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Analysis of α_{1L}-adrenoceptor pharmacology in rat small mesenteric artery

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1 To illuminate the controversy on α_{1A}- or α_{1L}-adrenoceptor involvement in noradrenaline-mediated contractions of rat small mesenteric artery (SMA), we have studied the effects of subtype-selective α_1-adrenoceptor agonists and antagonists under different experimental conditions.

2 The agonist potency order in rat SMA was: A61603 >> SKF89748-A > cirazoline > noradrenaline > ST-587 > methoxamine. Prazosin antagonized all agonists with a low potency (pA_2: 8.29–8.80) indicating the involvement of α_{1L}- rather than α_{1A}-adrenoceptors.

3 The putative α_{1L}-adrenoceptor antagonist JTH-601, but not the α_{1B}-adrenoceptor antagonist chloroethylclonidine (10 μM) antagonized noradrenaline-induced contractions of SMA. The potency of the selective α_{1D}-adrenoceptor antagonist BMY 7378 against noradrenaline ($pA_2 = 6.16 \pm 0.13$) and of the selective α_{1A}-adrenoceptor antagonist RS-17053 against noradrenaline ($pK_B = 8.35 \pm 0.10$) and against the selective α_{1A}-adrenoceptor agonist A-61603 ($pK_B = 8.40 \pm 0.09$) were too low to account for α_{1D}- and α_{1A}-adrenoceptor involvement.

4 The potency of RS-17053 (pK_B/pA_2's = 7.72–8.46) was not affected by lowering temperature, changing experimental protocol or inducing myogenic tone *via* KCl or U46619.

5 Selective protection of a putative α_{1A}-adrenoceptor population against the irreversible action of phenoxybenzamine also failed to increase the potency of RS-17053 ($pA_2 = 8.25 \pm 0.06$ against A61603).

6 Combined concentration-ratio analysis demonstrated that tamsulosin, which does not discriminate between α_{1A}- and α_{1L}-adrenoceptors, and RS-17053 competed for binding at the same site in the SMA.

7 In summary, data obtained in our experiments in rat SMA indicate that the α_1-adrenoceptor mediating noradrenaline-induced contraction displays a distinct α_{1L}-adrenoceptor pharmacology. This study does not provide evidence for the hypothesis that α_{1L}-adrenoceptors represent an affinity state of the α_{1A}-adrenoceptor in functional assays. Furthermore, there is no co-existing α_{1A}-adrenoceptor in the SMA.

Keywords: A61603; α_1-adrenoceptors; BMY 7378; chloroethylclonidine; noradrenaline; resistance vessels; phenoxybenzamine; prazosin; RS-17053; small mesenteric artery (rat)

Abbreviations: 5-HT, 5-hydroxytryptamine creatine sulphate; A61603, N-[5-(4,5-dihydro-1H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl] methanesulphonamide hydrobromide; BMY 7378, 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride; E/[A], concentration-effect; JTH-601, N-(3-hydroxy-6-methoxy-2,4,5-trimethylbenzyl)-N-methyl-2-(4-hydroxy-2-isopropyl-5-methyl-phenoxy) ethylamine hemifumarate; KHS, Krebs-Henselheit solution; RS-17053, *N*-[2-(2-cyclopropylmethoxyphenoxy)ethyl]-5-chloro-α, α-dimethyl-1*H*-indole-3-ethamine hydrochloride; SCH-23390, R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride; SKF89748-A, 1-5-methylthio-8-methoxy-2-aminotehalin hydrochloride; SMA, small mesenteric artery; ST-587, 2-(2-chloro-5-trifluormethyl-phenylimino)-imidazolin nitrate; U46619, 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$

Introduction

Radioligand binding studies and molecular biology experiments have demonstrated the existence of at least three α_1-adrenoceptor subtypes, now referred to as α_{1A} (previously known as α_{1C}), α_{1B} and α_{1D} (previously also known as α_{1A} or $\alpha_{1A/D}$) (see Hieble *et al*., 1995). These subtypes have been cloned and all display high, subnanomolar, affinities for prazosin. However, functional studies have provided evidence for the existence of an additional α_1-adrenoceptor subtype (α_{1L}), displaying low affinity for prazosin ($pK_B < 9$) and some other α_1-adrenoceptor antagonists, including RS-17053 (Flavahan & Vanhoutte, 1986; Muramatsu *et al*., 1990; Ford *et al*., 1994, 1996). The α_{1L}-adrenoceptor has no molecular correlate, but seems to mediate constriction of the human (Ford *et al*., 1996) and rabbit (Van der Graaf *et al*., 1997; Kava *et al*., 1998) lower urinary tract and rabbit and guinea-pig aorta (Muramatsu *et al*., 1990).

In rat isolated small mesenteric arteries (SMAs; internal diameter 100–300 μm), Högestatt & Andersson (1984) and Nielsen & Mulvany (1990) demonstrated that prazosin antagonizes noradrenaline-mediated contractions with high affinity ($pA_2 = 9.58$–9.84 and 9.23, respectively). Accordingly, it has been suggested that α_{1A}-adrenoceptors predominantly mediate noradrenaline-induced contraction of rat SMA (Chen *et al*., 1996; Ipsen *et al*., 1997). However, Schild analysis demonstrated complex antagonism by prazosin with its potency (pA_2) ranging from 8.8–9.6 and, therefore, additional involvement of α_{1L}-adrenoceptors was suggested (Chen *et al*.,

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1996). Van der Graaf *et al*. (1996) found that despite significant correlation of antagonist affinity values with pK_i values at the cloned α_{1a}-adrenoceptor, the pA_2 value of prazosin in rat SMA (8.5) was more consistent with the profile of the pharmacologically-defined α_{1L}-subtype (Flavahan & Vanhoutte, 1986; McGrath & Wilson, 1988; Ford *et al*., 1994). Adding to the confusion was a recent report that α_{1B}-adrenoceptors mediated contraction in rat SMA (Piascik *et al*., 1997). Thus, the α_1-adrenoceptor subtypes involved in noradrenaline-induced contractions in rat SMA are still controversial.

Using several subtype-selective α_1-adrenoceptor agonists and antagonists in the present investigation, we provide further evidence that the α_1-adrenoceptors mediating contraction of rat SMA are of the α_{1L} subtype. Since Ford and co-workers (1997) have suggested that the α_{1L} subtype may represent a particular conformational state (pharmacological phenotype) of the α_{1A}-adrenoceptor gene product, we have attempted to elaborate on the nature of the observed α_{1L}-adrenoceptor pharmacology under different experimental conditions.

Methods

Rat small mesenteric artery preparation

Male Wistar rats (250–350 g) were anaesthetized (sodium pentobarbitone, 60 mg kg^{-1}, i.p.) and killed by cervical dislocation and the mesentery was removed and placed in ice-cold modified Krebs-Henseleit solution (KHS) of the following composition (mM): NaCl 119.0, $NaHCO_3$ 25.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 5.5, $CaCl_2$ 2.5 and EDTA 0.026. Arterial trees were dissected and cleared from surrounding adipose tissue. As described previously (Mulvany & Halpern, 1977), from each arterial tree a ring segment ($\sim$2 mm in length) was mounted in a myograph (J.P. Trading, Aarhus, Denmark) with separated 6 ml organ baths containing modified KHS at 37°C (or at 27°C for certain experiments; see below). The KHS was continuously gassed with 95% O_2 and 5% CO_2 and tissue responses were measured continuously as changes in isometric force.

Following a 30 min stabilization period, the internal diameter of each vessel was set to a tension equivalent to 0.9 times the estimated diameter at 100 mmHg effective transmural pressure (l_{100} = 200–300 μm) according to the standard procedure of Mulvany & Halpern (1977). The presence of the endothelium was then confirmed with 10 μM of methacholine after a pre-contraction with either 30 μM 5-hydroxytryptamine (5-HT) or 10 μM noradrenaline (see below). Tissues which responded with less than 60% relaxation were rejected.

In all experiments, 60 min prior to construction of each agonist concentration-effect (E/[A]) curve, cocaine (30 μM), timolol (6 μM) and SCH-23390 (10 nM) were added to the KHS to block neuronal uptake, β_1/β_2-adrenoceptors and D_1 receptors, respectively (Van der Graaf *et al*., 1995).

Experimental designs

Single curve design After normalization and a further 30 min stabilization period, a calibration contraction (12.8 $\pm$ 0.5 mN, n = 49) was obtained to 30 μM 5-hydroxytryptamine (5-HT). After confirming the presence of the endothelium, tissues were washed for 30 min and then incubated for 60 min with antagonist or vehicle. Subsequently, a single agonist E/[A] curve was obtained by cumulative dosing at quarter-log unit concentration increments. In the experiments where the antagonism of chloroethylclonidine was investigated, tissues were pre-incubated for 30 min with 10 μM of the drug, followed by a 30-min washout period (ten solution changes).

Paired curve design After standardization of the internal diameter, the preparations were challenged five times with noradrenaline (10 μM) with washouts after each challenge. As described above, the integrity of the endothelium was assessed after the first challenge of noradrenaline. After a first agonist E/[A] curve was obtained (see Results), each tissue segment was washed (30 min) and equilibrated (60 min) with vehicle or different concentrations of antagonist. Subsequently, another agonist E/[A] curve was constructed in the presence of vehicle or antagonist.

Determination of affinity of RS-17053 under different experimental conditions

The antagonist affinity of RS-17053 was determined under the following experimental conditions.

Low bath fluid temperature Single curve design was used at a temperature of 27°C.

Protocol according to Chen et al. *(1996)* The preparations were challenged once with KCl (125 mM) and subsequently three times with a combination of KCl (125 mM) and noradrenaline (10 μM), and once more with KCl (125 mM) with washouts after each challenge. After a first agonist E/[A] curve, each tissue segment was washed for 30 min and then equilibrated for 60 min with vehicle or different antagonist concentrations as described above under Paired curve design. Subsequently, another noradrenaline E/[A] curve was obtained and the responses were expressed as percentage of the fifth noradrenaline challenge which served as calibration contraction.

Depolarization with K^+ before and after incubation of RS-17053 The single curve design was conducted except that noradrenaline E/[A] curves were obtained after partial depolarization by KCl (20 mM). This depolarization by KCl was applied either after or before incubation of the tissues with RS-17053 (0.1 μM).

Pre-contraction with U46619 (10–25 nM) The single curve design was conducted except that after incubation with RS-17053 (0.1 μM), noradrenaline E/[A] curves were obtained on top of a threshold contraction with the thromboxane A_2-mimetic, U46619 (10–25 nM).

Selective protection of α_{1A}-adrenoceptors

In a set of four experiments, after five challenges with noradrenaline (as in the paired curve design) the SMAs were incubated with RS-17053 (2 nM) for 60 min to selectively protect α_{1A}-adrenoceptors. At this concentration, RS-17053 is expected to occupy $\sim$95% of the α_{1A}-adrenoceptor population (based on a pA_2 of 9.9 as observed in the perfused mesentery; Ford *et al*., 1996), whereas it would occupy only $\sim$30% of the α_{1L}-adrenoceptor population (based on a pA_2 of 8.35; see Results). In the presence of RS-17053, the alkylating agent, phenoxybezamine (1 nM), was added for 15 min followed by extensive washing (10 solution changes over 30 min). After a first A61603 E/[A] curve had been obtained, vessel segments were washed (30 min) and equilibrated (60 min) with vehicle or different concentrations of RS-17053 (10, 30 and 100 nM). Subsequently, a second A61603 E/[A] curve was obtained and

the responses were expressed as percentage of fifth noradrenaline challenge, which served as calibration contraction.

Analysis

Individual agonist curve data were fitted to the Hill equation using an iterative, least-squares method:

$$E = \frac{\alpha * [A]^{n_H}}{[A]_{50}^{n_H} + [A]^{n_H}}$$

to provide estimates of midpoint slope (n_H), midpoint location ($[A]_{50}$ estimated as logarithm) and upper asymptote (α). The effect of drug treatment on these parameters was assessed by one-way analysis of variance (ANOVA) or Student's *t*-test, as appropriate. Values of $P<0.05$ were considered to be significant.

When the minimum criteria for competitive antagonism were satisfied, that is the antagonist produced parallel rightward shift of the agonist E/[A] curves with no change in upper asymptote, antagonist affinity estimates were obtained by fitting the individual midpoint location values obtained in the absence ($\log[A]_{50}$) and presence ($\log[A]_{50B}$) of antagonist (B) to the following derivation of the Schild equation (Black *et al*., 1985):

$$\log[A]_{50B} = \log[A]_{50} + \log(1 + [B]^b / 10^{\log K_B}).$$

When the Schild plot slope parameter (b) was not significantly different from unity, then the data were re-fitted with b constrained to unity so that the antagonist dissociation equilibrium constant, K_B, could be estimated as log $K_B \pm$ s.e. (Jenkinson *et al*., 1995). When less than three different concentrations of antagonist were tested or the criteria of competitive antagonism were not completely satisfied, an empirical pA_2 value was estimated using the above equation, with b constrained to unity.

Combined concentration-ratio analysis

In order to test whether RS-17053 and tamsulosin acted at the same site (syntopically), a combined concentration-ratio analysis was performed according to the procedure developed by Shankley and co-workers (1988). Briefly, when two antagonists act syntopically, then their combined concentration-ratio is given by:

$$r_{B+C} = r_B + r_C - 1$$

where r_B and r_C are the concentration-ratios obtained independently in the presence of the antagonists B and C, respectively. This relationship can be re-written in terms of $\log[A_{50}]$ values of the agonist E/[A] curves in the presence and absence of antagonists B and C using the following equation:

$$S_A = \log[A_{50}]_{B+C} - \log([A_{50}]_B + [A_{50}]_C - [A_{50}]),$$

where S_A is the test statistic for the additive model. Thus, if the experimental data comply with the additive model, S_A should have a value of zero. In contrast, when two antagonists act at different sites, that is allotopically, their combined concentration-ratios multiply;

$$r_{B+C} = r_B \cdot r_C$$

and expressed in terms of log $[A_{50}]$ values;

$$S_M = \log[A_{50}]_{B+C} - \log[A_{50}]_B - \log[A_{50}]_C + \log[A_{50}],$$

where S_M is the test statistic for the multiplicative model. If the antagonists behave allotopically, S_M should have a value of zero.

Because the distributions of S_A and its standard estimator are unknown, there is no formal statistical method available to decide in which cases the additive model should be accepted or rejected. In the present study, the null hypotheses (H_0) was formulated as 'B + C act syntopically' and it was assumed that S_A and S_M and their associated standard error estimators are approximately normally distributed. Deviations of S_A and S_M from zero were tested for significance using two- and one-sided *t*-tests, respectively, and H_0 was accepted in cases when $S_A = 0$ and $S_M < 0$. In all other cases H_0 was rejected.

Compounds

Compounds were obtained from the following sources: A61603 (N-[5-(4,5-dihydro-1H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl] methanesulphonamide hydrobromide): Abbott Laboratories, North Chicago, IL, U.S.A.; cocaine hydrochloride, 5-HT, methacholine bromide, l-noradrenaline hydrochloride, methoxamine hydrochloride, phenoxybenzamine hydrochloride and timolol maleate, U46619 (9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$): all from Sigma, Zwijndrecht, The Netherlands; BMY 7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride), chloroethylclonidine dihydrochloride, cirazoline hydrochloride and SCH-23390 (R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride): all from Research Biochemicals Incorporated, Natick, MA, U.S.A.; JTH-601 (N-(3-hydroxy-6-methoxy-2,4,5-trimethylbenzyl)-N-methyl-2-(4-hydroxy-2-isopropyl-5-methyl-phenoxy) ethylamine hemifumarate): Japan Tobacco Company, Tokyo, Japan; RS-17053 (*N*-[2-(2-cyclopropylmethoxyphenoxy)ethyl]-5-chloro-α, α-dimethyl-1*H*-indole-3-ethamine hydrochloride): Roche Bioscience, Palo Alto, CA, U.S.A.; tamsulosin: Yamanouchi Pharmaceutical Co. Ltd., Ibaraki, Tsukuba, Japan; SKF89748-A (1-5-methylthio-8-methoxy-2-aminotehalin hydrochloride): Smith Kline Beecham Pharmaceuticals, King of Prussia, PA, U.S.A.; ST 587 (2-(2-chloro-5-trifluormethyl-phenylimino)-imidazolin nitrate): Boehringer Ingelheim Ltd., Bracknell, Berkshire, U.K. Noradrenaline was dissolved in stoichiometric ascorbic acid solution. Methacholine was dissolved in ethanol. JTH-601 was dissolved in dimethyl sulphoxide as a 10 μM stock solution and further diluted in distilled water. Phenoxybenzamine was dissolved in absolute ethanol. RS-17053 was dissolved in a mixture of 10% dimethylsulphoxide, 20% propylene glycol and 70% distilled water as a 10 μM stock solution and further diluted in distilled water. SKF89748-A was dissolved in a mixture of 50% distilled water and 50% ethanol as a 20 mM stock solution and further diluted in distilled water. U46619 was dissolved initially in 20% ethanol to give a 1 mM stock solution and subsequently diluted in distilled water. All other drugs were dissolved in distilled water.

Results

Potency rank order of α_1-adrenoceptor agonists and effect of the non-selective α_1-adrenoceptor antagonist prazosin

The antagonism of prazosin (30 nM) against several agonists was studied in a paired curve design. All α_1-adrenoceptor agonists used in this investigation contracted rat SMA, displaying either full (noradrenaline, cirazoline, methoxamine, A61603) or partial (SKF89748-A, ST-587) agonism (see Table

1). The potency order (pEC_{50}) of the agonists in rat SMA was: A61603 >> SKF89748-A = cirazoline > noradrenaline > ST-587 > methoxamine. Half of the ST-587 E/[A] curves obtained were fitted with a fixed Hill slope ($n_H = 5$), since these individual curves were extremely steep. Prazosin (30 nM) antagonized the responses to all six agonists and the affinity estimates of prazosin (pA_2: 8.29–8.80), which were consistently lower than those reported at α_{1A}-, α_{1B}- or α_{1D}-adrenoceptor subtypes (Burt *et al*., 1995; Ford *et al*., 1996, 1997), did not differ between agonists (Table 1).

Effect of adrenoceptor antagonists, chloroethylclonidine (α_{1B}) and BMY 7378 (α_{1D})

Noradrenaline produced concentration-dependent contractions of SMAs and the individual E/[A] curves were fitted to the Hill equation to provide estimates of the midpoint location (pEC_{50} = 5.92±0.11), Hill slope ($n_H = 3.1 \pm 0.5$) and upper asymptote ($\alpha = 162 \pm 16\%$ of the 5-HT calibration contraction). Pretreatment of the tissues with 10 μM chloroethylclonidine, a ligand known to irreversibly inactivate α_{1B}-adrenoceptors (see Hieble *et al*., 1995), had no significant effects on the Hill parameters of the noradrenaline E/[A] curve ($pEC_{50} = 5.71 \pm 0.09$, $n_H = 2.5 \pm 0.4$, $\alpha = 162 \pm 13\%$ of the 5-HT calibration contraction (Figure 1, left panel).

In a concentration (100 nM) that is selective for α_{1D}-adrenoceptors (see Goetz *et al*., 1995), BMY 7378 did not shift the E/[A] curves to noradrenaline (data not shown). However, higher concentrations (1 and 10 μM) of BMY 7378 produced a significant rightward shift of the noradrenaline curve (Figure 1, right panel), and a pA_2 value of 6.16±0.13 was estimated. This pA_2 value is much lower than that reported for the α_{1D}-adrenoceptor in rat aorta ($pA_2 = 8.9$; Goetz *et al*., 1995).

Table 1 Hill parameters of different α_{1A}-adrenoceptor agonists and affinity estimates for prazosin in rat SMA ($n = 4-6$)

Agonist	*α (% of 10 μM noradrenaline contraction)*	*pEC_{50}*	*n_H*	*pA_2 prazosin*
Noradrenaline	102±8	6.32±0.11	2.3±0.3	8.50±0.1*
Cirazoline	102±3	6.85±0.08	3.6±0.8	8.44±0.06
Methoxamine	94±4	5.03±0.15	4.6±0.7	8.32±0.11
SKF89748-A	90±4	7.15±0.25	3.9±1.1	8.58±0.15
A61603	109±4	8.15±0.05	2.3±0.3	8.80±0.08
ST-587	47±11	5.56±0.20	4.3±0.4	8.29±0.13

*Reported by Van der Graaf *et al*. (1996).

Effect of selective α_{1A}-adrenoceptor antagonist RS-17053 against noradrenaline and A61603 as agonists

The selective α_{1A}-adrenoceptor antagonist RS-17053 (10–300 nM; Ford *et al*., 1996) also produced concentration-dependent, parallel, rightward shifts of the noradrenaline E/[A] curves. The Schild plot slope parameter (1.14±0.11) was not significantly different from unity and a pK_B of 8.35±0.10 was estimated (Figure 2, upper panels).

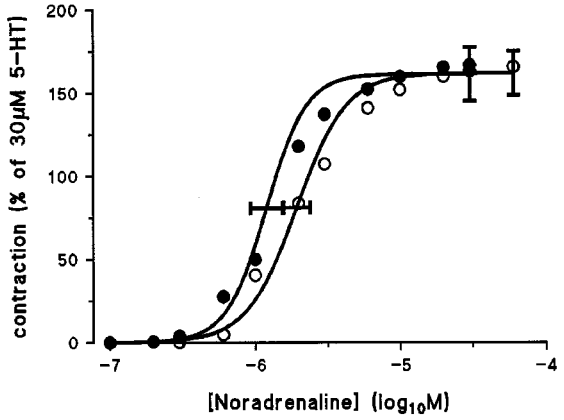
The selective α_{1A}-adrenoceptor agonist A61603 (Knepper *et al*., 1995) behaved as a full agonist with respect to noradrenaline and the Hill parameters were: $pEC_{50} = 7.82 \pm 0.12$, $n_H = 2.60 \pm 0.21$, $\alpha = 149 \pm 6\%$ of the 5-HT calibration contraction (Figure 2, lower panels). RS-17053 (10–300 nM) also competitively antagonized the A61603-induced contractions ($b = 1.14 \pm 0.09$) and a $pK_B = 8.40 \pm 0.09$ was estimated.

Effect of putative α_{1L}-adrenoceptor antagonist JTH-601 against noradrenaline as agonist

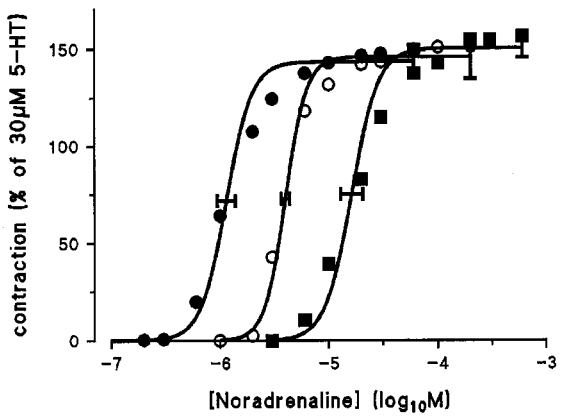
Previously, JTH-601 was demonstrated to have a ~10 times higher affinity than prazosin for the α_{1L}-adrenoceptor, whereas both compounds displayed equal binding affinities for the α_{1A} receptor subtype (Muramatsu *et al*., 1996). In the SMA, JTH-601 (3–100 nM) produced rightward shifts of the noradrenaline E/[A] curves (Figure 3). However, the shift did not occur in a concentration-dependent manner, since the concentration-ratios obtained with 10 and 30 nM JTH-601 were practically identical (Figure 3). From the shifts obtained with 3 and 10 nM a pA_2 value of 8.34±0.16 was estimated for the high affinity component.

Effect of experimental conditions on the affinity estimate of RS-17053

It was recently suggested that the α_{1L}-adrenoceptor, instead of being a distinct molecular entity, might represent a conforma-



0 (●); 10 (O) μM CEC



0 (●); 1 (O); 10 (■) μM BMY 7378

Figure 1 Concentration-effect curves to noradrenaline in rat small mesenteric artery in the absence or presence of chloroethylclonidine (left panel; $n = 3$) and BMY 7378 (right panel; $n = 4$). The lines shown superimposed on the mean data points were simulated using the Hill equation.

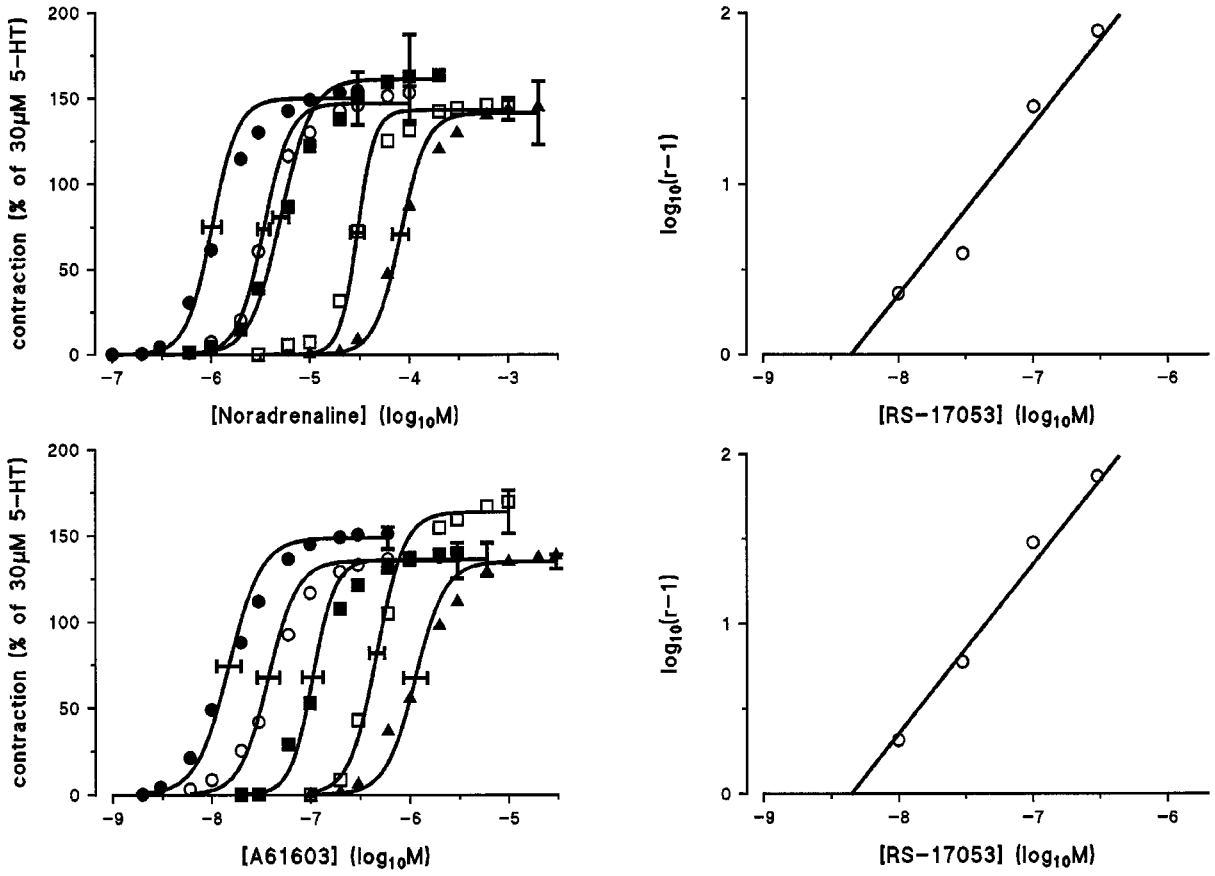


Figure 2 Left panels. Concentration-effect curves to noradrenaline (upper panel; $n=5$) and A61603 (lower panel; $n=5-6$) obtained on rat SMA in the absence or presence of RS-17053. The lines superimposed on the mean data points were simulated using the Hill equation. Right panels. Schild plots for the interaction of RS-17053 with noradrenaline (upper panel) and A61603 (lower panel). The solid lines superimposed on mean data points were simulated using the parameters obtained from the constrained model fits.

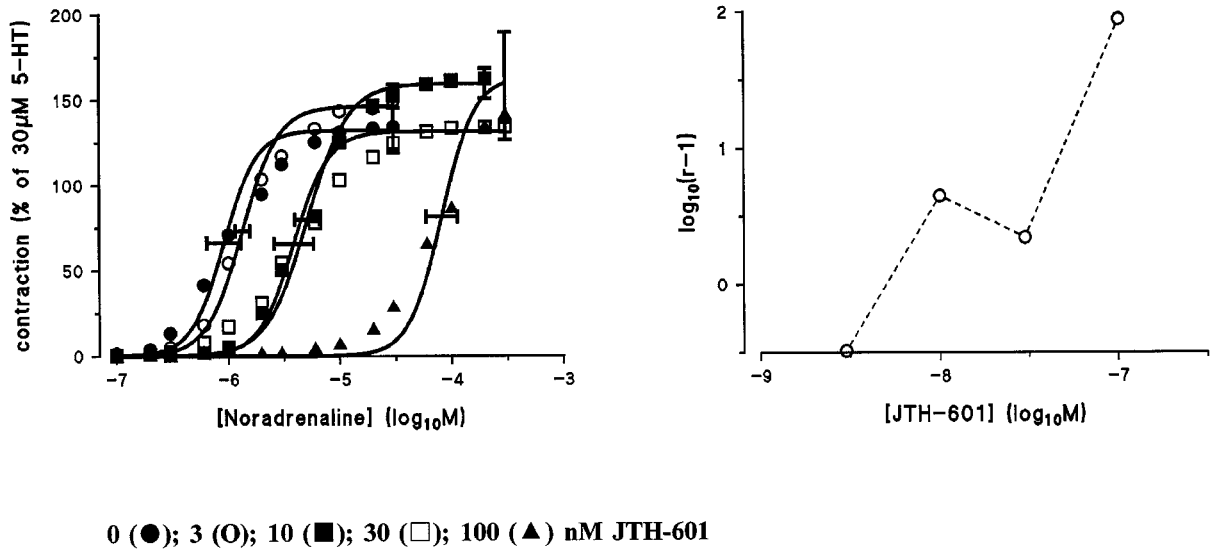


Figure 3 Left panel. Concentration-effect curves to noradrenaline obtained on rat SMA in the absence or presence of JTH-601 ($n=5$). The lines superimposed on the mean data points were simulated using the Hill equation. Right panel. Schild plot for the interaction of JTH-601 with noradrenaline.

tional affinity state of the α_{1A}-adrenoceptor and that it is possible to switch the pharmacological α_{1L}-adrenoceptor profile into an α_{1A}-profile by changing experimental conditions (Williams *et al.*, 1996). Therefore, we studied the antagonizing potency of RS-17053 under different experimental conditions (see Table 2).

Low bath fluid temperature When temperature was lowered to 27°C, noradrenaline still produced concentration-dependent contractions of the SMAs. RS-17053 (10–100 nM) behaved as a competitive antagonist ($b = 0.98 \pm 0.16$) with an estimated affinity ($pK_B = 8.42$) that was similar to that obtained under standard conditions (Table 2).

Protocol according to Chen et al. *(1996)* In a recent study, Chen *et al*. (1996), concluded that noradrenaline-induced contraction of the SMA involves predominantly α_{1A}-adrenoceptors. In experiments carried out according to their experimental protocol (see Methods for details), RS-17053 (10–100 nM) again caused a parallel rightward shift ($b = 0.95 \pm 0.23$) and displayed a similar affinity as under standard conditions ($pK_B = 8.46$; Table 2).

Depolarization with K^+ before and after incubation of RS-17053 Partial depolarization by KCl (20 mM) after pre-incubation with RS-17053 induced a threshold contraction of $4.7 \pm 0.7\%$ of the 5-HT calibration contraction. Under these conditions RS-17053 (0.1 μM) behaved as a competitive antagonist. The pA_2 value (7.72 ± 0.26; Table 2) was slightly lower compared to standard conditions, but due to a large between-tissue variability (95% confidence interval: ± 0.63) this difference was not statistically significant. The notable large variance could indicate perturbation of the equilibrium between antagonist and receptor by 20 mM KCl. Therefore, a threshold contraction ($6.1 \pm 1.1\%$ of 5-HT calibration contraction) by partial depolarization with KCl (20 mM) was induced before the 60 min pre-incubation with RS-17053 (0.1 μM). Co-equilibration of RS-17053 and KCl (20 mM) decreased the variance (95% confidence interval: ± 0.33), but did not significantly affect the affinity estimate of RS-17053 ($pA_2 = 8.31 \pm 0.16$; Table 2).

Pre-contraction with U46619 (10–25 nM) In the presence of a threshold contraction induced by 10–25 nM U46619 ($14.7 \pm 0.8\%$ of the 5-HT calibration contraction), RS-17053 (0.1 μM) unexpectedly caused a significant flattening of the noradrenaline E/[A] curve ($n_H = 0.9 \pm 0.1$ and 1.4 ± 0.1, respectively, with or without RS-17053; $P < 0.05$). However, the estimated pA_2 value (7.87 ± 0.33, Table 2) was not significantly different from the affinity of RS-17053 estimated under standard conditions (95% confidence interval: ± 0.80).

Selective protection of α_{1A}-adrenoceptors

If the α_{1A}- and α_{1L}-adrenoceptor are distinct subtypes, both might co-exist in rat SMA, but different experimental set-ups might favour the exhibition of one over the other type. After selective protection of the putative α_{1A}-adrenoceptor population from inactivation by phenoxybenzamine (see Methods), the affinity of RS-17053 against A61603 was assessed in a paired curve design. Hill slope parameters of the first A61603 E/[A] curve were: $n_H = 2.3 \pm 0.5$, $\alpha = 69.3 \pm 4.5\%$ of the calibration contraction, $pEC_{50} = 6.37 \pm 0.10$. RS-17053 (10–100 nM) caused a rightward shift of the A61603 E/[A] curve. Notwithstanding a significant steepening of the A61603 E/[A] curve ($n_H = 3.21 \pm 0.26$; $P < 0.05$) with RS-17053 (100 nM), Schild analysis was performed (Figure 4). The Schild slope parameter was not significantly different from unity ($b = 1.04 \pm 0.16$) and the estimated pA_2 (8.25 ± 0.06) was practically identical to the potency in untreated tissues ($pK_B = 8.40 \pm 0.09$; Figure 2).

Combination of RS-17053 and tamsulosin

The previously demonstrated susceptibility of the affinity estimate of RS-17053 but not of tamsulosin to experimental conditions (Williams *et al*., 1996) might indicate that RS-17053 and tamsulosin act at different sites of α_{1A}-adrenoceptors. A combined concentration-ratio analysis experiment was designed to test whether RS-17053 and tamsulosin act syntopically in rat SMA. As shown in Figure 5, both RS-17053 and tamsulosin produced a parallel rightward shift of the noradrenaline E/[A] curve (concentration-ratio = 17.5 ± 8.9

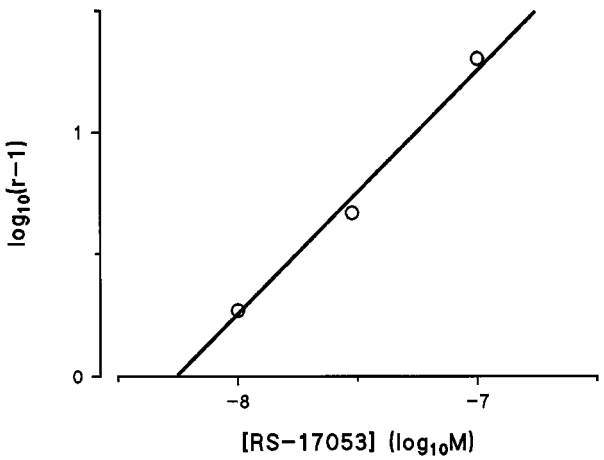


Figure 4 Schild plot for the interaction of RS-17053 with A61603 after selective protection of α_{1A}-adrenoceptors with RS-17053 (2 nM, 60 min) from inactivation by phenoxybenzamine (1 nM, 15 min); $n = 4$ (for details, see Methods). The solid line superimposed on mean data points was simulated using the parameters obtained from the constrained model fit. Please note that the E/[A] curves have been omitted from the figure because they showed considerable variability due to unpredictable extent of receptor inactivation by phenoxybenzamine in individual segments.

Table 2 Effect of experimental protocol on the Hill equation parameters of noradrenaline and affinity estimates for RS-17053 in rat SMA

Experimental protocol (see Methods for details)	*Pre-contraction (% 30 μM 5-HT)*	*Hill equation parameters noradrenaline* α *(% of 30 μM 5-HT or 10 μM noradrenaline†)*	pEC_{50}	n_H	pK_B (pA_2) *RS-17053*
Standard*	–	162±16	5.92±0.11	3.1±0.5	8.35±0.10
Low bath fluid temperature (27°C) ($n=4$)	–	142±8	5.86±0.10	3.8±0.8	8.42±0.11
Protocol according to Chen *et al.* (1996) ($n=3$)	–	99±1†	6.01±0.16	2.9±0.07	8.46±0.09
Depolarization with K^+ after RS-17053 ($n=5$)	4.7±0.7	120±6	6.12±0.14	1.2±0.1	(7.72±0.26)
Depolarization with K^+ before RS-17053 ($n=7$)	6.1±1.1	112±4	6.53±0.09	1.6±0.2	(8.31±0.16)
Pre-contraction with U46619 (10–25 nM) ($n=5$)	14.7±0.8	149±8	6.65±0.17	1.4±0.1	(7.87±0.33)

Data are mean±s.e.mean. *Data from Figure 2.

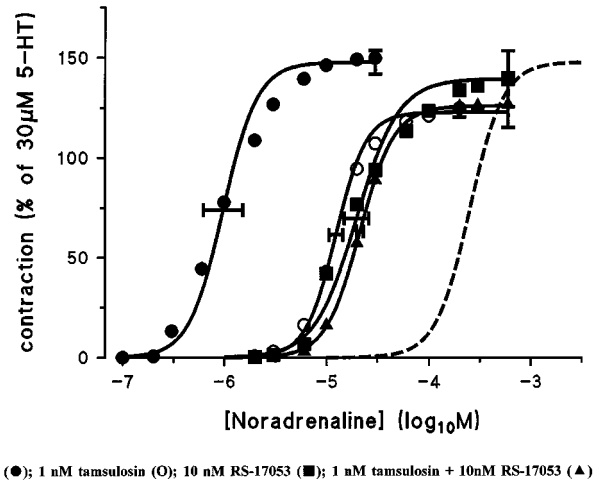


Figure 5 Combined concentration-ratio analysis: concentration-effect curves to noradrenaline obtained on rat SMA in the absence or presence of 100 nM RS-17053, 1 nM tamsulosin or both 100 nM RS-17053 and 10 nM tamsulosin ($n=3$ each). The lines shown superimposed on the mean data points were simulated using the Hill equation. The dashed line shows the location of the concentration-effect curve which was predicted by assuming that the antagonists acted allotopically.

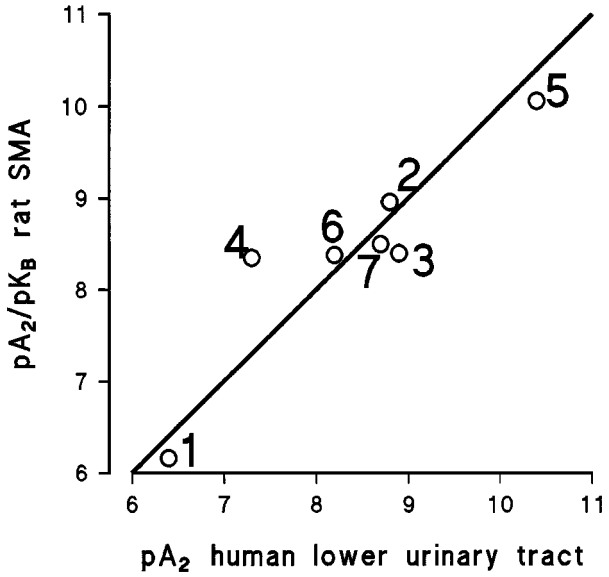


Figure 6 Relation between pA_2 estimates in human lower urinary tract (Ford *et al*., 1996) and pK_B/pA_2 estimates in rat SMA, determined against noradrenaline (this study and Van der Graaf *et al*., 1996) for (1) BMY 7378, (2) HV 723, (3) prazosin, (4) RS-17053, (5) tamsulosin, (6) 5-methylurapidil, (7) WB4101. The solid line represents the line of identity.

and 20.8 ± 3.4, respectively; $pA_2 = 8.29 \pm 0.22$ and 10.06 ± 0.20, respectively). The potency of tamsulosin was in accordance with a previous reported value in rat SMA (9.8; Van der Graaf *et al*., 1996) and with its reported affinity for α_{1L} and α_{1A}-adrenoceptors (10–10.5; Van der Graaf *et al*., 1996). Combined concentration-ratio analysis indicated that RS-17053 (100 nM) and tamsulosin (1 nM) competed for binding to the same site, since the test statistic S_A for the additive model ($S_A = -0.16 \pm 0.09$) was not significantly different from 0 ($P > 0.05$), whereas the test statistic S_M for the multiplicative model was significantly smaller than 0 ($S_M = -1.07 \pm 0.24$; $P < 0.05$).

Discussion

The official nomenclature of α_1-adrenoceptors recognizes α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors, which have all been cloned and which all display high, subnanomolar affinity for prazosin (Hieble *et al*., 1995). Based on functional studies, an alternative classification scheme exists, which recognizes α_{1H}- and α_{1L}-adrenoceptors displaying high (α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors) and low affinity for prazosin, respectively (Flavahan & Vanhoutte, 1986; McGrath & Wilson, 1988; Muramatsu *et al*., 1990; Ford *et al*., 1994). Because of the reported high (>9.2) or low (<8.5) affinity of prazosin, the involvement of either α_{1A}- or α_{1L}-adrenoceptors in rat isolated SMA, which is believed to represent resistance vessels (Mulvany & Aalkjaer, 1990; Christensen & Mulvany, 1993; Fenger-Gron *et al*., 1995), is controversial (Högestätt & Andersson, 1984; Nielsen & Mulvany, 1990; Chen *et al*., 1996; Van der Graaf *et al*., 1996). The present study has further examined this controversy using prazosin and several recently discovered, selective α_1-adrenoceptor antagonists under different experimental conditions.

Involvement of α_{1L}-adrenoceptor in the contraction of rat SMA

The low affinity of prazosin ($pA_2 = 8.29 - 8.80$) in rat SMA proved to be agonist independent (Table 1) and indicated α_{1L}-adrenoceptor involvement (Muramatsu *et al*., 1990). It may be noted that the affinity of prazosin in our experiments with intact endothelium did not differ from that found in rat SMA denuded of endothelium ($pA_2 = 8.5$; Van der Graaf *et al*., 1996). The potency rank order of the agonists SKF89748-A > cirazoline > noradrenaline > ST-587 > methoxamine (Table 1) was similar to that observed for the cloned α_{1a}-subtype (Minneman *et al*., 1994), except for SKF89748-A which was less potent than both cirazoline and noradrenaline at the α_{1a}-adrenoceptor. A lack of effect of chloroethylclonidine (10 μM), which in this concentration inactivates rat α_{1B}-adrenoceptors (Michel *et al*., 1993; Sugden *et al*., 1996), and the low potency of the potent and selective α_{1D}-adrenoceptor antagonist BMY 7378 (pK_i for rat cloned α_{1d}-adrenoceptors = 8.2; Goetz *et al*., 1995) excluded the involvement of α_{1B}- and α_{1D}-adrenoceptors, respectively, in the noradrenaline-induced contraction of rat SMA (Figure 1). Moreover, the affinity of another putative α_{1B}-adrenoceptor antagonist (+)-cyclazosin (Giardina *et al*., 1996) in rat SMA ($pK_B = 7.78$) did not indicate α_{1B}-adrenoceptor involvement either (Stam *et al*., 1998).

The affinity of the selective α_{1A}-adrenoceptor antagonist RS-17053 (Ford *et al*., 1996) against noradrenaline ($pK_B = 8.35$) and against the selective α_{1A}-adrenoceptor agonist A61603 ($pK_B = 8.40$) was too low (see Figure 2) to account for α_{1A}-adrenoceptor involvement (pK_i for α_{1A}-adrenoceptors in rat submaxillary gland = 9.1 and pA_2 in the perfused mesentery = 9.9; Ford *et al*., 1996). Interestingly, JTH-601 caused a complex shift of the noradrenaline E/[A] curve (Figure 3) in rat SMA. However, functional data for JTH-601 on α_{1A}-adrenoceptors are required in order to assess the nature of this complex behaviour.

Is the α_{1L}-adrenoceptor a conformational state of α_{1A}-adrenoceptor?

The affinity of RS-17053 in rat SMA was 35 fold lower in the present experiments than that reported by Ford and colleagues for antagonizing pressor responses to noradrena-

line in the perfused mesentery ($pK_B = 9.9$; Ford *et al*., 1996); the latter being in agreement with functional affinity estimates for α_{1A}-adrenoceptors in rat perfused kidney ($pA_2 = 9.8$; Ford *et al*., 1996) and rat vas deferens ($pA_2 = 9.5$; Marshall *et al*., 1996). Therefore, it appears that α_{1A}-adrenoceptors mediate the pressor response in rat perfused mesentery, whereas noradrenaline-induced contraction in rat isolated SMA is mediated by a different type of α_1-adrenoceptor, possibly α_{1L}. One explanation for this discrepancy is that the pressor response in the perfused mesentery to noradrenaline reflects resistance changes in distal arterioles, which were shown to co-determine vascular resistance (Fenger-Gron *et al*., 1997).

Alternatively, the α_{1L}-adrenoceptor in the SMA assay might be a pharmacological phenotype of the α_{1A}-adrenoceptor subtype (Ford *et al*., 1997). Functional studies in rat vas deferens (Ohmura *et al*., 1992; Prins *et al*., 1992; Burt *et al*., 1995; Guh *et al*., 1995; Chess-Williams *et al*., 1996; Muramatsu *et al*., 1996), portal vein (Digges & Summers, 1983; Chess-Williams *et al*., 1996; Green *et al*., 1996) and human lower urinary tract (see Hieble & Ruffolo, 1996), where the presence of both α_{1A}- and α_{1L}-adrenoceptor has been claimed on the basis of prazosin affinity, have now produced a range of affinities for RS-17053. The high affinity for RS-17053 in rat vas deferens ($pK_B = 9.5$; Marshall *et al*., 1996) and perfused mesentery ($pA_2 = 9.9$; Ford *et al*., 1996) indicated α_{1A}-adrenoceptor involvement. However, an α_{1L}-adrenoceptor displaying a 250 fold lower potency for RS-17053 was found in rat portal vein ($pK_B = 7.1$; Marshall *et al*., 1996), human lower urinary tract ($pA_2 = 7.3$; Ford *et al*., 1996) and prostate ($pA_2 = 7.2$; Marshall *et al*., 1996). Interestingly, apart from RS-17053, the affinity estimates of different antagonists in the SMA are in good agreement with those determined in human lower urinary tract (Figure 6). The affinity of RS-17053 in rat SMA ($pK_B = 8.35$) is more in accordance with an intermediate affinity value demonstrated in the prostatic portion of rat vas deferens by Burt and colleagues ($pA_2 = 8.3$; 1998). Furthermore, accumulation of [^{3}H]-inositol phosphates by cells expressing the human α_{1a}-adrenoceptor was antagonized by RS-17053 with similar intermediate affinity ($pA_2 = 8.3$; Ford *et al*., 1997). Consequently, the authors postulated that this α_{1L}-adrenoceptor was an affinity state of the α_{1A}-adrenoceptor gene product. Taken together, these observations indicate that the structurally defined α_{1A}-adrenoceptor either presents itself functionally as, or consists of, at least three different subtypes which can be discriminated by RS-17053. Indeed, in radioligand binding studies a complete switch from an α_{1L}-adrenoceptor pharmacological profile into an α_{1A}-adrenoceptor profile could be induced by changing experimental conditions, which included (i) a decrease in temperature from 37 to 20°C, (ii) the use of TRIS/EDTA buffer instead of Ham's buffer and (iii) the disruption of cells into membranes (Williams *et al*., 1996).

Therefore, we found it of interest to study whether a switch in the state of affinity of RS-17053 can be established in functional studies with rat SMA (see Table 2). For obvious reasons, in such studies one cannot employ TRIS/EDTA buffer or cell membranes as used in the radioligand binding assay (Williams *et al*., 1996). However, we determined the affinity of RS-17053 at a lower bath temperature. The pK_B estimate of RS-17053 in the SMA was unaffected by decreasing the temperature from 37 to 27°C. Experiments carried out according to the protocol of Chen and co-workers (1996) demonstrated simple competitive antagonism and also yielded an affinity estimate for RS-17053 similar to that obtained under standard conditions and thus incompatible with the suggested α_{1A}-adrenoceptor involvement (Chen *et al*., 1996). Interestingly, high affinities for RS-17053 have been estimated in perfused assays, like rat kidney ($pA_2 = 9{,}8$ Ford *et al*., 1996), mesentery ($pA_2 = 9.9$, Ford *et al*., 1996) or hind limb ($pA_2 = 9.47$; Zhu *et al*., 1997). The spontaneous development of myogenic tone in perfused vessels might be a major experimental difference with the SMA preparation (Dunn *et al*., 1994). We induced myogenic tone in rat SMA by either partial depolarization with KCl (20 mM) or by a threshold contraction with the thromboxane A_2-mimetic, U46619. U46619 was selected, since thromboxane A_2 is produced by the endothelium, a tissue which function varies upon perfusion (Furchgott & Vanhoutte, 1989). Interestingly, the induction of myogenic tone modified the shape and location of the noradrenaline E/[A] curves similar to that observed in the rat and rabbit pressurized perfused SMA (Buus *et al*., 1994; Dunn *et al*., 1994), but did not affect the antagonizing potency for RS-17053 (Table 2).

Do low affinity (α_{1L}-) and high affinity (α_{1A}-adrenoceptors) sites co-exist in rat SMA

By selective inactivation of the α_{1L}-adrenoceptors with phenoxybenzamine while protecting α_{1A}-adrenoceptors, we attempted to unmask a putative α_{1A}-adrenoceptor population in rat SMA. However, Schild analysis demonstrated a single receptor again displaying low affinity for RS-17053 ($pA_2 = 8.25$). Therefore, it is unlikely that α_{1A}- and α_{1L}-adrenoceptors co-exist as distinct subtypes in rat SMA.

Observations from previous reports led to the idea that the α_{1A}-adrenoceptor antagonists, tamsulosin and RS-17053, might act at different sites at the α_1-adrenoceptor, which display differential susceptibility for affinity changes. For example, experimental conditions influenced the binding affinities of, among others, RS-17053 and prazosin, whereas that of tamsulosin and indoramin remained unaffected (Williams *et al*., 1996). Accordingly, tamsulosin displayed similar affinities for functional α_{1A}-adrenoceptors and α_{1L}-adrenoceptors (Ford *et al*., 1996). Combined concentration-ratio analysis, however, indicated that RS-17053 and tamsulosin compete for binding to the α_1-adrenoceptor site in rat SMA, which indicates that both α_1-adrenoceptor antagonists act syntopically.

In summary, data obtained in our experiments in rat SMA indicate that (i) the α_1-adrenoceptor mediating noradrenaline-induced contraction displays a distinct α_{1L}-adrenoceptor pharmacology, where both prazosin and RS-17053 have a low affinity; (ii) the affinity of α_{1L}-adrenoceptor for RS-17053 is not affected by changes in experimental conditions; (iii) it is unlikely that there is a co-existing α_{1A}-adrenoceptor population and (iv) tamsulosin, which does not discriminate between α_{1A}- and α_{1L}-adrenoceptors, acts at the same site as RS-17053. Overall, this study does not provide evidence for the hypothesis that α_{1L}-adrenoceptors represent an affinity state of the α_{1A}-adrenoceptor in functional assays (Ford *et al*., 1997).

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