline, and clonidine) are generally known as agonists at α -adrenoceptors with greater potency than either noradrenaline or adrenaline, but possess varying selectivity for the sub-types (McGrath *et al.*, 1989). Compounds in this class are used clinically as nasal decongestants, anti-hypertensive agents and anti-spasticity agents, and to reduce symptoms of opiate withdrawal. Third is the finding that anal sphincter smooth muscle possesses both α_1 - and α_2 -adrenoceptor binding sites (see above). Thus, seven different imidazoline derivatives were examined in competition binding studies to determine their relative affinity at α_1 - and α_2 -adrenoceptors in the anal sphincter.

With the exception of xylometazoline and oxymetazoline, all of the imidazoline derivatives exhibited a greater selectivity (>3-fold) for α_2 -adrenoceptor binding sites over α_1 -adrenoceptor binding sites. In general, these compounds possessed a higher affinity (3- to 300-fold) at α_2 -adrenoceptor binding sites than either L-erythro-methoxamine or noradrenaline. For the majority of compounds examined, the affinity at α_2 -adrenoceptor binding sites in the sheep IAS agrees with the known activity at recombinant human α_{2A} / α_{2D} -adrenoceptors expressed in HEK cells (Jasper *et al.*, 1998). In contrast, at α_1 -adrenoceptor binding sites, the imidazoline derivatives can be divided into two groups: those with an affinity comparable to L-erythro-methoxamine and noradrenaline (tizanidine, rilmenidine and moxonidine) and those that

possessed a 10-fold higher affinity (naphazoline, clonidine, oxymetazoline and xylometazoline).

Contraction-based experiments revealed that all the agents examined elicited concentration-dependent responses. A characteristic feature of the results is that the maximum responses for the imidazoline compounds were approximately 30% of that elicited by noradrenaline, an effect similar to that observed in other smooth muscle preparations that possess α_1 -adrenoceptors, such as rat aorta (Ruffolo and Waddell, 1982), porcine splenic artery (Barbieri *et al.*, 1998) and human nasal mucosa (Johannssen *et al.*, 1997). Also, with the exception of moxonidine, L-erythro-methoxamine and noradrenaline, the potency of the compounds was more closely related to the affinity detected against the α_1 -adrenoceptor binding sites. Confirmation of the predominant role of α_1 -adrenoceptors was obtained when examining the effect of sub-type-selective antagonists, prazosin and RX-811059, against L-erythro-methoxamine and clonidine-induced contractions. In each case, prazosin (100 nM) caused an approximate 30-fold rightward displacement of the concentration–response curve. However, while the selective α_2 -adrenoceptor antagonist RX-811059 (100 nM) was inactive against L-erythro-methoxamine-induced contractions, it caused a threefold rightward displacement of clonidine-induced contractions. The latter observation provides unambiguous evidence for the presence of α_2 -adrenoceptors on the smooth muscle of the sheep isolated internal sphincter and raises the possibility of a synergistic interaction with α_1 -adrenoceptors.

A functional role for α_2 -adrenoceptors in the control of defaecation has largely been confined to the enteric nervous system; by reducing the rate of gastrointestinal transit (Scheibner *et al.*, 2002), interference in the ano-rectal inhibitory response in the opossum (Yamato and Rattan, 1990) and modulation of colonic and rectal compliance in man (Viramontes *et al.*, 2001; Camilleri *et al.*, 2003). The finding that α_2 -adrenoceptors are also present on the sphincter muscle and have the potential to enhance α_1 -adrenoceptor responses, may explain the observation in the anaesthetized opossum that naphazoline causes both increased IAS pressure and inhibited the recto-anal inhibitory response (Yamato and Rattan, 1990).

The original basis for examining the potential of L-erythro-methoxamine for treating faecal incontinence was the finding that it possessed approximately fourfold greater potency than phenylephrine (Jones *et al.*, 2003). With the exception of moxonidine, all of the imidazoline derivatives examined in this study were more potent than L-erythro-

methoxamine, but significantly less ‘efficacious’. The impact of the latter property on their therapeutic potential is unclear, but it is noteworthy that many imidazoline derivatives have been successfully used as potent decongestants in man despite possessing significantly low constrictor ‘efficacy’ at α -adrenoceptors in nasal blood vessels (Johannssen *et al.*, 1997; Corboz *et al.*, 2003).

In conclusion, we have demonstrated the presence of α_{1A} - and α_{2D} -adrenoceptors on the sheep IAS muscle. Although α_{1A} -adrenoceptors appear to make the major contribution to the constrictor effect of known α -adrenoceptor agonists, in the case of selective α_2 -adrenoceptor imidazoline derivatives, there is the possibility of synergistic interaction between receptor sub-types. Because many imidazoline derivatives (e.g. clonidine) have greater potency at both α -adrenoceptor sub-types compared to existing selective α_1 -adrenoceptor agonists, these agents could be effective at lower doses, thereby reducing the propensity for local or systemic side effects. In light of this, some imidazoline derivatives would be worthy candidates for investigating in the treatment of faecal incontinence.

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Conflict of interest

None to declare.

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