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RESEARCH PAPER

Effects of ρ-Da1a a peptidic α_{1A}-adrenoceptor antagonist in human isolated prostatic adenoma and anaesthetized rats

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Part of this work has been presented in abstract form.

S Palea, N Gilles, V Guilloteau, M Guérard, P Lluel. Comparison of the effects of AdTx1 and tamsulosin on the increase in intra-urethral and arterial pressures induced by phenylephrine in anesthetized male rats. World Congress of Basic and Clinical Pharmacology, Copenhagen (Denmark) July 2010.

Keywords
anaesthetized rats; intraurethral pressure; arterial pressure; human prostatic adenoma; snake toxin; benign prostatic hyperplasia; α_{1A}-adrenoceptors

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BACKGROUND AND PURPOSE
ρ-Da1a, a 65 amino-acid peptide, has subnanomolar affinity and high selectivity for the human α_{1A}-adrenoceptor subtype. The purpose of this study was to characterize the pharmacological effects of ρ-Da1a on prostatic function, both *in vivo* and *in vitro*.

EXPERIMENTAL APPROACH
ρ-Da1a was tested as an antagonist of adrenaline-induced effects on COS cells transfected with the human α_{1A}-adrenoceptor as well as on human isolated prostatic adenoma obtained from patients suffering from benign prostatic hyperplasia. Moreover, we compared the effects of ρ-Da1a and tamsulosin on phenylephrine (PHE)-induced increases in intra-urethral (IUP) and arterial pressures (AP) in anaesthetized rats, following i.v. or p.o. administration.

KEY RESULTS
On COS cells expressing human α_{1A}-adrenoceptors and on human prostatic strips, ρ-Da1a inhibited adrenaline- and noradrenaline-induced effects. In anaesthetized rats, ρ-Da1a and tamsulosin administered i.v. 30 min before PHE significantly antagonized the effects of PHE on IUP. The pK_B values for tamsulosin and ρ-Da1a for this effect were similar. With regards to AP, ρ-Da1a only reduced the effect of PHE on AP at the lowest dose tested (10 $\mu g \cdot kg^{-1}$), whereas tamsulosin significantly reduced PHE effects at doses between 10 and 150 $\mu g \cdot kg^{-1}$.

CONCLUSIONS AND IMPLICATIONS
ρ-Da1a exhibited a relevant effect on IUP and a small effect on AP. In contrast, tamsulosin antagonized the effects of PHE on both IUP and AP. We conclude that ρ-Da1a is more uroselective than tamsulosin. ρ-Da1a is the most selective peptidic antagonist for α_{1A}-adenoceptors identified to date and could be a new treatment for various urological diseases.

Abbreviations

AP, arterial pressure; BPH, benign prostatic hyperplasia; CRC, concentration-response curves; DR, dose ratio; IUP, intra-urethral pressure; PHE, phenylephrine

Introduction

Toxins active on GPCR are only found in two venomous animals. Marine cone snails produce toxins acting on vasopressin, neurotensin and adrenoceptors (Nielsen *et al.*, 1994; Craig *et al.*, 1999; Sharpe *et al.*, 2001). Snakes produce sarafotoxins, which are active at endothelin receptors, muscarinic toxins, which act on muscarinic receptors or β-cardiotoxin, which acts on β-adrenoceptors (Servent and Fruchart-Gaillard, 2009). Since animal venoms are likely to represent a novel source of new GPCRs ligands, we screened mamba snake venom for peptides binding to α_1-adrenoceptors. Using a combination of liquid chromatography and binding experiments, we identified ρ-Da1a (previously known as AdTx1) the first peptide to show high affinity and selectivity for the human α_{1A}-adrenoceptor subtype (Quinton *et al.*, 2010). Adrenoceptors are divided into three classes (α_1, α_2 and β), and α_1-adrenoceptors into three subtypes (α_{1A}, α_{1B} and α_{1D}). These receptors are involved in the regulation of urogenital tissue muscle tone (α_{1A}), myocardial contractility (α_{1B}) and vascular tone (α_{1D}; Koshimizu *et al.*, 2003). Due to its potency and selectivity, ρ-Da1a is a new pharmacological tool, which could be useful to clarify the physiological functions of α_1-adrenoceptors in animal models. Moreover, this peptide could be the basis for the synthesis of new α_1-adrenoceptor antagonists that could be used to treat lower urinary tract symptoms secondary to benign prostatic hyperplasia (BPH).

Following an in depth biochemical characterization and a limited functional study on rabbit isolated prostatic smooth muscle (Quinton *et al.*, 2010), the first aim of this study was to test the antagonistic potency of ρ-Da1a on human isolated prostatic adenoma contractility. Moreover, we compared the *in vivo* activity of ρ-Da1a in anaesthetized rats on intra-urethral (IUP) and arterial blood pressure (AP) increases induced by i.v. administration of phenylephrine (PHE). As a reference compound, we used tamsulosin, one of the most potent α_{1A}-adrenoceptor antagonists known and the gold standard for the symptomatic treatment of lower urinary tract symptoms secondary to BPH in humans (Chapple and Andersson, 2002).

Methods

Safety panel of ρ-Da1a

ρ-Da1a (1 μM) was tested as an agonist and as an antagonist on 78 human GPCRs and on eight ion channels implicated in cardiac activity (hERG, hHCN4, hKCNQ1/minK, hKir2.1, hKv1.5, hKv4.3/hKChIP2, Human L-type Calcium, hNav1.5). These tests were performed in duplicate by Millipore (Dundee, UK).

Functional tests on recombinant α_1-adrenoceptor subtypes

Functional tests were performed in 96-well plates on COS cells (100,000 cells per well) transfected using calcium phosphate precipitation by α_{1A}-adrenoceptor receptor plasmid (pRK5, 0.1 μg per well, kindly provided by Michael Brownstein, Craig Venter Institute, Rockville, MD, USA). Forty-eight hours after transfection, cells were incubated for 1 h at 37°C with 10 μL of 10X solutions of the various compounds tested and 100 μL of dye solution (FLIPR Calcium Kit buffer, Molecular Devices, Sunnyvale, CA, USA) supplemented with 2.5 mM probenecid. Changes in fluorescence induced by adrenaline were measured at room temperature using a Flexstation II (Molecular Devices). The resulting activation curves obtained were analysed using Softmax Pro software (Molecular Devices, Sunnyvale, CA, USA). Receptor activation for the different experimental conditions was evaluated using maximum and minimum fluorescence values and analysed with GraphPad Prism® (GraphPad Software, La Jolla, CA, USA).

Antagonism of NA-induced contractions on human isolated prostatic smooth muscle

Human prostatic adenoma specimens were obtained from four male patients (age range 68–76 years). These patients (negative for HIV1-2, HTLV1-2, hepatitis B-C and syphilis) had undergone transvesical prostatectomy for BPH in the Urology Department of Rangueil Hospital (Toulouse, France) or Foch Hospital (Suresnes, France). These tissues were donated following patients written informed consent. UROsphere obtained, from the Urology Department, an anonymous patient sheet containing information on patient's age, sex, body weight, height, anaesthetics used during surgery, nature of drugs administered in the 1 month period before hospitalization.

The specimens, which appeared macroscopically healthy, were placed in a cold storage solution (Custodiol®, OPi-France, Limonest, France) immediately after surgery and transported to UROsphere's laboratories in a box maintained at 4°C. The shipment between Hospital and UROsphere's laboratories was carried out by a French courier (Trans-Medical-Services, Montreuil, France). Upon receipt, tissues were stored at 4°C until the start of the experiment (maximum 24 h after surgery).

Prostatic strips (approximate size 8 mm × 3 mm) from the transition zone (12 from each patient) were dissected and mounted, under 1.0 g of initial tension, in organ baths containing a Krebs–Henseleit solution of the following composition (in mM): NaCl 114, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.7 and ascorbic acid 1.1 (pH 7.4, gassed with 95%O_2 and 5%CO_2 at 37°C). The Krebs also contained propranolol (1 μM), desipramine (0.1 μM), deoxycorticosterone (3 μM) and normetanephrine (1 μM) in order to block β-adrenoceptors, neuronal and extraneuronal uptake and catechol-O-methyltransferase respectively.

Five experimental groups (n = 5–10 per group) in four patients were performed, one for the vehicle and four for ρ-Da1a-treated strips, at four different concentrations. A total of 43 strips were used for the experiments.

After 60 min of equilibration, smooth muscle strips were exposed to 30 μM NA to measure their viability. Strips having a contractile response <0.3 g were discarded (n = 5). After washout and 60 min of re-equilibration, ρ-Da1a at four concentrations (10, 30, 100 and 300 nM) or its vehicle (distilled water) were incubated for 3 h, then a cumulative concentration-response curve (CRC) to NA (0.1–1000 μM) was performed. Only one concentration of ρ-Da1a was tested on each single strip.

In separate experiments, the strips were pre-incubated with tamsulosin (10 nM) or its solvent for 1 h before obtaining a cumulative CRC to NA.

Effects of ρ-Da1a and tamsulosin on the increase in IUP and AP induced by PHE in anaesthetized rats

All experimental protocols were carried out in accordance with guidelines for animal care published by the European Community Council Directive 86/609/EEC and the French veterinary service. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Sixty-seven adult male Wistar rats (Charles River Laboratories, L'Arbresle, France), weighing 275–375 g at the beginning of the experiments, were used. They were delivered to the laboratory at least 5 days before the experiments in order to be acclimatized to laboratory conditions. They were housed five per cage and given food (Teklad Global 16% Protein Rodent Diet, Harlan, Gannat, France) and water *ad libitum*. Sawdust (Lignocel 3–4, Harlan, Gannat, France) bedding in rodent cages was changed twice weekly. The animal room was maintained with a 12/12 h alternating light-dark cycle (light phase 07:00–19:00 h).

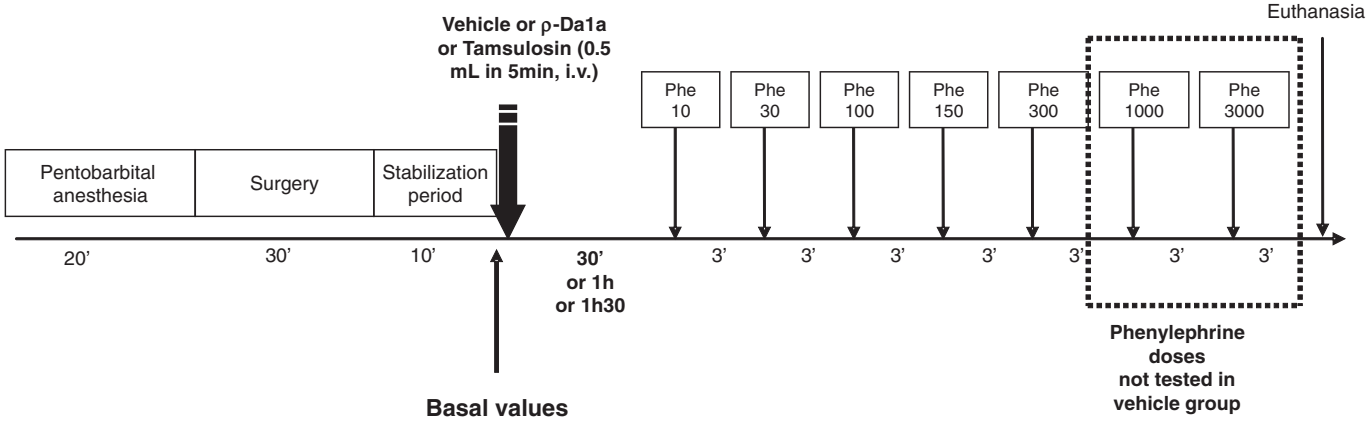
Surgical procedures. The experimental protocol for testing ρ-Da1a and tamsulosin was derived from a paper describing the effects of some α_1-adrenoceptors antagonists on IUP in anaesthetized rats (Akiyama *et al.*, 1999). Rats were anaesthetized with pentobarbital 40 mg kg^{-1}, i.p. (Centravet, Lapalisse, France), with a supplementary dose of 10 mg kg^{-1}, s.c., to maintain anaesthesia as necessary during the experiment. The depth of anaesthesia was assessed by pinching the rat hindpaw and by visual control of respiration rate during the experiment. A jugular catheter was inserted for drug administration and another into the carotid artery to record arterial pressure. After a midline incision of the abdomen, a polyethylene catheter was inserted into the urethra via an incision in the bladder. The urethral catheter was held in place by a suture on the bladder wall.

Measurements of urethral and arterial pressures. The urethral catheter was connected via a T-tube to a strain gauge and a syringe injection pump. Saline at room temperature was continuously infused into the urethra at a flow rate of 0.5 mL·h^{-1}.

I.v. administration (protocol 1). After a stabilization period of at least 10 min, ρ-Da1a (0.1, 0.3 or 1 mg·kg^{-1}), tamsulosin (0.01 mg·kg^{-1}) or the common vehicle (physiological saline) was administered i.v. and 30, 60 or 90 min later, the first dose of PHE was administered.

The different doses of PHE (from 10 to 3000 μg·kg^{-1}) were administered i.v. in a volume of 1 mL·kg^{-1} (bolus) with a 3 min interval between each dose. Baseline values were measured 1 min before administration of the test compounds.

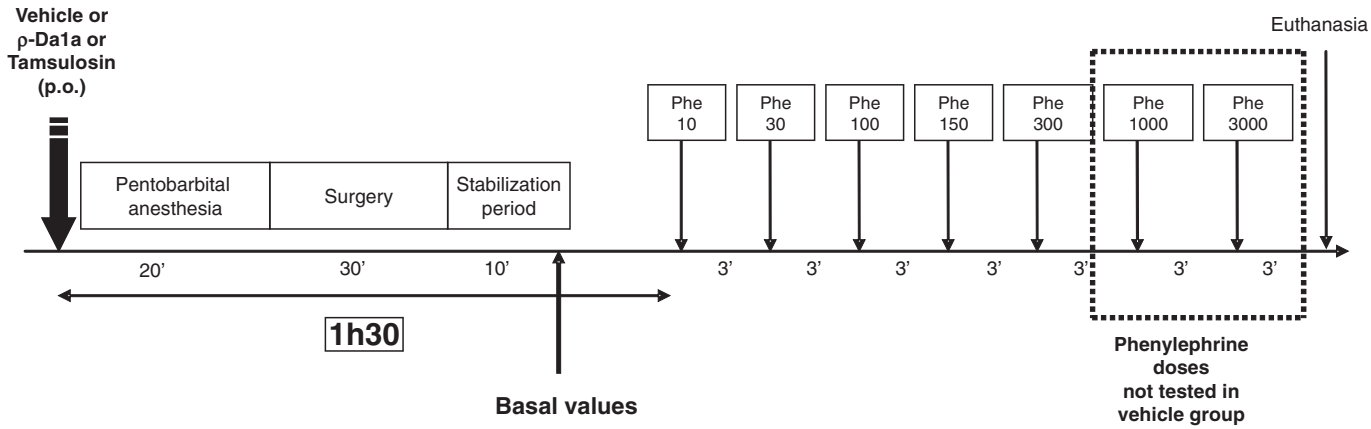
Protocol 1



Phe=phenylephrine (μg/kg, i.v.)

Oral administration (protocol 2). Rats were deprived of food (fasted) for at least 12 h before the start of this experiment. Vehicle, ρ-Da1a (10 mg·kg^{-1}) or tamsulosin (0.3 mg·kg^{-1}) was administered p.o. before anaesthesia. Ninety minutes after gavage, the first dose of PHE was administered; the different doses of PHE (10 to 3000 μg·kg^{-1}) were administered i.v. in a volume of 1 mL·kg^{-1} (bolus) with a 3 min interval between each dose. Baseline values were measured after a stabilization period.

Protocol 2



Phe=phenylephrine (μg/kg, i.v.)

Expression of data and statistical analysis

In vitro *studies*. In COS cells, results are expressed as mean ± SD with *n*, number of independent experiments. In the presence of non-competitive antagonism (depression of maximal response to adrenaline but without change in pEC_{50}), the Gaddum method for non-competitive antagonist was used (see Kenakin, 2009). Briefly, equi-active concentrations of adrenaline in the absence or in the presence of 10 nM ρ-Da1a were compared in a linear regression. The slope of this regression was used to estimate the K_B for the antagonist with the equation K_B = [antagonist]/(slope – 1).

On human isolated prostatic adenoma, contractile responses to NA were expressed as % of the maximal effect (E_{max}) obtained with the initial contraction to 30 μM NA. CRCs were fitted, using mean results, by non-linear regressions (GraphPad Prism® version 4.0) to obtain the following parameters: E_{max}, maximal contraction induced by NA; EC_{50}, NA concentration, which induces 50% of the maximum effect, expressed as pEC_{50} (-log EC_{50}). Mean CRCs for controls and treated strips were fitted in parallel and statistically compared. The first fitting was used to compare E_{max} values and when these values were not statistically significantly different, a second fit was performed, sharing E_{max}, in order to obtain pEC_{50} values for each pair of curves. Differences were considered statistically significant when the null hypothesis could be rejected at a risk α of less than 0.05.

Finally, concentration-ratios (ratios of pEC_{50} values) for NA in the absence and presence of various concentrations of ρ-Da1a were calculated and used to estimate antagonist potency.

Due to non-parallel shifts of the CRCs to NA, ρ-Da1a antagonist potency was calculated, using the data obtained following incubation with 100 nM ρ-Da1a, from the following equation: pK_B = –log[antagonist] + log(dose ratio – 1).

In vivo *studies*. Increases in IUP and AP (mmHg) were calculated for each animal and each dose of PHE (10, 30, 100, 300, 1000 and 3000 μg·kg^{-1}, i.v.). Increases in IUP and AP (Δ-IUP and Δ-AP) corresponded to the maximal values for IUP and AP after PHE administration minus basal value measured at the end of the stabilization period (1 min before PHE administration).

One-way ANOVA followed by Dunnett's test was used to compare different treated groups to vehicle group. A $P < 0.05$ was accepted for statistical significance.

In order to facilitate comparison of ρ-Da1a and tamsulosin potencies in *in vivo* experiments, PHE doses were expressed as mol·kg^{-1} and data plotted on a logarithmic scale.

In the presence of competitive antagonism, pK_B was calculated from the mean pEC_{50} values of PHE in the presence and absence of antagonist using the following equation:

$$pK_B = -\log[\text{antagonist}] + \log(DR - 1)$$

where antagonist concentration is expressed as mol·kg^{-1} and dose ratio (DR) = EC_{50}(with antagonist)/EC_{50}(without antagonist).

In the present paper, this equation was used for the calculation of ρ-Da1a potencies (0.1 mg·kg^{-1}, i.v., 30 min) and tamsulosin (0.01 mg·kg^{-1}, i.v., 30 or 90 min post-administration; Figures 4 and 7).

When antagonism was considered as non-competitive (depression of maximal response to PHE but without change in pEC_{50}), equi-active concentrations of PHE were compared in a linear regression. The slope of this regression was used to estimate the K_B for the antagonist from the equation K_B = [antagonist]/(slope – 1).

This equation was used to calculate antagonist potency values for ρ-Da1a (0.3 mg·kg^{-1}, i.v., 90 min post-administration; Figure 7).

When antagonism was considered as insurmountable (depression of maximal response to agonist with an associated shift to the right of the curve), an equi-active DR for PHE concentrations was calculated at a level of 30% of the maximal response of the dose-response curve. The pA_2 was calculated from the following equation:

$$pA_2 = -\log[\text{antagonist}] + \log(DR - 1).$$

This equation was used to calculate antagonist potency values for tamsulosin (0.3 mg·kg^{-1}, p.o., 90 min post-administration; Figure 9).

Results

In vitro *studies*

Selectivity of ρ-Da1a. ρ-Da1a was tested as an agonist as well as an antagonist on 78 different GPCRs, comprising muscarinic M_1, M_2 and M_3 receptors, as well as α_{2A}, β_1 and β_2 adrenoceptors. This peptide showed no significant activity on these receptors at the concentration of 1 μM (data not shown). In addition, ρ-Da1a showed no activity on the eight principal ion channels implicated in cardiac activity (data not shown).

Non-competitive antagonist properties of ρ-Da1a on recombinant α_1-adrenoceptor subtypes. The effect of ρ-Da1a on α_{1A}-adrenoceptors was investigated in mammalian cells using a fluorescent probe that measured increases in calcium concentration, which reflects receptor activation. Stimulation of α_{1A}-adrenoceptors by adrenaline was evaluated in the presence of increasing concentrations of ρ-Da1a (1–10–50–200 nM). Adrenaline activated α_{1A}-adrenoceptors with EC_{50} of 9.1 ± 3.0 μM (n = 3). Pre-incubation with 1 nM ρ-Da1a had no effect on the response of the cells to adrenaline. At higher ρ-Da1a concentrations, adrenaline efficacy decreased dramatically and was almost completely abolished at 50 nM (Figure 1).

The non-competitive nature of ρ-Da1a's antagonism meant that its antagonist properties could not be estimated from a Schild plot. Hence, we used the Gaddum method for non-competitive antagonists to estimate a pK_B value equal to 9.04 for ρ-Da1a.

Insurmountable antagonist properties of ρ-Da1a on human isolated prostatic smooth muscle. The magnitudes of contractions induced by 30 μM NA were 0.96 ± 0.10 g, 0.96 ± 0.20 g, 0.90 ± 0.11 g, 0.95 ± 0.18 g and 0.85 ± 0.09 g, for strips used to test solvent or ρ-Da1a at 10, 30, 100 and 300 nM respectively.

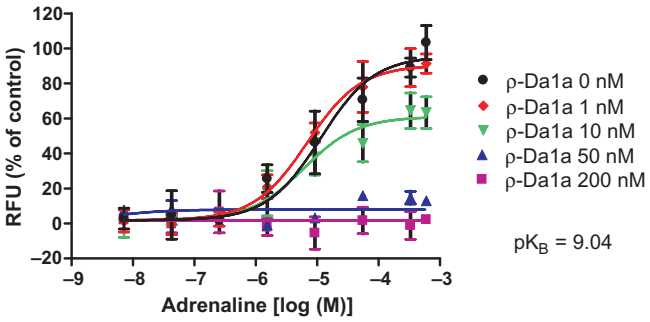


Figure 1

Functional characterization of ρ-Da1a on COS cells expressing the human α_{1A}-adrenoceptor. Concentration-response curves for adrenaline (n = 3) were obtained in the presence of increasing concentrations of ρ-Da1a.

These values were not significantly different from each other (P > 0.05 by one-way ANOVA and Kruskall–Wallis test).

In strips pre-incubated with ρ-Da1a solvent (distilled water), NA (range 0.0001–1 mM) induced concentration-dependent contractions of the isolated human prostate with pEC_{50} = 5.26 ± 0.09 and a maximal effect of 100.2 ± 11.5% (n = 8, Figure 2).

At the lowest concentrations of 10 nM (n = 6) and 30 nM (n = 9), ρ-Da1a had no significant effect on the CRC to NA (Figure 2A and B) and pEC_{50} values in the presence of ρ-Da1a at 10 and 30 nM (5.03 ± 0.09 and 5.15 ± 0.08, respectively) were not significantly different from the value obtained in strips treated with solvent. At a concentration of 100 nM (n = 10), ρ-Da1a antagonized the CRC to NA (Figure 2C). It decreased both NA potency (pEC_{50} = 4.64 ± 0.24) and NA efficacy, with a maximal effect of 26.1 ± 8.2%. At the highest concentration tested (300 nM; n = 5), ρ-Da1a almost completely antagonized the response to NA (maximal contraction of 9.2 ± 5.5%; Figure 2D).

Due to non-parallel shifts of the CRC to NA, the antagonist potency of ρ-Da1a was calculated, using the data obtained with 100 nM ρ-Da1a, by the use of the equation described in the Methods section. A pK_B value of 7.54 was calculated, which represents an estimation of ρ-Da1a potency on human prostatic α_{1A}-adrenoceptors. Tamsulosin 10 nM (n = 5) markedly reduced the maximal response to NA; pK_B = 10.3 (calculated from the same equation as that used to obtain the pK_B for ρ-Da1a).

In vivo *studies*

Effects of vehicle (i.v.), ρ-Da1a (0.1 and 0.3 mg·kg^{-1}, i.v.) and tamsulosin (0.01 mg·kg^{-1}, i.v.) when administered 30, 60 and 90 min before PHE. Basal values, obtained just before administration of the α_{1A}-adrenoceptor antagonists were compared to those obtained 30, 60 and 90 min following administration of the compounds. Neither ρ-Da1a nor tamsulosin or the common vehicle modified IUP and AP (data not shown).

Effects of 30 min pretreatment with vehicle, ρ-Da1a and tamsulosin (i.v.) on Δ-IUP and Δ-AP induced by PHE. No difference between basal values of IUP and AP for each group was observed (data not shown). The effects of increasing doses of PHE in rats pretreated with vehicle (n = 12) were compared to PHE effects in rats pretreated with different doses of ρ-Da1a or a single dose of tamsulosin (Figure 3).

When administered 30 min before the first dose of PHE, tamsulosin (0.01 mg·kg^{-1}, i.v.; n = 6) and ρ-Da1a at 0.1–0.3 mg·kg^{-1} i.v. (both at n = 6) and 1 mg·kg^{-1}, i.v. (n = 3) significantly reduced the increase in IUP induced by PHE at 100–150 and 300 μg·kg^{-1}, i.v (one-way ANOVA followed by Dunnett's test) but had no effect on lower doses of PHE (Figure 3).

With regard to AP, tamsulosin (0.01 mg·kg^{-1}, i.v.; n = 6) strongly antagonized the dose-response curve to PHE (Figure 3). This effect was statistically significant at all PHE doses except 300 μg·kg^{-1} (P < 0.01, one-way ANOVA followed by Dunnett's test). In contrast, ρ-Da1a (n = 6–3) was without significant effect (Figure 3) on the increase in AP induced by PHE, except at the lowest dose of PHE tested (10 μg·kg^{-1}, i.v.; P < 0.05, one-way ANOVA and Dunnett's test).

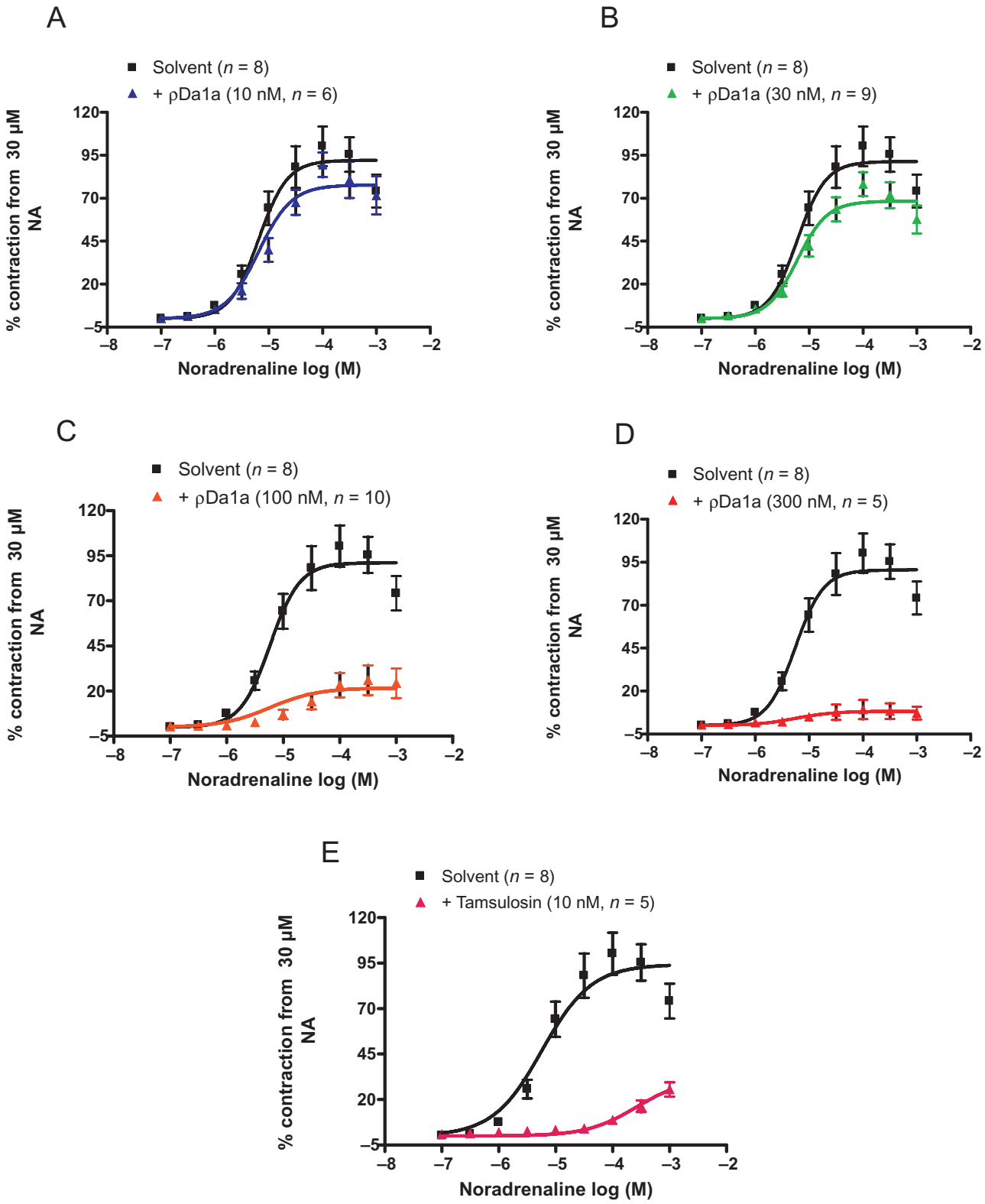


Figure 2

Concentration-response curves to NA on human isolated prostatic muscle incubated for 180 min with solvent or one concentration (A, 10 nM; B, 30 nM; C, 100 nM; D:, 300 nM) of ρ-Da1a or with 10 nM tamsulosin incubated for 60 min (E). The Krebs contained propranolol (1 µM), desipramine (0.1 µM), deoxycorticosterone (3 µM) and normetanephrine (1 µM) in order to block β-adrenoceptors, neuronal and extraneuronal uptakes and catechol-O-methyltransferase respectively. Contractile responses to the agonist are expressed as % of the maximal tension obtained with 30 µM NA. Data are means ± SEM of 5 to 10 strips obtained from four patients.

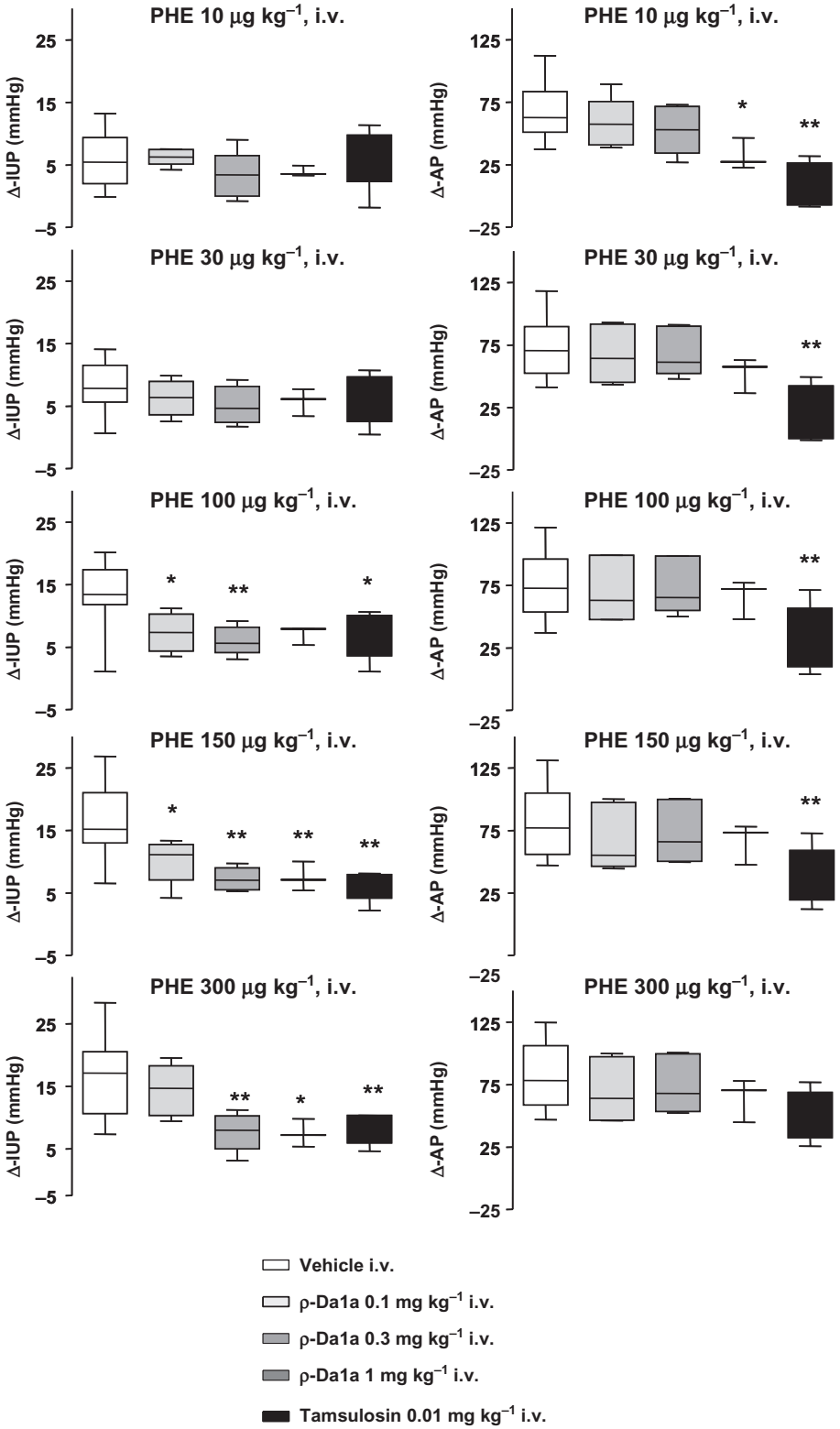


Figure 3

Effect of a 30 min i.v. pretreatment with vehicle (n = 12), ρ-Da1a (0.1–0.3 and 1 mg·kg^{-1}; n = 6–3), or tamsulosin (0.01 mg·kg^{-1}; n = 6) on the increases in IUP and AP induced by phenylephrine (PHE; 10–300 µg·kg^{-1}, i.v.) in anaesthetized male rats.

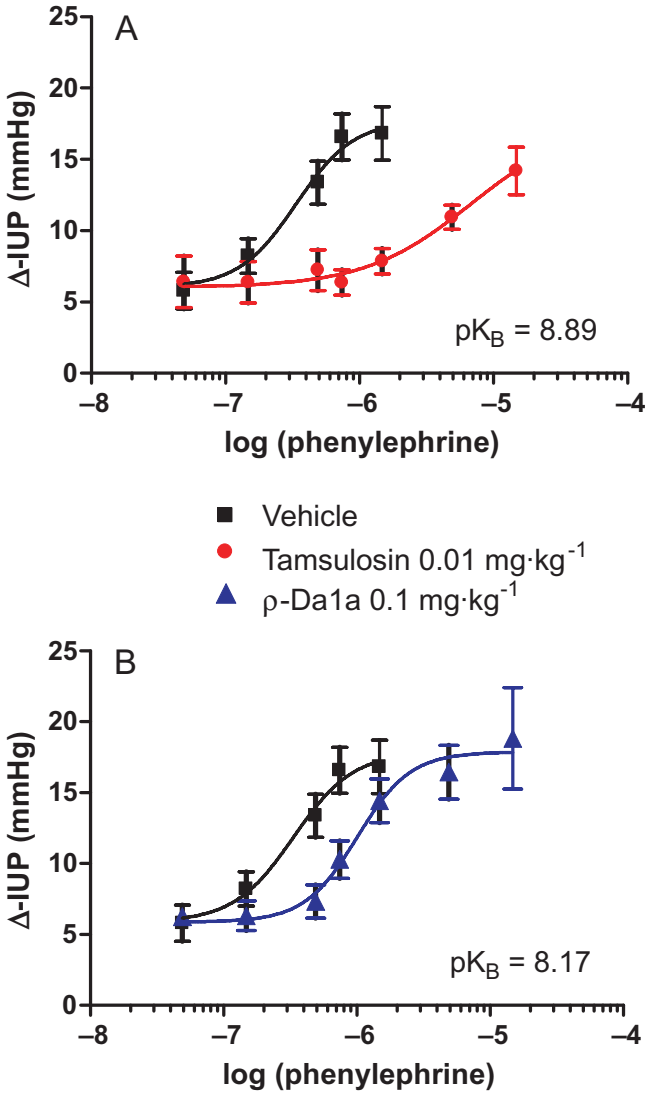


Figure 4

GraphPad fitting of dose-response curves to phenylephrine (PHE) on increase in IUP in the presence of (A) vehicle ($n = 12$) and tamsulosin (0.01 mg·kg^{-1}; $n = 6$) or (B) vehicle ($n = 12$) and r-Da1a (0.1 mg·kg^{-1}, i.v.; $n = 6$) 30 min post-administration of each antagonist.

In order to easily compare the potency of ρ-Da1a versus tamsulosin, PHE doses were also expressed as mol·kg^{-1} and data plotted on a logarithmic scale. Using data obtained from experiments with ρ-Da1a at 0.1 mg·kg^{-1}, i.v., and tamsulosin at 0.01 mg·kg^{-1}, i.v., the antagonism appeared competitive in nature. Using the equation described in the Methods section, the pK$_B$ values for tamsulosin and ρ-Da1a were calculated to be 8.89 and 8.17 respectively (Figure 4).

Effects of 60 min pretreatment with vehicle and ρ-Da1a (i.v.) on increases in IUP and AP induced by PHE. No difference between basal values of IUP and AP for each group was observed (data not shown). The effects of increasing doses of PHE in rats pretreated with vehicle ($n = 12$) were compared to those in rats pretreated with a single dose of ρ-Da1a (Figure 5).

When administered 60 min before the first dose of PHE, ρ-Da1a 0.3 mg·kg^{-1}, i.v. ($n = 6$) significantly reduced the increase in IUP induced by PHE at 100–150 and 300 μg·kg^{-1}, i.v (one-way ANOVA and Dunnett's test) but had no effect on lower doses of PHE (Figure 5). However, ρ-Da1a was without significant effect (Figure 5) on the increase in AP induced by PHE, except at the lowest dose of PHE tested (10 μg·kg^{-1}, i.v.; $P < 0.05$, one-way ANOVA and Dunnett's test).

Effects of 90 min pretreatment (i.v. route) with vehicle, ρ-Da1a and tamsulosin on increases in IUP and AP induced by PHE. No difference between basal values of IUP and AP for each group was observed (data not shown). The effects of increasing doses of PHE in rats pretreated with vehicle ($n = 12$) were compared to those in rats pretreated with a single dose of ρ-Da1a or tamsulosin ($n = 6$ for each) (Figure 6).

On IUP, ρ-Da1a (0.3 mg·kg^{-1}, i.v.) and tamsulosin (0.01 mg·kg^{-1}, i.v.), administered 90 min prior the first dose of PHE, significantly inhibited the effect of PHE at 150 μg·kg^{-1}, i.v. (Figure 6). The potency of tamsulosin, was reduced more than 10 times with respect to the potency observed following its administration 30 min before PHE (pK$_B$ = 7.74 vs. 8.89). On the other hand, the potency of ρ-Da1a was only slightly reduced with respect to that obtained after it was administered 30 min before PHE (pK$_B$ = 7.73 vs. 8.17; Figure 7).

When administered 90 min before the first dose of PHE, ρ-Da1a (0.3 mg·kg^{-1}, i.v.) and tamsulosin (0.01 mg·kg^{-1}, i.v.) significantly reduced AP only for the lowest dose of PHE tested (10 μg·kg^{-1}, i.v.; Figure 6; $P < 0.05$ one-way ANOVA and Dunnett test).

Effects of 90 min pretreatment (oral route) of vehicle, ρ-Da1a and tamsulosin on increases in IUP and AP induced by PHE. No difference between basal values of IUP and AP for each group was observed (data not shown). The effects of increasing doses of PHE in rats pretreated with vehicle ($n = 6$) were compared to those in rats pretreated with a single dose of ρ-Da1a ($n = 4$) or tamsulosin ($n = 6$) (Figure 8).

When administered 90 min before the first dose of PHE, tamsulosin (0.3 mg·kg^{-1}, p.o.) significantly reduced the increase in IUP for each dose of PHE tested with a pK$_B$ value of 7.13, whereas ρ-Da1a had no effect (Figure 8). Neither ρ-Da1a nor tamsulosin had any effect on the increase in AP in response to any dose of PHE (Figure 8). To estimate the potency of p.o. tamsulosin, the data were also plotted as a dose-response curve using a log scale and fitted by a non-linear regression and a pK$_B$ = 7.13 was calculated (Figure 9).

Discussion

In vitro *studies*

Screening of ρ-Da1a versus 78 human GPCR and eight ion channels revealed an impressive selectivity of this peptide for α_{1A}-adrenoceptors. Unfortunately, as far as we know, the selectivity of the non-peptidic α_{1A}-adrenoceptors antagonists was not demonstrated on such large panels of targets, making a direct comparison with ρ-Da1a unfeasible.

The pEC$_{50}$ value for NA-induced contractions in the human isolated prostate was equal to 5.26 ± 0.09, which is

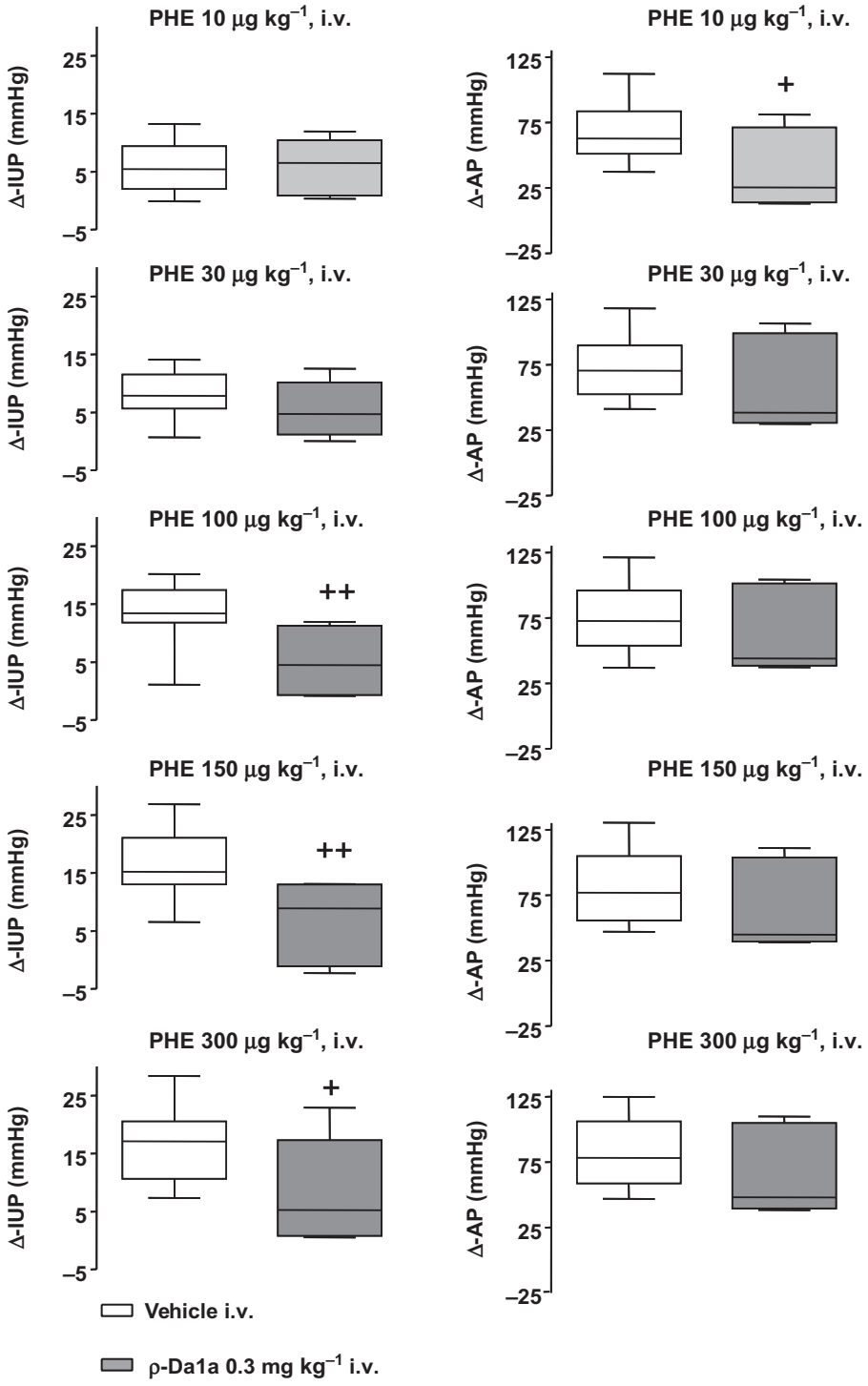


Figure 5

Effect of a 60 min i.v. pretreatment with vehicle ($n = 12$) or ρ–Da1a (0.3 mg·kg^{-1}; $n = 6$) on the increases in IUP and AP induced by phenylephrine (PHE; 10–300 μg·kg^{-1}) in anaesthetized male rats.

similar to the value previously obtained for NA in the human prostatic adenoma (5.48 ± 0.11; Palea *et al.*, 2000). The antagonist potency of ρ-Da1a versus NA in human isolated prostate ($pK_B = 7.54$) was smaller than the potency exhibited in functional studies on COS cells expressing the human α_{1A}-adrenoceptors ($pK_B = 9.04$). The hypothesis that the contractility of human prostate is essentially mediated by activation of α_{1A}-adrenoceptors was advanced, leading to the clinical development of silodosin, an extremely selective α_{1A}-adrenoceptor antagonist (Marks *et al.*, 2009). However, the

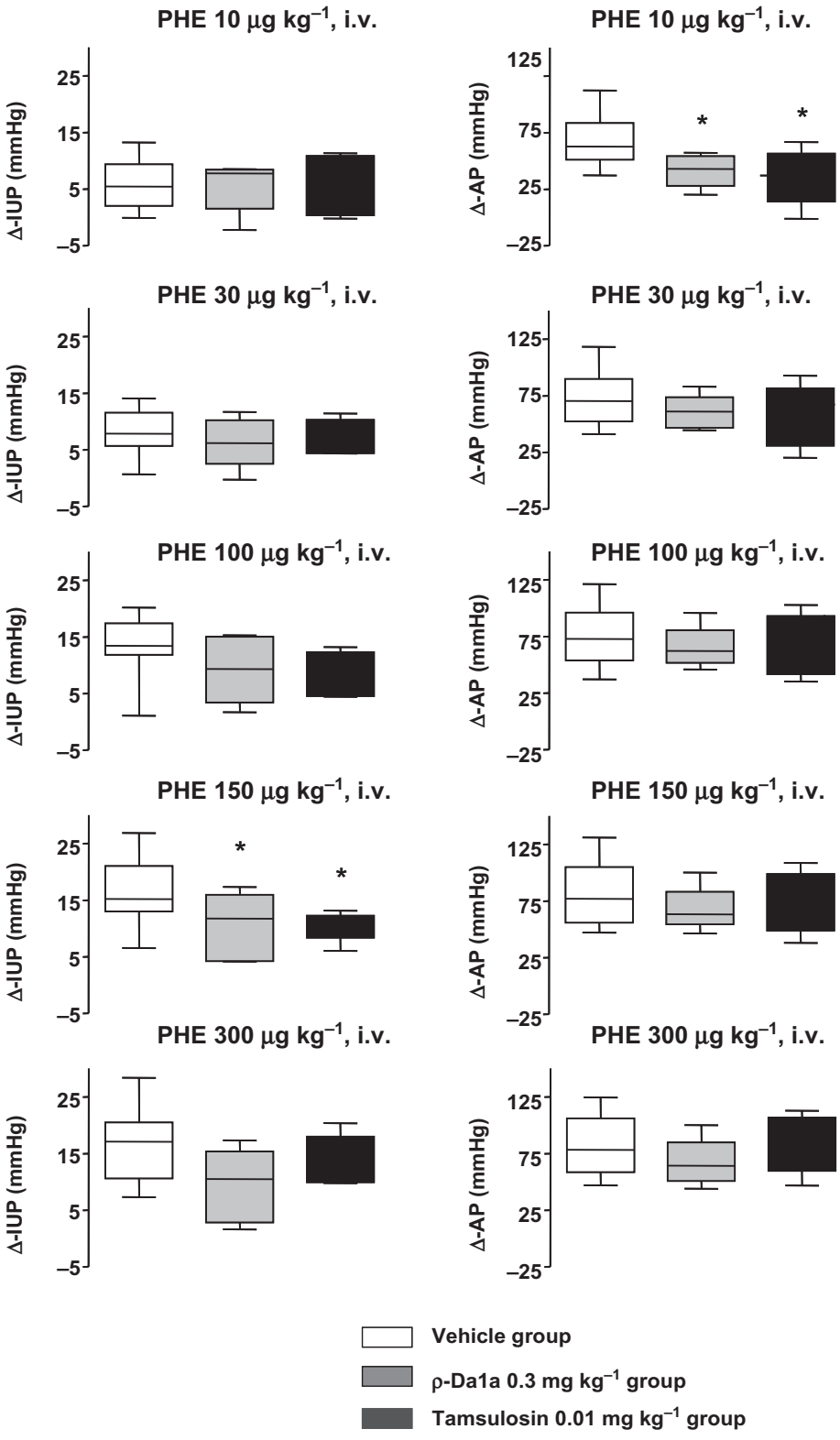


Figure 6

Effect of a 90 min i.v. pretreatment with vehicle (n = 12), ρ-Da1a (0.3 mg·kg^{-1}; n = 6) or tamsulosin (0.01 mg·kg^{-1}, i.v.; n = 6) on the increases in IUP and AP induced by phenylephrine (PHE; 10–300 μg·kg^{-1}, i.v.) in anaesthetized male rats.

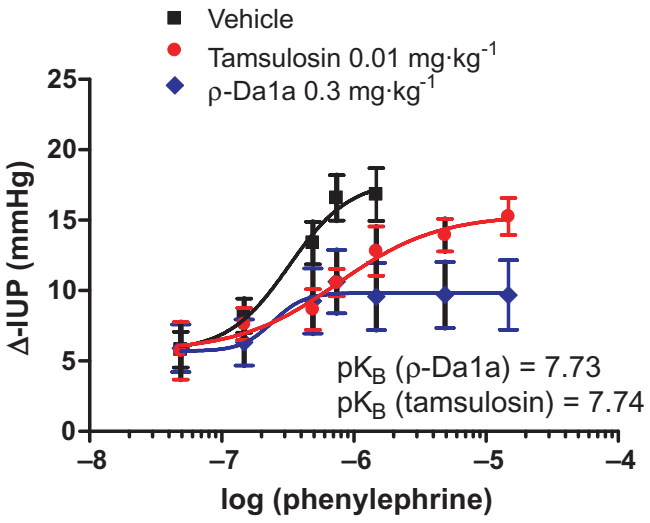


Figure 7

GraphPad fitting of dose-response curves for phenylephrine (PHE)-induced increases in IUP in the presence of vehicle (n = 12), tamsulosin (0.01 mg·kg^{-1}, i.v.; n = 6) or ρ-Da1a (0.3 mg·kg^{-1}; n = 6) 90 min post-administration of each antagonist.

antagonistic potency of ρ-Da1a versus NA in the human isolated prostate is quite lower with respect to the corresponding value obtained on recombinant human α_{1A}-adrenoceptors, which is in sharp contrast with this hypothesis. This discrepancy could be due to a variety of factors, the more straightforward being related to the abnormal pharmacology of α_{1A}-adrenoceptors in prostatic and urethral tissues. In the past, observations such as these justified the hypothesis for the existence of the α_{1L}-adrenoceptor subtype which, however, is now recognized to be a product of the α_{1A}-adrenoceptor gene (Gray *et al.*, 2008; Muramatsu *et al.*, 2008). The α_{1L}-adrenoceptor subtype is characterized by a low affinity for prazosin and other α_{1A}-adrenoceptor antagonists (with the exception of tamsulosin) when compared with recombinant α_{1A}-adrenoceptors. Another potential explanation for the differences in potencies values between recombinant receptors and native receptors in the human prostate could be the presence of a heterogeneous population of α_1-adrenoceptor subtypes in the hyperplastic tissue.

Nevertheless, we have shown here that ρ-Da1a is an insurmountable antagonist of α_{1A}-adrenoceptors in human prostatic tissue, and this accords with results obtained in rabbit isolated prostate (Quinton *et al.*, 2010). Tamsulosin, the most potent α_{1A}-adrenoceptor antagonist on the market, was a potent antagonist in our hands, having a pK_B value of 10.3. This value is similar to the potency previously calculated on human isolated prostate by another group (Noble *et al.*, 1997). Therefore, ρ-Da1a is approximately 100 times less potent than tamsulosin on human isolated prostate. Nevertheless, it should be noted that the potency of ρ-Da1a on human prostate (pK_B = 7.54) is quite similar to potencies displayed by drugs frequently used to treat lower urinary tract symptoms, such as alfuzosin (pK_B = 7.37; Palea *et al.*, 2000), terazosin (pA_2 = 8.17; Chueh *et al.*, 2002) and doxazosin (pA_2 = 8.43; Hatano *et al.*, 1996). However, unlike ρ-Da1a, alfuzosin, terazosin and doxazosin are competitive antagonists in human isolated prostate. Tamsulosin, in our hands, strongly depressed the maximal response to NA in human tissue but, unlike ρ-Da1a, was not able to inhibit completely the agonist response (see Figure 2 but also Noble *et al.*, 1997). Therefore, we presume that the nature of the antagonism induced by ρ-Da1a differs from that of tamsulosin, which is supported by the strong difference in chemical structure between these two compounds as well as by the half-life of almost 4 h of the ρ-Da1a/α_{1A}-adrenoceptor complex (Quinton *et al.*, 2010).

In vivo *studies*

Pretreatment with ρ-Da1a and tamsulosin (as well as the vehicle) was ineffective at reducing basal values of AP, suggesting that these molecules are not likely to produce hypotension when administered alone in animals or humans. This finding is consistent with the lack of effect of tamsulosin on AP in a large cohort of patients suffering from BPH (Chapple *et al.*, 1997).

ρ-Da1a (0.1, 0.3 and 1 mg·kg^{-1}, i.v.), administered 30 min before PHE, dose-dependently antagonized the effects of PHE on IUP. In the presence of 1 mg·kg^{-1} ρ-Da1a, the effect of PHE on IUP was almost totally abolished. This result is consistent with the non-competitive antagonism observed on the rabbit isolated prostate (Quinton *et al.*, 2010) and human prostatic tissue (present study). Hence, we suggest that ρ-Da1a is an insurmountable antagonist not only *in vitro* but also *in vivo*.

The antagonist effect of ρ-Da1a on IUP at 0.3 mg·kg^{-1} (i.v.) was found to be similar to that of tamsulosin at 0.01 mg·kg^{-1}, i.v. Therefore, expressed in mg·kg^{-1}, tamsulosin seems to be approximately 30 times more potent than ρ-Da1a. However, in order to obtain a better comparison of the relaxant effects of ρ-Da1a and tamsulosin on rat urethra *in vivo*, we decided to express their potency as pK_B, a method classically used to quantify antagonist potency *in vitro* but can also be applied to *in vivo* pharmacological results when an exogenous agonist of a particular receptor is administered systemically in the absence and presence of a selective antagonist for the same receptor (Dykstra *et al.*, 1988; Negus *et al.*, 1993).

The antagonism induced by 0.1 mg·kg^{-1} ρ-Da1a was competitive in nature and its antagonistic potency (pK_B = 8.17) was slightly less than that of tamsulosin (pK_B = 8.89). Interestingly, the potency of ρ-Da1a *in vivo* was similar to its potency on rabbit isolated prostate (pA_2 = 8.38; Quinton *et al.*, 2010), whereas tamsulosin potency *in vivo* was similar to the potency value calculated in the rat isolated prostate (pK_B = 9.23; Pulito *et al.*, 2000). Based on these pK_B values calculated *in vivo*, we conclude that tamsulosin is only three times more potent than ρ-Da1a when administered by the i.v. route in anaesthetized rats.

Importantly, we found that ρ-Da1a has a long duration of action since its effect on IUP was statistically significant 60 min after its i.v. administration. Similar to tamsulosin, a small inhibitory effect was even observed 90 min post-administration of ρ-Da1a. However, the potency of tamsulosin declined over time, pK_B being 8.89 and 7.74 at 30 and 90 min post-administration respectively, whereas the potency of ρ-Da1a was only slightly reduced between 30 and 90 post-administration (from 8.17 to 7.73). Hence, we suggest that ρ-Da1a has a longer duration of action than tamsulosin when administered by the i.v. route.

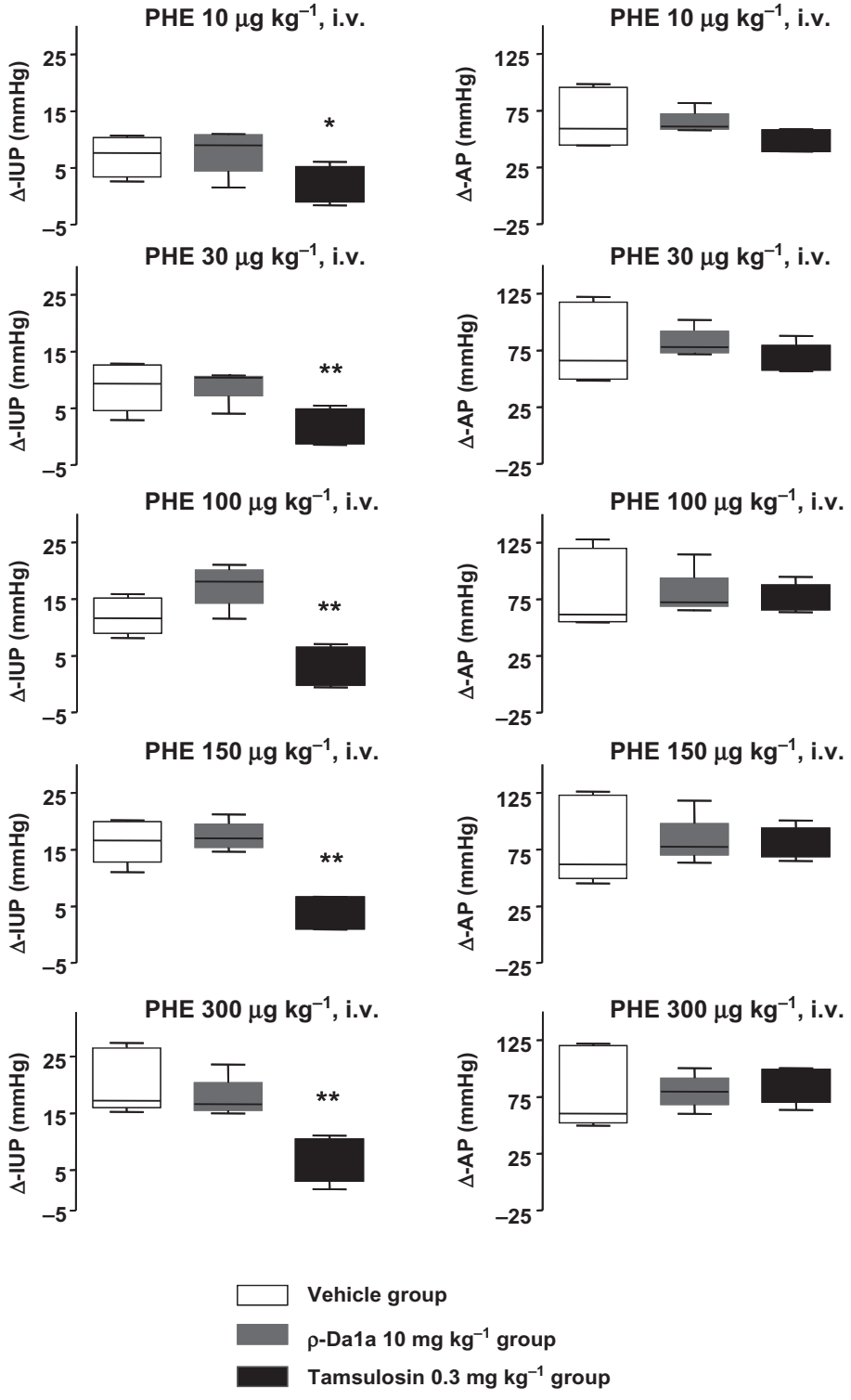


Figure 8

Effect of a 90 min pretreatment with vehicle ($n = 6$), ρ-Da1a (10 mg·kg^{-1}; $n = 4$) or tamsulosin (0.3 mg·kg^{-1}; $n = 6$), all administered p.o., on the increases in IUP and AP induced by phenylephrine (10–300 μg·kg^{-1}, i.v.) in anaesthetized male rats.

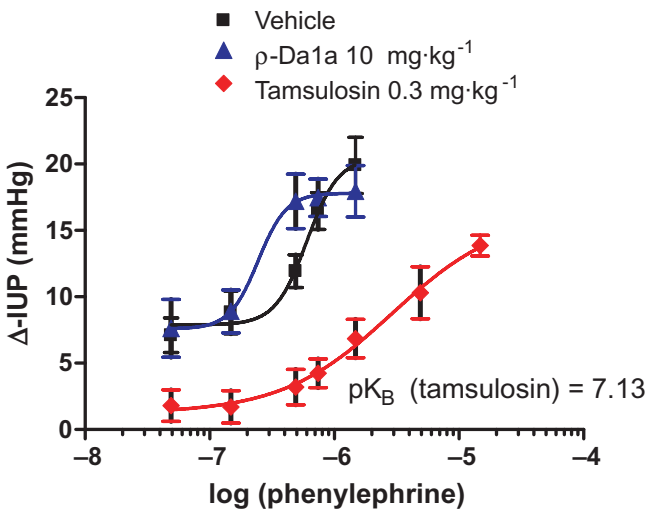


Figure 9

GraphPad fitting of dose-response curves for phenylephrine (PHE)-induced increases in IUP in the presence of vehicle ($n = 6$), tamsulosin (0.3 mg·kg^{-1}, p.o.; $n = 6$) or ρ-Da1a (10 mg·kg^{-1} p.o.; $n = 4$) 90 min post-administration of each antagonist.

With regard to the effects of the two antagonists on AP 30 min post-administration, we found that ρ-Da1a, even at 1 mg·kg^{-1} (i.v.), was ineffective at blocking the effects of PHE on AP, whereas tamsulosin at 0.01 mg·kg^{-1} (i.v.) markedly antagonized the effects of PHE on AP. This suggests that tamsulosin is at least 100 times more potent than ρ-Da1a on α_1-adrenoceptors activated by PHE in the resistance arteries (probably the α_{1B}-adrenoceptor subtype). These findings also confirm results obtained in binding studies where it was shown that the affinity of ρ-Da1a for α_{1B}-adrenoceptors is 100 times less than for α_{1A}-adrenoceptors (Quinton *et al.*, 2010).

When administered p.o., ρ-Da1a at 10 mg·kg^{-1} was ineffective, as expected. However, it was not possible to test the effect of ρ-Da1a at 30 mg·kg^{-1} because of limited amounts of the peptide available. Tamsulosin, on the other hand, at 0.3 mg·kg^{-1}, p.o., was able to antagonize the effect of PHE on IUP but was ineffective on AP, confirming its uroselectivity when administered orally; an effect previously observed in anaesthetized dogs (Sato *et al.*, 2001).

Perspectives

The ability of ρ-Da1a to act as an insurmountable antagonist on α_{1A}-adrenoceptors represents an advantage compared to classical α_1-blockers on the market, because it is able to exert a stronger antagonism of the receptor even in the presence of high concentrations of the agonist. In addition, the long duration of action of ρ-Da1a, linked to the half-life of almost 4 h of the complex ρ-Da1a/α_{1A}-adrenoceptor (Quinton *et al.*, 2010), is a real advantage for chronic diseases. Coupled with *ad hoc* depot formulations, administration of the drug can be envisaged to be compatible with the long duration of the treatment. Moreover, ρ-Da1a displayed an impressive selectivity for the recombinant α_{1A}-adrenoceptor and a clear uroselectivity *in vivo*, therefore decreasing the chance of potential cardiovascular side effects (hypotension). Finally, due to its completely new chemical structure, ρ-Da1a could lack some of the side effects associated with the use of tamsulosin, in particular intra-operative floppy iris syndrome (Chang and Campbell, 2005). This syndrome is specific to tamsulosin and not related to a class effect of α_1-adrenoceptors (Bell *et al.*, 2009).

At present, all therapeutic peptides are administered by the parenteral route, because of insufficient absorption from the gastrointestinal tract. Peptide drugs are usually indicated for chronic conditions, and the use of injections on a daily basis has obvious drawbacks, whereas the oral route offers the advantages of self-administration. The main reasons for the low oral bioavailability of peptide drugs are enzymatic degradation and poor penetration of the intestinal mucosa. A considerable amount of research has focused on overcoming the challenges presented by absorption barriers to provide effective oral delivery of peptides (Dünnhaupt *et al.*, 2012). Therefore, if the problems of oral absorption could be solved by new technologies, ρ-Da1a has the potential to be a new drug for the pharmacological treatment of urinary tract dysfunctions in humans.

Apart from the classical use of α_{1A}-adrenoceptor antagonists for the pharmacological treatment of BPH, this class of compound could be used for other urological disorders. α_{1A}-Adrenoceptors are functionally expressed by capsaicin-sensitive, nociceptive, primary sensory neurons of the rat urinary tract, and their activation may contribute to the signalling of irritative and nociceptive responses arising from the urinary tract (Trevisani *et al.*, 2007). Interestingly pre-treatment with alfuzosin (10 mg·kg^{-1}day^{-1}) was able to suppress bladder overactivity induced by cyclophosphamide in conscious rats (Lluel *et al.*, 2008) suggesting that ρ-Da1a has the potential to decrease bladder inflammation. This property could be useful for controlling the symptoms of pain and urinary frequency in patients suffering from painful bladder syndrome.

Furthermore, tamsulosin and other α_{1A}-adrenoceptor antagonists have been successfully used in recent years to facilitate the passage of renal stones from the ureter to the urinary bladder (Yilmaz *et al.*, 2005; Porpiglia *et al.*, 2006). A clinical investigation monitoring ureteric pressure during ureteroscopy for stone disease showed that oral administration of tamsulosin significantly reduced ureteric pressure (Davenport *et al.*, 2007). Therefore, administration of ρ-Da1a could be very effective in facilitating expulsion of ureteral stones. The advantage of using ρ-Da1a rather than tamsulosin would be its longer duration of action of α_{1A}-adrenoceptor blockade *in vivo*, as demonstrated in the present study in rats (by i.v. route).

We conclude that ρ-Da1a could be a new treatment for lower urinary tract symptoms, painful bladder syndrome and urolithiasis. Local administration of ρ-Da1a (i.e. by intraprostatic, intravesical or intra-ureteral routes) could be used to circumvent its lack of oral bioavailability.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

References

Akiyama K, Hora M, Tatemichi S, Masuda N, Nakamura S, Yamagishi R *et al*. (1999). KMD-3213, a uroselective and long-acting alpha(1a)-adrenoceptor antagonist, tested in a novel rat model. J Pharmacol Exp Ther 291: 81–91.

Bell CM, Hatch WV, Fischer HD, Cernat G, Paterson JM, Gruneir A *et al*. (2009). Association between tamsulosin and serious ophthalmic adverse events in older men following cataract surgery. JAMA 301: 1991–1996.

Chang DF, Campbell JR (2005). Intraoperative floppy iris syndrome associated with tamsulosin. J Cataract Refract Surg 31: 664–673.

Chapple CR, Andersson KE (2002). Tamsulosin: an overview. World J Urol 19: 397–404.

Chapple CR, Baert L, Thind P, Höfner K, Khoe GS, Spångberg A (1997). Tamsulosin 0.4 mg once daily: tolerability in older and younger patients with lower urinary tract symptoms suggestive of benign prostatic obstruction (symptomatic BPH). The European Tamsulosin Study Group. Eur Urol 32: 462–470.

Chueh SC, Chern JW, Choong CM, Guh JH, Teng CM (2002). Characterization of some novel alpha 1-adrenoceptor antagonists in human hyperplastic prostate. Eur J Pharmacol 445: 125–131.

Craig AG, Norberg T, Griffin D, Hoeger C, Akhtar M, Schmidt K *et al*. (1999). Contulakin-G, an O-glycosylated invertebrate neurotensin. J Biol Chem 274: 13752–13759.

Davenport K, Timoney AG, Keeley FX Jr (2007). Effect of smooth muscle relaxant drugs on proximal human ureteric activity in vivo: a pilot study. Urol Res 35: 207–213.

Dünnhaupt S, Barthelmes J, Iqbal J, Perera G, Thurner CC, Friedl H *et al*. (2012). In vivo evaluation of an oral drug delivery system for peptides based on S-protected thiolated chitosan. J Control Release 160: 477–485.

Dykstra LA, Bertalmio AJ, Woods JH (1988). Discriminative and analgesic effects of mu and kappa opioids: in vivo pA2 analysis. Psychopharmacol Ser 4: 107–121.

Gray K, Short J, Ventura S (2008). The alpha1A-adrenoceptor gene is required for the alpha1L-adrenoceptor-mediated response in isolated preparations of the mouse prostate. Br J Pharmacol 155: 103–109.

Hatano A, Tang R, Walden PD, Lepor H (1996). The alpha-adrenoceptor antagonist properties of the enantiomers of doxazosin in the human prostate. Eur J Pharmacol 313: 135–143.

Kenakin T (2009). A Pharmacology Primer: Theory, Application and Methods. Elsevier Academic Press: London, pp. 101–127.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). NC3Rs Reporting Guidelines Working Group. Br J Pharmacol 160: 1577–1579.

Koshimizu TA, Tanoue A, Hirasawa A, Yamauchi J, Tsujimoto G (2003). Recent advances in alpha1-adrenoceptor pharmacology. Pharmacol Ther 98: 235–244.

Lluel P, Guilloteau V, Palea S (2008). Alfuzosin reverses bladder overactivity induced by cyclophosphamide in conscious rats. J Urol 79 (4 Suppl.): 371.

McGrath J, Drummond G, Kilkenny C, Wainwright C (2010). Guidelines for reportingexperiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Marks LS, Gittelman MC, Hill LA, Volinn W, Hoel G (2009). Rapid efficacy of the highly selective alpha1A-adrenoceptor antagonist silodosin in men with signs and symptoms of benign prostatic hyperplasia: pooled results of 2 phase 3 studies. J Urol 181: 2634–2640.

Muramatsu I, Morishima S, Suzuki F, Yoshiki H, Anisuzzaman AS, Tanaka T *et al*. (2008). Identification of alpha 1L-adrenoceptor in mice and its abolition by alpha 1A-adrenoceptor gene knockout. Br J Pharmacol 155: 1224–1234.

Negus SS, Burke TF, Medzihradsky F, Woods JH (1993). Effects of opioid agonists selective for mu, kappa and delta opioid receptors on schedule-controlled responding in rhesus monkeys: antagonism by quadazocine. J Pharmacol Exp Ther 267: 896–903.

Nielsen DB, Dykert J, Rivier JE, McIntosh JM (1994). Isolation of Lys-conopressin-G from the venom of the worm-hunting snail, Conus imperialis. Toxicon 32: 845–848.

Noble AJ, Chess-Williams R, Couldwell C, Furukawa K, Uchyiuma T, Korstanje C *et al*. (1997). The effects of tamsulosin, a high affinity antagonist at functional alpha 1A- and alpha 1D-adrenoceptor subtypes. Br J Pharmacol 120: 231–238.

Palea S, Barras M, Deplanne V, Vallancien G (2000). Antagonistic effects of alfuzosin on concentration-response curves to phenylephrine and noradrenaline in human prostatic adenoma. Neurourol Urodyn 19: 431–433.

Porpiglia F, Vaccino D, Billia M, Renard J, Cracco C, Ghignone G *et al*. (2006). Corticosteroids and tamsulosin in the medical expulsive therapy for symptomatic distal ureter stones: single drug or association? Eur Urol 50: 339–344.

Pulito VL, Li X, Varga SS, Mulcahy LS, Clark KS, Halbert SA *et al*. (2000). An investigation of the uroselective properties of four novel alpha(1a)-adrenergic receptor subtype-selective antagonists. J Pharmacol Exp Ther 294: 224–229.

Quinton L, Girard E, Maiga A, Rekik M, Lluel P, Masuyer G *et al*. (2010). Isolation and pharmacological characterization of AdTx1, a natural peptide displaying specific insurmountable antagonism of the alpha1A-adrenoceptor. Br J Pharmacol 159: 316–325.

Sato S, Ohtake A, Matsushima H, Saitoh C, Usuda S, Miyata K (2001). Pharmacological effect of tamsulosin in relation to dog plasma and tissue concentrations: prostatic and urethral retention possibly contributes to uroselectivity of tamsulosin. J Pharmacol Exp Ther 296: 697–703.

Servent D, Fruchart-Gaillard C (2009). Muscarinic toxins: tool for the study of the pharmacological and functional properties of muscarinic receptors. J Neurochem 109: 1193–1202.

Sharpe IA, Gehrmann J, Loughnan ML, Thomas L, Adams DA, Atkins A *et al*. (2001). Two new classes of conopeptides inhibit the alpha1-adrenoceptor and noradrenaline transporter. Nat Neurosci 4: 902–907.

Trevisani M, Campi B, Gatti R, André E, Materazzi S, Nicoletti P *et al*. (2007). The influence of alpha1-adrenoreceptors on neuropeptide release from primary sensory neurons of the lower urinary tract. Eur Urol 52: 901–908.

Yilmaz E, Batislam E, Basar MM, Tuglu D, Ferhat M, Basar H (2005). The comparison and efficacy of 3 different alpha1-adrenergic blockers for distal ureteral stones. J Urol 173: 2010–2012.