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RESEARCH PAPER

Characterization of the postjunctional α_{2C}-adrenoceptor mediating vasoconstriction to UK14304 in porcine pulmonary veins

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Background and purpose: In terms of postjunctional α_2-adrenoceptors in the pulmonary circulation, no evidence is available with regard to the receptor subtypes mediating vasoconstriction. Therefore, we characterized the α_2-adrenoceptor subtypes mediating contraction in isolated porcine pulmonary veins.
Experimental approach: α-adrenoceptor-mediated vasoconstriction was studied using a tissue bath protocol. mRNA profile and relative quantification of α_2-adrenoceptor subtypes were determined in porcine pulmonary veins using reverse-transcriptase polymerase chain reaction (RT-PCR) and real-time PCR.
Key results: In porcine pulmonary veins, noradrenaline, phenylephrine (α_1-adrenoceptor agonist), UK14304 and clonidine (α_2-adrenoceptor agonists) caused concentration-dependent contractions. The rank order of agonist potency was: NA $\approx$ UK14304 $\approx$ clonidine $>$ phenylephrine. UK14304 responses were antagonised by MK912 (noncompetitive antagonist parameter pD'$_2$: 10.1), rauwolscine (pK$_B$: 9.5), yohimbine (pK$_B$: 9.1), WB4101 (pK$_B$: 8.7), ARC239 (pK$_B$: 7.5), prazosin (pK$_B$: 7.1) and BRL44408 (pK$_B$: 7.0). Antagonist potencies fitted best with radioligand binding data (pK$_i$) at the human recombinant α_{2C}-adrenoceptor ($r^2 = 0.96$, $P = 0.0001$). Correlation with α_{2B}-adrenoceptors was lower ($r^2 = 0.74$, $P > 0.01$) and no correlation was obtained with α_{2A}-adrenoceptors. Moreover, RT-PCR studies in porcine pulmonary veins showed mRNA signals for α_{2A}- and α_{2C}-adrenoceptors, but not for α_{2B}-adrenoceptors, whilst real-time PCR studies indicated a prominent expression of α_{2C}-adrenoceptor mRNA.
Conclusions and Implications: Postjunctional α_{2C}-adrenoceptors mediated contraction in porcine pulmonary veins. α_1-Adrenoceptors also seem to be present in this tissue. Since α_2-adrenoceptor responsiveness is increased when pulmonary vascular tone is elevated, α_{2C}-adrenoceptor antagonists may be beneficial in diseases such as pulmonary hypertension or congestive heart failure.
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Keywords: α_2-adrenoceptors; α-adrenoceptor antagonists; porcine pulmonary veins; UK14304; vasoconstriction

Abbreviations: ARC239, 2-[2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl]-4,4-dimethyl-1,3-(2*H*,4*H*)-isoquinolindione dihydrochloride; BRL44408, 2-[(4,5-dihydro-1*H*-imidazol-2-yl)methyl]-2,3-dihydro-1-methyl-1*H*-isoindole maleate; CRC, concentration–response curve; KHS, Krebs-Henseleit solution; MK912, (2*S*-*trans*)-1,3,4,5′,6,6′,7,12b-octahydro-1′,3′-dimethyl-spiro[2*H*-benzofuro[2,3-a]quinolizine-2,4′(1′*H*)-pyrimidin]-2′(3′*H*)-one hydrochloride; PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase polymerase chain reaction; UK14304, brimonidine tartrate; WB4101, 2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane hydrochloride

Introduction

Postjunctional vascular α_1- and α_2-adrenoceptors have been shown to coexist in systemic arterial and venous circulatory beds mediating vasoconstriction (Docherty, 1998; Guimarães and Moura, 2001). On the basis of conjunction of structural, transductional and operational criteria, α_1-adrenoceptors have been classified into α_{1A}-, α_{1B}- and α_{1D}-adrenoceptor subtypes, whereas α_2-adrenoceptors have been classified into α_{2A}-, α_{2B}- and α_{2C}-adrenoceptor subtypes (Bylund *et al.*, 1994). Whereas postjunctional α_{1A}- and

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α_{1D}-adrenoceptor subtypes are mainly involved in the contractions induced by α_1-adrenoceptor agonists, there is relatively little evidence available as to the subtypes mediating vascular contractions via postjunctional α_2-adrenoceptors (Docherty, 1998; Guimarães and Moura, 2001). In this respect, several studies have demonstrated vasoconstriction mediated by (i) α_{2A}-adrenoceptors in canine saphenous vein and mesenteric artery (MacLennan *et al*., 1997; Paiva *et al*., 1999) as well as in porcine ciliary artery (Wikberg-Matsson and Simonsen, 2001); (ii) α_{2C}-adrenoceptors in human saphenous vein (Gavin *et al*., 1997) and (iii) both α_{2A}- and α_{2C}-adrenoceptors in porcine nasal mucosa strips (Corboz *et al*., 2003) and in the canine external carotid arterial bed (Willems *et al*., 2001a, b).

In vivo and *in vitro* studies suggest that the pulmonary circulation is also endowed with both postjunctional α_1- and α_2-adrenoceptors which mediate vasoconstriction (Hyman and Kadowitz, 1985; Shebuski *et al*., 1986, 1987; Ohlstein *et al*., 1989). Interestingly, α_2-adrenoceptor responsiveness is selectively enhanced when the pulmonary vascular tone is elevated (Hyman and Kadowitz, 1986; Shebuski *et al*., 1987). However, no data are available on the α_2-adrenoceptor subtype(s) mediating contraction in pulmonary blood vessels.

Pronounced α_2-adrenoceptor-mediated contractile responses in veins, but not in arteries, have previously been described in the canine pulmonary circulation. Thus, it has been hypothesized that pulmonary vascular α_2-adrenoceptors may be located preferentially on the venous side of the pulmonary circulation (Shebuski *et al*., 1987; Ohlstein *et al*., 1989). Consequently, this study has focused on the characterization of the postjunctional α_2-adrenoceptor subtype(s) in pulmonary veins. In contrast to peripheral veins, pulmonary veins have a specific feature because they carry oxygenated blood. However, pulmonary veins macroscopically have a thinner wall thickness because they are exposed to lower blood pressure than the pulmonary arteries. We used porcine veins to examine the pharmacological characteristics of α_2-adrenoceptor-mediated contraction because (i) pigs and humans share anatomical, physiological, histological and biochemical similarities (Pound and Houpt, 1978); (ii) pigs are favoured as potential donors for xenotransplantation (Platt, 2001); and (iii) the pig α_2-adrenoceptor subtypes are pharmacologically more related to those of humans than to those of rodents (Wikberg-Matsson *et al*., 1995). In tissue bath studies, we used α-adrenoceptor agonists such as noradrenaline (a non-selective adrenoceptor agonist), phenylephrine (α_1-adrenoceptor agonist), clonidine and brimonidine tartrate (UK14304) (both α_2-adrenoceptor agonists). In addition, we examined the blocking properties of the antagonists 2-[(4,5-dihydro-1*H*-imidazol-2-yl)methyl]-2,3-dihydro-1-methyl-1*H*-isoindole maleate (BRL44408) (α_{2A}; Young *et al*., 1989), (2*S-trans*)-1,3,4,5′,6,6′,7,12b-octahydro-1′,3′-dimethyl-spiro[2*H*-benzofuro[2,3-a]quinolizine-2,4′(1′*H*)-pyrimidin]-2′(3′*H*)-one hydrochloride (MK912) (α_{2C}; Uhlén *et al*., 1992), 2-[2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl]-4,4-dimethyl-1,3-(2*H*,4*H*)-isoquinolindione dihydrochloride (ARC239) ($\alpha_{2B/2C}$; Bylund *et al*., 1988), 2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane hydrochloride (WB4101) (α_{1A}/α_{2C}; Uhlén *et al*., 1994) as well as prazosin (α_1, α_{2B}/α_{2C}; Bylund *et al*., 1994), yohimbine and rauwolscine (non-selective α_2-adrenoceptor antagonists; Table 1) against the contractile response to UK14304. In addition, we used both reverse-transcriptase polymerase chain reaction (RT-PCR) and real-time polymerase chain reaction techniques to identify and to quantify the mRNA being expressed in porcine pulmonary veins. Our results suggest that UK14304-induced vasoconstriction in porcine pulmonary veins is mediated by postjunctional α_{2C}-adrenoceptors. Functional studies rule out the involvement of α_{2A}-adrenoceptors, both functional studies and RT-PCR exclude the presence of the α_{2B}-subtype and real-time PCR suggests the predominant occurrence of the α_{2C}-subtype in porcine pulmonary veins.

Table 1 Binding affinities (pK_i values) of antagonists for α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors and antagonist affinity estimates (pK_B values) against the contractile response to UK14304 in porcine pulmonary veins

Antagonists	*α_{2A} (pK_i)*	*α_{2B} (pK_i)*	*α_{2C} (pK_i)*	*Pulmonary vein (pK_B)*
ARC239	5.88	7.71	7.50	7.48±0.03 (4–7)
BRL44408	8.25	6.19	6.82	7.02±0.08 (4–8)
MK912	8.90	8.87	10.07	10.05±0.04 (5)[a]
Rauwolscine	8.43	8.34	9.11	9.53±0.08 (4)[b]
Prazosin	5.65	6.94	7.24	7.06±0.06 (4)
Yohimbine	8.43	7.87	8.52	9.09±0.05 (4–5)
WB4101	7.84	7.11	8.50	8.65±0.05 (4–6)

Binding affinities (cloned human α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors expressed in Cos-7 cells; [^{3}H]MK912), data from Uhlén *et al*. (1994). The slopes of the Schild plots were as follows: ARC239, 0.92±0.05; BRL44408, 1.00±0.11; prazosin, 1.09±0.11; yohimbine, 1.12±0.08; WB4101, 0.97±0.09. The slopes were not significantly different from unity. Antagonist affinity estimates are means±s.e.m. for *n* animals in parentheses.
[a]Noncompetitive antagonist parameter pD'_2.
[b]Apparent pK_B.

Methods

Isolated tissues

Lungs and brains from pigs were obtained from the local slaughterhouse (Lehr- und Versuchsanstalt für Tierzucht und Tierhaltung, Teltow-Ruhlsdorf, Germany). During the transportation to the laboratory, lungs and brains were placed in ice-cold Krebs-Henseleit solution (KHS) of the following composition (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$, 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and D -glucose 10 (pH 7.4). The solution was aerated with 95% O_2/5% CO_2. Small branches of intralobar pulmonary veins were dissected and placed in KHS. After removal of parenchyma and connective tissue, blood vessels were stored overnight at 4°C in previously gassed KHS. Preliminary experiments had shown that overnight storage of tissues did not impair the contractility of the smooth muscle. On the following day, the veins were cut into rings (3 mm long, 1.5 mm wide) which were horizontally suspended between two L-shaped stainless-steel hooks (150 μm diameter) and mounted in a water-jacketed 20 ml organ bath filled with KHS. The solution was continuously aerated with 95% O_2/5% CO_2 and warmed to a constant temperature of 37°C (pH 7.4). For continuous measurement

of force changes, preparations were connected to an isometric force transducer (W Fleck, Mainz, Germany) attached to a TSE 4711 transducer coupler and a Siemens C1016 compensograph. Initial resting tension was adjusted to 10 mN at the beginning of each experiment.

Measurement of vascular tone

The venous rings were stabilized for 90 min with bath fluid replacement every 30 min. During a subsequent equilibration period of 180 min, vessels were stimulated first with 45 mM KCl and then four times with 0.3 μM of the α_2-adrenoceptor agonist UK14304 with washings after each contractile challenge. This procedure was considered to yield stable and reproducible contractions. Tension was repeatedly readjusted to 10 mN and remained unchanged after the third UK14304 stimulation.

In a first set of experiments, we checked whether removal of the endothelium had an influence on cumulative concentration–response curves (CRCs) to UK14304. To rub off the endothelium, dental floss (Oral-B, Superfloss) was used by pulling it gently through the lumen of the blood vessel. The absence of endothelium was verified by the failure of carbachol (10 μM) to cause relaxation following the third contraction with UK14304. Cumulative CRCs to UK14304 were not different in the absence or presence of endothelium in porcine pulmonary veins (not shown). Therefore, no attempt was made to remove the endothelium in further experiments.

Agonists. To determine the contractile effects of agonists (noradrenaline, phenylephrine, clonidine and UK14304), a cumulative CRC to each agonist was constructed by increasing the agonist concentration cumulatively by half-log increments until a maximum response was observed. Cocaine (10 μM) and propranolol (1 μM) were present in the bath fluid to block neuronal uptake and β-adrenoceptors, respectively.

Antagonists. The participation of α_2-adrenoceptors in the contractile response to UK14304 in porcine pulmonary veins was further assessed using a series of antagonists, which show relative selectivity for α_2-adrenoceptors (ARC239, BRL44408, MK912, WB4101, prazosin, yohimbine and rauwolscine). Antagonists were generally added to the bath fluid 60 min before the construction of a cumulative CRC to UK14304. MK912 and rauwolscine (0.3 and 1 nM) were added 120 min before the construction of a CRC. UK14304 control curves showed no difference in E_{max} and pEC_{50} irrespective of an incubation time of 60 or 120 min. In this set of experiments, cocaine and propranolol were not present in the bath fluid, as both drugs had no influence on the CRCs to UK14304 (not shown).

RT-PCR

Small branches of porcine pulmonary veins were obtained as described above. To obtain a positive control of receptor expression in RT-PCR studies, pieces of cerebral cortex (*cortex cerebri*) of two pigs were isolated. Presence of mRNA in the central nervous system for the three subtypes of the α_2-adrenoceptor has been reported in different animal species (Wikberg-Matsson *et al*., 1995; Saunders and Limbird, 1999). Veins and brain pieces were stored at −80°C. The frozen tissue was homogenized in guanidium thiocyanate solution using a Mikro-Dismembrator U (Braun, Melsungen, Germany) and the total RNA was extracted as described earlier (Chomczynski and Sacchi, 1987). Possible contaminating genomic DNA was removed by DNase treatment of the RNA sample. Subsequently, total RNA was purified using the GeneMATRIX Universal RNA Purification Kit (EURx Ltd, Gdañsk, Poland). RNA concentration was measured by UV absorbance at 260 nm using a GeneRay UV photometer (Biometra, Goettingen, Germany). Following denaturation of the RNA (2 μg) at 70°C, the first strand of cDNA was synthesized in a reaction volume of 25 μl reverse transcription buffer (50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; 10 mM dithiothreitol) supplemented with 0.5 mM dNTPs, ribonuclease inhibitor (25 U) and M-MLV reverse transcriptase (200 U) (60 min, 37°C). To prove the absence of contamination with genomic DNA, a control was similarly prepared, except that the M-MLV reverse transcriptase was omitted (negative control). Two microlitres of the cDNA thus synthesized were diluted to a total volume of 50 μl containing the following components: Taq DNA Polymerase (1.25 U; EURx Ltd, Gdañsk, Poland), 200 μM of each dATP, dTTP, dGTP and dCTP, PCR buffer, 10 × AmpliBuffer B (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 0.01% Triton X-100; EURx Ltd., Gdańsk, Poland) and 1 μM of the following forward and reverse oligonucleotide primers (TiBMolBiol, Berlin, Germany): 5′-ATC ATT GCC GTG TTC ACA AGC and 5′-AAG AAG GAG CCG ATG CAA GAC for the pig α_{2A}-adrenoceptor (GenBank entry NM214400, nucleotides 163–617); 5′-AAC TGG CCA CTG CTG GAG AG and 5′-TTC AGC CTC CTC CTC TGG TG for the pig α_{2B}-adrenoceptor (NM 001037148, nucleotides 681–859); and 5′-CCA ACG AGC TCA TGG CCT AC and 5′-GAG ATG ACG GCC GAG ATG AG for the bovine α_{2C}-adrenoceptor (AJ 488281, nucleotides 85–304). On the basis of the nucleotide sequence of porcine glyceraldehyde-3-phosphate dehydrogenase (GAPDH; AF 017079, nucleotides 513–895; positive control), the following primer pair (forward and reverse) was constructed (TiBMolBiol, Berlin, Germany): 5′-GTC AAG GCT GAG AAT GGG and 5′-GTC TTC TGG GTG GCA GTG ATG. To establish the appropriate primer pairs the following software was used: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi (Rozen and Skaletsky, 2000). A control was included that contained all the components of the PCR except the template DNA. The PCR was performed in a Stratagene Robocycler Gradient 40 (Stratagene Europe, Amsterdam, The Netherlands) under the following conditions: cDNA was denaturated for 60 s at 95°C and annealed to the primers for 60 s at 55°C with the reaction extended for 90 s at 72°C and this procedure was repeated for 40 cycles. The amplified PCR products (fragment size of 455, 179, 220 and 383 bp for the α_{2A}-, α_{2B}-, α_{2C}-adrenoceptor and GAPDH, respectively) were separated on 2% TAE-agarose gel, treated with ethidium bromide for 15 min, briefly differentiated with water and photographed.

Real-time PCR

Real-time PCR assays were carried out using SYBR Green PCR Master Mix on ABI PRISM 7900HT Sequence Detection System according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Amplification was performed in 10 μl reactions (Primer concentration 250 nM, 1 × SYBR Green Master Mix) containing 2 μl cDNA (equivalent to 10 ng of total RNA) in 40 cycles of 95°C, 15 s, 60°C, 1 min. Total RNA of three different sets of tissue was used to analyse receptor expression. Data normalization was performed using GAPDH as a reference gene. The following primer sequences were used for pig GAPDH (GenBank entry AF017079, nucleotides 156–399) (TiBMolBiol, Berlin, Germany): 5′-CAA ATT CCA CGG CAC AGT CA and 5′-CAT GCC CAT CAC AAA CAT GG. Relative mRNA expression was quantified using the comparative CT method according to the ABI manual. The following primer sequences were used (TiBMolBiol, Berlin, Germany): 5′-CGC TTG TCA TCC CTT TCT CG and 5′-CTC TAT GGC CTG GGT GAT GG for the pig α_{2A}-adrenoceptor (NM 214400, nucleotides 251–420); 5′-AAC TGG CCA CTG CTG GAG AG and 5′-TTC AGC CTC CTC CTC TGG TG for the pig α_{2B}-adrenoceptor (NM 001037148, nucleotides 681–859); and 5′-CCA ACG AGC TCA TGG CCT AC and 5′-GAG ATG ACG GCC GAG ATG AG for the bovine α_{2C}-adrenoceptor (AJ 488281, nucleotides 85–304). Nucleotide sequences were retrieved and respective primer pairs constructed as mentioned above.

Data analysis and presentation

Data are presented as mean ± s.e.m. for tissues from n animals. CRCs were fitted to the Hill equation using an iterative, least-squares method (GraphPad Prism 4.0, GraphPad Software, San Diego, CA, USA) to provide estimates of the maximum response E_{max} (contractile response in % relative to the effect of the fourth UK14304 (0.3 μM)-induced contraction) and the half-maximum effective concentration pEC_{50} (the negative logarithm of the molar concentration of the agonist producing 50% of the maximal response). In these calculations, the bottom of the curves was fixed at 0 (i.e., 0% contraction over basal); the Hill slopes (n_H) were kept variable.

Antagonist affinities were generally expressed as either an apparent pK_B or a full pK_B value. Apparent pK_B values were calculated from the equation $pK_B = -\log[B] + \log(r-1)$, where [B] is the molar concentration of the antagonist and r the ratio of agonist EC_{50} measured in the presence and absence of antagonist (Furchgott, 1972). In the case of competitive antagonism (i.e., the antagonist produced parallel rightward shifts of the CRC without attenuation in the maximum response), antagonist affinities (full pK_B) were estimated using the method of Arunlakshana and Schild (1959). If the Schild regression line had a slope not differing significantly from 1.00, the slope was constrained to unity. The intercept on the −log antagonist concentration axis provided the estimate of pK_B (Jenkinson *et al*., 1995). For the noncompetitive antagonist MK912, a pD'_2 value was calculated according to van Rossum (1963). pD'_2 was defined as the negative logarithm of the molar concentration of antagonist which caused a 50% depression of the maximum response to the agonist: $pD'_2 = -\log[B] + \log(E_{max}/E_{max}{}^* - 1)$, where E_{max} is the maximum response to the agonist in the absence of antagonist and E_{max}* the maximum response to the agonist in the presence of antagonist. Student's t-test (unpaired, two-tailed) was used to assess differences between two mean values with $P<0.05$ being considered as significant.

Drugs

The drugs used in the present study were obtained from the sources indicated: UK14304; gift from Allergan Pharmaceuticals, Westport, Co Mayo, Ireland; cocaine hydrochloride (Merck, Darmstadt, Germany); (*R*)-phenylephrine hydrochloride and yohimbine hydrochloride (Janssen, Beerse, Belgium); carbachol, clonidine hydrochloride, MK912, noradrenaline bitartrate, prazosin hydrochloride, (*R*,*S*)-propranolol hydrochloride and rauwolscine hydrochloride (Sigma-Aldrich, Taufkirchen, Germany); ARC239, BRL44408 and WB4101 (Tocris, Bristol, UK).

All drugs were dissolved in distilled water to a 10 mM stock solution, except for prazosin, which was dissolved in 50% ethanol. Stock solutions were stored at –18°C and freshly diluted in distilled water before the beginning of the experiment. The final organ bath concentration of ethanol (which had no quantifiable effects) did not exceed 0.05%.

Results

Effects of agonists in porcine pulmonary veins

The purpose of these experiments was to study the contractile response to a series of agonists noradrenaline ($pEC_{50} = 7.27 \pm 0.07$, $E_{max} = 254 \pm 17\%$, $n = 8$), UK14304 ($pEC_{50} = 7.16 \pm 0.06$, $E_{max} = 118 \pm 10\%$, $n = 4$), clonidine ($pEC_{50} = 7.04 \pm 0.08$, $E_{max} = 50 \pm 7\%$, $n = 4$) and phenylephrine ($pEC_{50} = 5.92 \pm 0.07$, $E_{max} = 150 \pm 10\%$, $n = 8$) produced concentration-dependent contractile responses in porcine pulmonary veins (Figure 1). The E_{max} of noradrenaline was significantly higher when compared to that of the other compounds, whereas the E_{max} of UK14304 and phenylephrine did not significantly differ from each other. The ability of the selective α_2-adrenoceptor agonists, UK14304 and clonidine, to contract porcine pulmonary veins indicates that this tissue is endowed with α_2-adrenoceptors. As the selective α_1-adrenoceptor agonist phenylephrine also contracted these blood vessels, α_1-adrenoceptors may be present in this tissue.

Effects of α_2-adrenoceptor antagonists in porcine pulmonary veins

To determine the subtype(s) of α_2-adrenoceptors mediating the contractile response in porcine pulmonary veins, we examined the effect of a series of relatively selective α_2-adrenoceptor antagonists against UK14304. Antagonist effects of these drugs are summarized in Table 1. With the exception of MK912 and rauwolscine, all compounds caused a concentration-dependent rightward shift of the CRC to UK14304 with little or no effect on the maximum response. A four-point or five-point Schild regression for these data revealed competitive antagonism (Figure 2). The slopes of the Schild plots were not significantly different from unity

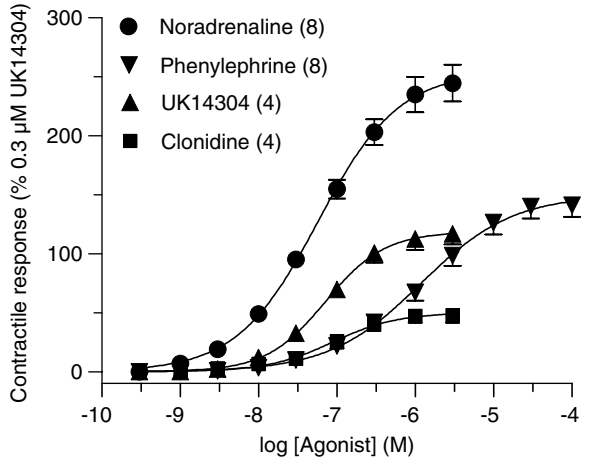

Figure 1 Cumulative log CRCs to α-adrenoceptor agonists in rings of porcine pulmonary veins. Values are mean ± s.e.m. (vertical bars) from *n* animals as indicated in parentheses.

(Table 1). In the case of rauwolscine, an apparent pK_B value of 9.53 (Table 1) was determined from a single concentration of the drug (1 nM), as higher concentrations of rauwolscine led to insurmountable antagonism (i.e., depression of the maximum response and rightward shift of the UK14304 curve) (Figure 3a), precluding Schild regression analysis. MK912 (0.1 nM) slightly, but significantly, shifted the CRC to UK14304 to the right (log $r = 0.42 \pm 0.11$, $n = 5$) and induced a powerful depression of the maximum response (Figure 3b). Thus, MK912 fulfilled the criteria for non competitive antagonism and the noncompetitive antagonist parameter pD'_2 was calculated, according to van Rossum (1963) (Table 1).

Attempts were made to correlate the affinity parameters determined in functional tests in pig pulmonary veins with their affinities (pK_i values) at cloned human α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors (Uhlén *et al.*, 1994). Affinities found in porcine pulmonary veins fitted best with radioligand binding data at the human α_{2C}-adrenoceptor (Figure 4). Correlation with α_{2B}-adrenoceptors was lower and no significant correlation was obtained with α_{2A}-adrenoceptors (Figure 4). Re-calculation of the correlations excluding the affinities for rauwolscine and MK912, as both drugs failed to show competitive antagonism of the UK14304 response (see above), yielded a highly significant correlation for α_{2C}-adrenoceptors ($r^2 = 0.94$, $P < 0.01$) and nonsignificant correlations for α_{2A}-adrenoceptors ($r^2 = 0.32$, $P > 0.30$) and α_{2B}-adrenoceptors ($r^2 = 0.40$, $P > 0.25$).

RT-PCR and real-time PCR studies

Using RT-PCR, we analysed the mRNAs for the three α_2-adrenoceptor subtypes (α_{2A}, α_{2B} and α_{2C}) expressed in porcine pulmonary veins. The amplification of GAPDH cDNA ensured the quality of the samples and the level of transcription. Control experiments of RT-PCR confirmed three different α_2-adrenoceptor mRNAs, α_{2A}, α_{2B} and α_{2C} in pig brain cortex. In porcine pulmonary veins only the expression of mRNA for α_{2A}- and α_{2C}-adrenoceptors was detectable (Figure 5). Real-time PCR indicated that the relative mRNA amount of α_2-adrenoceptor subtypes was $\alpha_{2C} >> \alpha_{2A}$. In agreement with the RT-PCR results, real-time analysis of PCR-products confirmed the absence of mRNA for the α_{2B}-adrenoceptor in porcine pulmonary veins (Figure 6).

Discussion

The primary objective of the present study was to examine the pharmacological characteristics of the postjunctional α_2-adrenoceptor mediating contraction in porcine pulmonary veins. We could show that a number of adrenoceptor agonists, noradrenaline, phenylephrine, UK14304 and clonidine, elicited contractile responses in this tissue. Noradrenaline, a non-selective adrenoceptor agonist, exhibited a greater maximum response than the α_1-adrenoceptor agonist phenylephrine and the α_2-adrenoceptor agonists, UK14304 and clonidine, respectively; a finding that may be attributable to a simultaneous stimulation of both α_1- and α_2-adrenoceptors. In accordance with these findings, a mixed population of contractile α_1- and α_2-adrenoceptors has also been shown in canine pulmonary veins as well as in porcine palmar lateral vein and marginal ear vein (Ohlstein *et al.*, 1989; Blaylock and Wilson, 1995; Wright *et al.*, 1995).

It should be emphasized that agonist potencies alone did not allow definitive receptor identification and classification (Hoyer and Boddeke, 1993). Therefore, we used a number of antagonists to characterize the α_2-adrenoceptor subtype(s) mediating the contractile response to UK14304 which exhibits selectivity for α_2-adrenoceptors over α_1-adrenoceptors (Cambridge, 1981). Although selective antagonists are now being developed to differentiate between the α_2-adrenoceptor subtypes, there are, at present, no highly selective ligands available for the α_2-adrenoceptor subtypes. Thus, pharmacological characterization is based on a limited range of compounds, which exhibit different affinities for the subtypes. The most valuable antagonists are: (i) BRL44408, with 30–120-fold selectivity for α_{2A}-adrenoceptors versus α_{2B}- and α_{2C}-adrenoceptors; (ii) ARC239, with 40–70-fold selectivity for $\alpha_{2B/2C}$-adrenoceptors versus the α_{2A}-adrenoceptor; and (iii) MK912 with 15-fold selectivity for α_{2C}-adrenoceptor versus α_{2A}- and α_{2B}-adrenoceptors (Uhlén *et al.*, 1994). Interestingly, ARC239 displayed a 52-fold higher antagonist affinity in pulmonary veins than in porcine ciliary arteries (Wikberg-Matsson and Simonsen, 2001). This argues against a role for α_{2A}-adrenoceptors in UK14304-induced contraction in porcine pulmonary veins. Furthermore, the relatively low antagonist activity for BRL44408 (pK_B 7.0), the high antagonist activities for MK912 (pD'_2 10.1), ARC239 (pK_B 7.5), WB4101 (pK_B 8.7) and prazosin (pK_B 7.1), and the absence of mRNA for the α_{2B}-adrenoceptor (see below) argue for an α_{2C}-adrenoceptor-mediated component of contraction in porcine pulmonary veins. This observation is supported by the correlation analysis between the affinity estimates for seven antagonists in porcine pulmonary veins and human binding data (Uhlén *et al.*, 1994). Our study shows that antagonist potencies fitted best

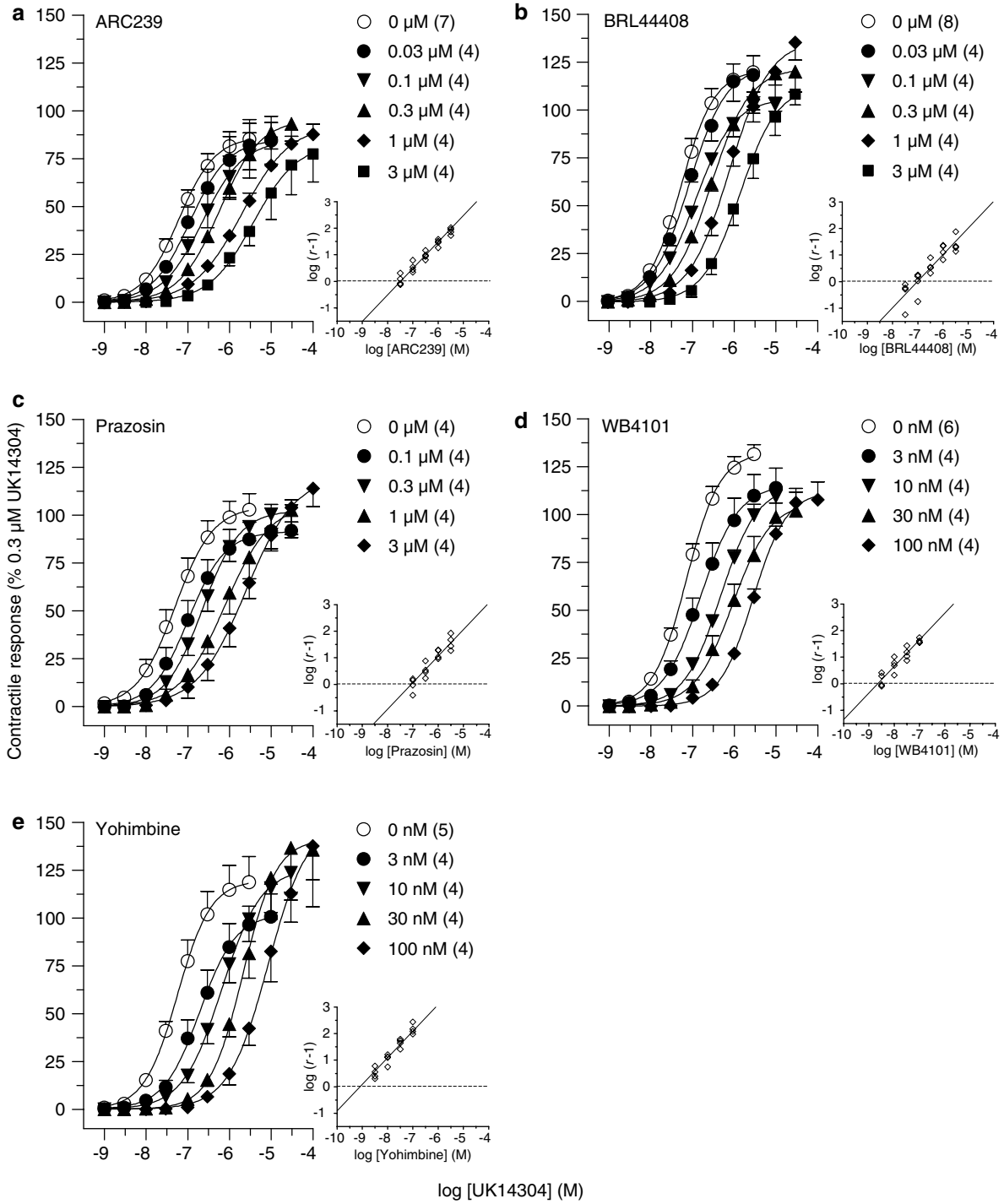

Figure 2 Cumulative log CRCs to UK14304 in the absence or presence of ARC239 (**a**) BRL44408 (**b**) prazosin (**c**) WB4101 (**d**) and yohimbine (**e**) in rings of porcine pulmonary veins. The insets represent the Schild regression analysis of the respective CRCs. Values are means ± s.e.m. (vertical bars) from *n* animals as indicated in parentheses.

with binding affinity estimates (pK_i) obtained for these antagonists at the human recombinant α_{2C}-adrenoceptor. Correlation with α_{2B}-adrenoceptors was less favourable, and no correlation was obtained with α_{2A}-adrenoceptors. It is worth mentioning that, with the exception of rauwolscine and MK912, the rest of antagonists used behaved as competitive antagonists. Schild regression lines with slopes not significantly different from unity for ARC239, BRL44408, prazosin, yohimbine and WB4101 against UK14304 argue for a single receptor population (Kenakin, 1993) mediating the contractile response in porcine pulmonary veins. Rauwolscine induced a depression of the maximal UK14304 response, whereas MK912 behaved as a non competitive antagonist.

RT-PCR experiments using blood vessels with endothelium indicated an absence of mRNA for the α_{2B}-adrenoceptor but the presence of mRNA for both α_{2A}- and α_{2C}-adrenoceptors in porcine pulmonary veins. Quantitative measurement by real-time PCR showed a dominant expression of the mRNA for the α_{2C}-adrenoceptor indicating a prominent role for the α_{2C}-adrenoceptor in this vessel. The presence of mRNA for the α_{2A}-adrenoceptor, showing a lower expression level than that for the α_{2C}-adrenoceptor in real-time PCR, may result from the existence of prejunctional inhibitory α_{2A}-adrenoceptors in the adventitia and/or relaxant α_{2A}-adrenoceptors in the endothelium. The α_{2A}-adrenoceptor has been shown to regulate neurotransmitter release from sympathetic neurons in almost all species and tissues (Trendelenburg *et al*., 1997). Moreover, the α_{2A}-adrenoceptor mediates endothelium-dependent relaxation of porcine coronary arteries, although the endothelium of this vessel contains both α_{2A}- and α_{2C}-adrenoceptors (Bockman *et al*., 1993). α_{2A}-Adrenoceptors also appear to play the same functional role in the endothelium of other species (Guimarães and Moura, 2001). There is no reason to assume that neuronal α_2-adrenoceptors (namely α_{2A}-adrenoceptors) are involved in contractions in the present studies using porcine pulmonary venous rings. In addition, our functional studies clearly show that endothelial α_{2A}-adrenoceptors are not involved in the contraction. The contractile smooth muscle α_2-adrenoceptor in porcine pulmonary veins is of the α_{2C}-type whereas the porcine endothelial relaxant α_2-adrenoceptor is of the α_{2A}-type. Porcine endothelial and smooth muscle cells are endowed with α_{2A}- and α_{2C}-adrenoceptors, but functionally, both subtypes are linked to different and opposite vascular actions. Furthermore, our data show that the presence of α_{2A}-adrenoceptor mRNA in a particular blood vessel cannot be taken as evidence that the receptor is necessarily involved in the contraction of that vessel (Piascik *et al*., 1995; Civantos Calzada and de Artiñano, 2001).

Contractile α_2-adrenoceptors have recently been identified in porcine nasal veins (Corboz *et al*., 2007). α_{2A}- and α_{2C}-Adrenoceptor subtypes have been shown to be present in porcine nasal mucosa mediating contraction (Corboz *et al*., 2003). Using histological studies, it has also been demonstrated that the contraction in nasal mucosa was due to activation of veins in this tissue (Corboz *et al*., 2007). Thus, both subtypes, α_{2A}- and α_{2C}-adrenoceptors, are present in nasal mucosal veins and in pulmonary veins as well. However, in contrast to nasal mucosa veins from upper airway tissue, in veins from lower airway tissue, pulmonary

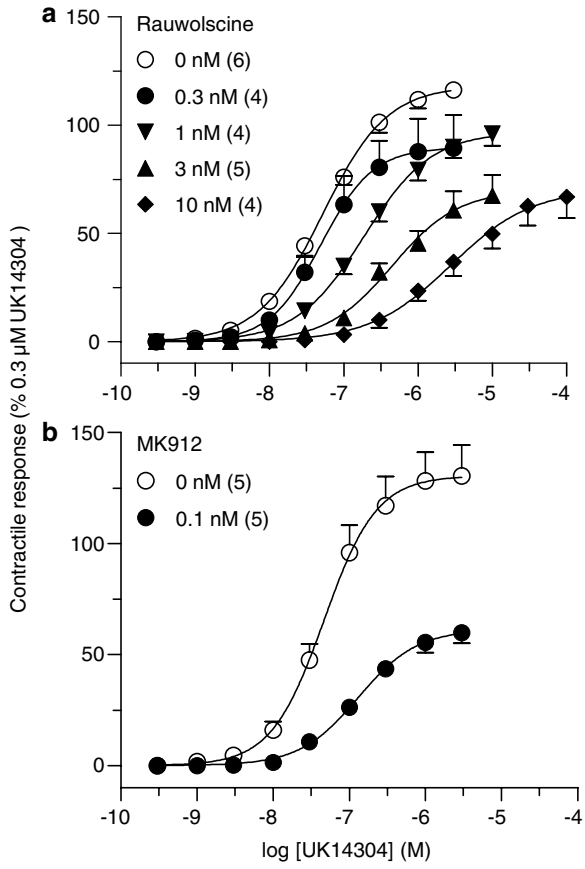

Figure 3 Cumulative log CRCs to UK14304 in the absence or presence of rauwolscine (**a**) and MK912 (**b**) in rings of porcine pulmonary veins. Values are means±s.e.m. (vertical bars) from *n* animals as indicated in parentheses.

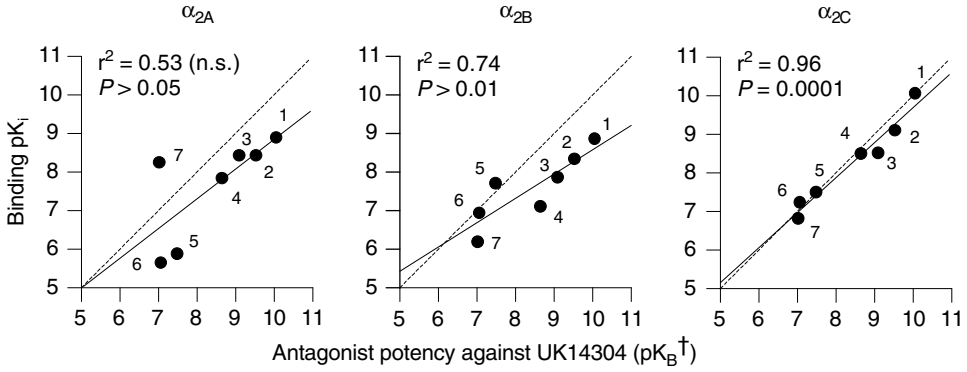

Figure 4 Correlation of antagonist affinity estimates (pK_B; $^{\dagger}pD'_2$ in the case of MK912) against UK14304 at the contractile α_2-adrenoceptor in porcine pulmonary veins and binding affinity (pK_i) at human recombinant α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors. pK_i values were taken from Uhlén *et al*. (1994). The dashed line represents the line of identity and the solid line, the regression line of the plotted points. The numbering of the drugs is as follows: MK912 (1), rauwolscine (2), yohimbine (3), WB4101 (4), ARC239 (5), prazosin (6) and BRL44408 (7).

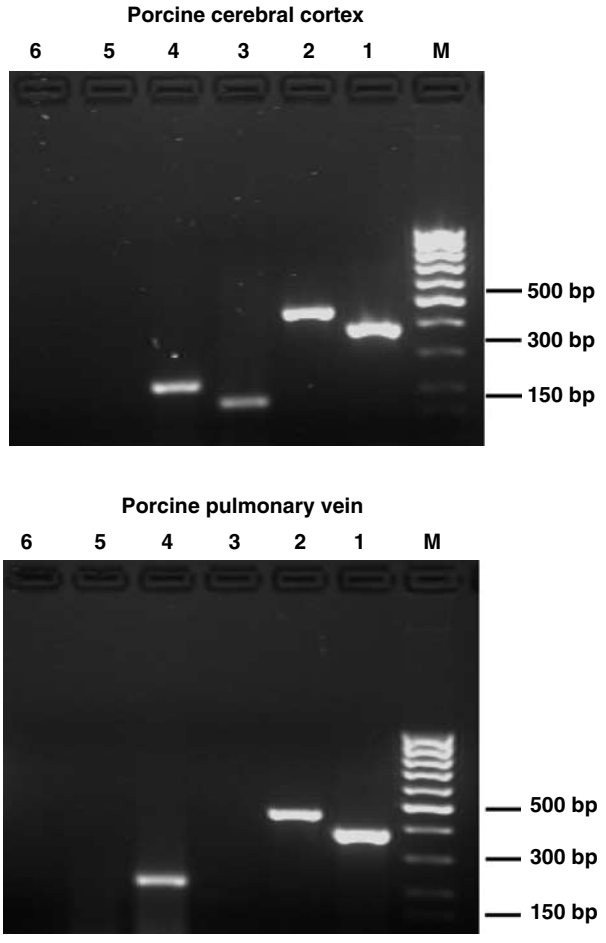

Figure 5 Agarose gel electrophoresis of RT-PCR products showing the presence of mRNA for α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors in pig cerebral cortex (upper panel) and the presence of mRNA for α_{2A}- and α_{2C}-adrenoceptors in porcine pulmonary veins (lower panel). The marked lanes denote: 100 bp DNA ladder (M), positive control showing RT-PCR product of 383 bp using GAPDH primers (1), RT-PCR product of 455 bp obtained using forward and reverse primers of α_{2A}-adrenoceptor (2), RT-PCR product of 179 bp obtained using forward and reverse primers of α_{2B}-adrenoceptor (3), RT-PCR product of 220 bp obtained using forward and reverse primers of α_{2C}-adrenoceptor (4), negative control, that is, a sample without reverse transcriptase to monitor genomic contamination (5) and negative control, that is, a PCR sample without template to monitor contamination of the reaction mixture (6). The size (bp) of three marker bands is indicated in the right margin.

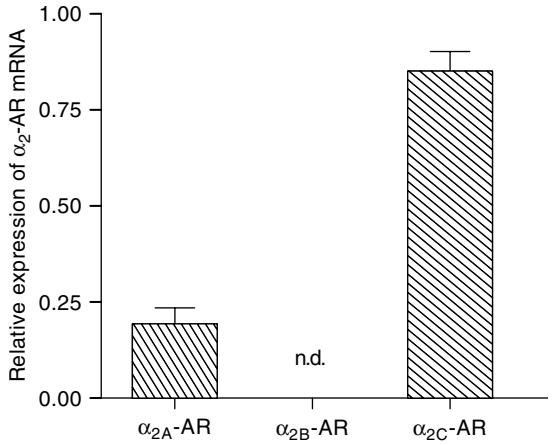

Figure 6 Real-time PCR results showing the relative expression of mRNA for α_{2A}- and α_{2C}-adrenoceptors (AR) compared to GAPDH mRNA. Note that mRNA for the α_{2B}-adrenoceptor was not detectable (n.d.), confirming the RT-PCR results. Values are means$\pm$s.e.m. (vertical bars) from three independent experiments.

veins in the present study, contraction is mediated solely by the α_{2C}-adrenoceptor subtype.

In summary, the present data provide evidence that postjunctional α_{2C}-adrenoceptors play a dominant role in mediating contraction to UK14304 in porcine pulmonary veins; functional and PCR studies rule out an involvement of α_{2A}- and α_{2B}-ARs. Moreover, a component of the contraction induced by noradrenaline or phenylephrine in these blood vessels may be mediated by α_1-adrenoceptors, which should be characterized in further experiments which fall beyond the scope of the present study. As α_2- adrenoceptor-mediated responsiveness is selectively enhanced under conditions of elevated pulmonary vascular tone (Hyman and Kadowitz, 1986; Shebuski *et al.*, 1987), it would be of great interest to ascertain whether selective α_{2C}-adrenoceptor antagonists are beneficial in the treatment of pulmonary venous hypertension and congestive heart failure.

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Conflict of interest

The authors state no conflict of interest.

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