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RESEARCH PAPER

Pharmacological profile of the clonidine-induced inhibition of vasodepressor sensory outflow in pithed rats: correlation with $\alpha_{2A/2C}$-adrenoceptors

CM Villalón[1], JA Albarrán-Juárez[1], J Lozano-Cuenca[1], HH Pertz[2], T Görnemann[2] and D Centurión[1]

[1]*Departamento de Farmacobiología, Cinvestav—Coapa, Tlalpan, México D.F., México and* [2]*Institut für Pharmazie, Freie Universität Berlin, Berlin (Dahlem), Germany*

Background and purpose: Resistance blood vessels are innervated by sympathetic and primary sensory nerves, which modulate vascular tone through the release of noradrenaline and calcitonin gene-related peptide (CGRP), respectively. Moreover, electrical stimulation of the perivascular sensory outflow in pithed rats results in vasodepressor responses which are mainly mediated by CGRP release. The present study has investigated the role of α_2-adrenoceptors in the inhibition of these vasodepressor responses.
Experimental approach: 144 pithed male Wistar rats were pretreated with hexamethonium (2 mg kg^{-1} min^{-1}) followed by i.v. continuous infusions of either methoxamine (15 and 30 μg kg^{-1} min^{-1}) or clonidine (3, 10 and 30 μg kg^{-1} min^{-1}). Under these conditions, electrical stimulation (0.56–5.6 Hz; 50 V and 2 ms) of the spinal cord (T_9–T_{12}) resulted in frequency-dependent decreases in diastolic blood pressure.
Key results: The infusion of clonidine (10 μg kg^{-1} min^{-1}), as compared to those of methoxamine (15 or 30 μg kg^{-1} min^{-1}), inhibited the vasodepressor responses to electrical stimulation without affecting those to i.v. bolus injections of α-CGRP (0.1–1 μg kg^{-1}). This inhibition by clonidine was: (i) antagonized by 300 μg kg^{-1} rauwolscine ($\alpha_{2A/2B/2C}$), 300 and 1000 μg kg^{-1} BRL44408 (α_{2A}), or 10 and 30 μg kg^{-1} MK912 (α_{2C}); and (ii) unaffected by 1 ml kg^{-1} saline, 100 μg kg^{-1} BRL44408, 3000 and 10000 μg kg^{-1} imiloxan (α_{2B}) or 3 μg kg^{-1} MK912.
Conclusions and implications: The inhibition produced by 10 μg kg^{-1} min^{-1} clonidine on the vasodepressor (perivascular) sensory outflow in rats may be mainly mediated by prejunctional α_{2A}/α_{2C}-adrenoceptors.
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Keywords: α_2-adrenoceptors; BRL44408; α-CGRP; imiloxan; MK912; pithed rat; sensory nerves; vasodilatation

Abbreviations: BRL44408, (2-[2H-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole maleate; CGRP, calcitonin gene-related peptide; KO, knockout; MK912, (2*S*,12bs)1′,3′ dimethylspiro(1,3,4,5′,6,6′,7,12b-octahydro-2H-benzo[b]furo [2,3-a] quinazoline)-2,4′-pyrimidin-2′one hydrochloride; NANC, non-adrenergic, non-cholinergic

Introduction

Resistance blood vessels are innervated by sympathetic (see Hoffman, 2001) and primary sensory (Taguchi *et al*., 1992) nerves which modulate the vascular tone. C fibres are primary sensory nerves originating from the spinal cord (Julius and Basbaum, 2001) and, upon stimulation, cause a non-adrenergic, non-cholinergic (NANC) vasodilatation via the release of vasodilator neurotransmitters, particularly calcitonin gene-related peptide (CGRP; Kawasaki *et al*., 1988). CGRP is predominantly located in sensory neurons and perivascular nerves surrounding blood vessels, where it is colocalized with other neuropeptides, such as substance P and neurokinin A (Van Rossum *et al*., 1997).

Indeed, Taguchi *et al*. (1992) have shown that electrical stimulation of the thoracic (T_9–T_{12}) spinal cord in pithed rats receiving infusions of hexamethonium (to block autonomic outflow) and methoxamine (for a sustained increase in blood pressure) caused vasodepressor responses which were as follows: (i) blocked by tetrodotoxin or capsaicin; (ii) unaffected after atropine, propranolol or pyrilamine + cimetidine; and (iii) antagonized by $CGRP_{(8–37)}$, a $CGRP_{1/2}$ receptor antagonist. Thus, these NANC vasodepressor

Correspondence: Professor CM Villalón, Departamento de Farmacobiología, Cinvestav-Coapa, Calzada de los Tenorios 235, Col. Granjas-Coapa, Deleg. Tlalpan, Mexico D.F 14330, Mexico.
E-mail; cvillalon@cinvestav.mx
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Table 1 Binding affinity constants (pK_i) of several antagonists for cloned human α_2-adrenoceptor subtypes

Antagonist	α_{2a}	α_{2b}	α_{2c}
Rauwolscine	9.5[a]	9.4[a]	9.9[a]
	8.9[b]	8.9[b]	9.3[b]
BRL44408	8.2[c]	6.2[c]	6.8[c]
	7.6[d]	6.0[d]	6.4[d]
Imiloxan	5.8[d]	6.9[d]	6.0[d]
	6.5[b]	7.2[b]	6.8[b]
MK912	8.9[c]	8.9[c]	10.2[c]
	9.1[b]	9.1[b]	10.2[b]

Data taken from: [a]Bylund (1992); [b]Jasper *et al.* (1998); [c]Uhlén *et al.* (1994); [d]Devedjian *et al.* (1994).

responses are mainly mediated by CGRP release from primary sensory nerves (Taguchi *et al.*, 1992).

Interestingly, the electrically induced vasodilator responses in the rat mesenteric bed precontracted with noradrenaline are smaller than those elicited in the same vascular bed precontracted with methoxamine (Kawasaki *et al.*, 1988, 1990), an α_1-adrenoceptor agonist (Richer *et al.*, 1987). This suggests that circulating or neuronally released noradrenaline inhibits CGRP release via prejunctional α_2-adrenoceptors located on primary sensory nerves. Nevertheless, no study has analysed in pithed rats whether specific α_2-adrenoceptor subtypes located on primary sensory nerves inhibit these NANC vasodepressor responses.

On this basis, and considering that α_2-adrenoceptors exist in three pharmacologically and structurally distinguishable subtypes, namely α_{2A}, α_{2B} and α_{2C} (see Bylund *et al.*, 1994; Docherty, 1998; Alexander *et al.*, 2007), the present study set out to investigate in pithed rats: (a) whether clonidine, an α_2-adrenoceptor agonist with antihypertensive properties (Velasco and Luchsinger, 1995), is capable of inhibiting the vasodepressor responses induced by either stimulation of the perivascular sensory outflow (Taguchi *et al.*, 1992) or i.v. bolus injections of exogenous α-CGRP; and (b) the specific α_2-adrenoceptor subtypes involved in this clonidine-induced inhibition by analysing the effects of the antagonists rauwolscine ($\alpha_{2A/2B/2C}$), BRL44408 (α_{2A}), imiloxan (α_{2B}) and MK912 (α_{2C}) (see Table 1). Our results suggest that clonidine-induced inhibition of the vasodepressor sensory outflow may be mainly mediated by prejunctional α_{2A}- and α_{2C}-adrenoceptors. Preliminary results have been reported to the 15th IUPHAR World Congress of Pharmacology (Albarrán-Juárez *et al.*, 2006).

Materials and methods

Animals

All animal procedures and the protocols of the present investigation were approved by the Institutional Ethics Committee on the use of animals in scientific experiments. Male Wistar normotensive rats (300–350 g) were maintained at a 12/12-h light/dark cycle (with light beginning at 0700 hours) and housed in a special room at constant temperature (22 ± 2 °C) and humidity (50%), with food and water freely available in their home cages.

General methods

Experiments were carried out in a total of 144 rats. After anaesthesia with ether and cannulation of the trachea, the rats were pithed by inserting a stainless steel rod through the orbit and foramen magnum into the vertebral foramen (Gillespie *et al.*, 1970). Then, the animals were artificially ventilated with room air using a model 7025 Ugo Basile pump (56 strokes per min; stroke volume = 20 ml kg^{-1}), as established by Kleinman and Radford (1964). After bilateral vagotomy, catheters were placed in: (i) the left and right femoral and jugular veins, for the continuous infusions of agonists (methoxamine or clonidine) and i.v. administration of antagonists, respectively; and (ii) the left carotid artery, connected to a Grass pressure transducer (P23XL), for the recording arterial blood pressure. Heart rate was measured with a tachograph (7P4, Grass Instrument Co., Quincy, MA, USA) triggered from the blood pressure signal. Both blood pressure and heart rate were recorded simultaneously by a model 7 Grass polygraph (Grass Instrument Co., Quincy, MA, USA). At this point, the 144 rats were initially divided into two main sets, so that the effects produced by the continuous infusions of methoxamine and clonidine under different treatments could be investigated on the vasodepressor responses induced by either: (i) electrical stimulation of the perivascular (vasodepressor) sensory outflow (set 1; $n = 120$) or (ii) i.v. bolus injections of exogenous α-CGRP (set 2; $n = 24$). The vasodepressor stimulus–response curves and dose–response curves elicited by electrical stimulation and exogenous α-CGRP, respectively, were completed in about 50 min, and each response was elicited under unaltered values of resting blood pressure. The electrical stimuli (0.56, 1, 1.8, 3.1 and 5.6 Hz) as well as the dosing with α-CGRP (0.1, 0.18, 0.31, 0.56 and 1 μg kg^{-1}) were given using a sequential schedule at 5–10 min intervals (see below). The body temperature of each pithed rat was maintained at 37 °C by a lamp and monitored with a rectal thermometer.

Experimental protocols

Protocol 1. Electrical stimulation of the perivascular (vasodepressor) sensory outflow. In the first set of rats ($n = 120$), the pithing rod was replaced by an electrode enamelled except for 1.5 cm length 9 cm from the tip, so that the uncovered segment was situated at T_9–T_{12} of the spinal cord, and an indifferent electrode was placed dorsally (Gillespie *et al.*, 1970; Taguchi *et al.*, 1992). Before electrical stimulation, the animals received (i.v.): (i) a bolus injection of gallamine (25 mg kg^{-1}) to avoid electrically induced muscular twitching; and (ii) 10 min later, a continuous infusion of hexamethonium (2 mg kg^{-1} min^{-1}) to block electrically induced vasopressor responses that are produced by stimulation of the preganglionic sympathetic vasopressor outflow (Villalón *et al.*, 1998). After 10 min, baseline values of diastolic blood pressure (a more accurate indicator of peripheral vascular resistance) and heart rate were determined. Then, this set of rats was divided into four groups ($n = 12$, 18, 30 and 60, respectively).

The first group ($n=12$) was subdivided into two subgroups ($n=6$ each) that received an i.v. continuous infusion of, respectively, 15 and 30 $\mu g\,kg^{-1}\,min^{-1}$ methoxamine (control experiments); whereas the second group ($n=18$) was subdivided into three subgroups ($n=6$ each) that received an i.v. continuous infusion of, respectively, 3, 10 and 30 $\mu g\,kg^{-1}\,min^{-1}$ clonidine. Thirty minutes later, the values of diastolic blood pressure and heart rate were determined once again in both groups; under these conditions, a sustained increase in diastolic blood pressure (maintained at around 135 mm Hg; see the Results section) was produced. Then, the perivascular sensory outflow was electrically stimulated during the infusions of methoxamine or clonidine to elicit vasodepressor responses (Taguchi *et al*., 1992) by applying 10-s trains of monophasic, rectangular pulses (2 ms, 50 V), at increasing frequencies of stimulation (0.56, 1, 1.8, 3.1 and 5.6 Hz). When diastolic blood pressure had returned to baseline levels, the next frequency was applied. This procedure was systematically performed until the stimulus–response curve had been completed.

The third group ($n=30$) received an i.v. continuous infusion of methoxamine (15 $\mu g\,kg^{-1}\,min^{-1}$) and, 20 min later, was subdivided into five subgroups ($n=6$ each) comprising i.v. bolus injections of, respectively: (i) saline (1 $ml\,kg^{-1}$); (ii) rauwolscine (300 $\mu g\,kg^{-1}$); (iii) BRL44408 (300 $\mu g\,kg^{-1}$); (iv) imiloxan (3000 $\mu g\,kg^{-1}$) and (v) MK912 (30 $\mu g\,kg^{-1}$). After 10 min, a stimulus–response curve was constructed as described above during the infusion of methoxamine to determine the effect of these antagonists *per se*.

The fourth group ($n=60$) received an i.v. continuous infusion of clonidine (10 $\mu g\,kg^{-1}\,min^{-1}$) and, 20 min later, was subdivided into 10 subgroups ($n=6$ each dose) comprising i.v. bolus injections of, respectively: (i) saline (1 $ml\,kg^{-1}$); (ii) rauwolscine (300 $\mu g\,kg^{-1}$); (iii) BRL44408 (100, 300 and 1000 $\mu g\,kg^{-1}$); (iv) imiloxan (3000 and 10000 $\mu g\,kg^{-1}$) and (v) MK912 (3, 10 and 30 $\mu g\,kg^{-1}$). Ten minutes later, a stimulus–response curve was constructed as described above, during the infusion of clonidine. Only one stimulus–response curve was carried out per animal since tachyphylaxis of the vasodepressor responses was observed when eliciting a second stimulus–response curve (data not shown).

Protocol 2. Administration of exogenous α-CGRP. The second set of rats ($n=24$) was prepared as described above, but the pithing rod was left throughout the experiment and the administration of both gallamine and hexamethonium was omitted. Then, this set of rats was divided into two groups ($n=12$ each). The first group was subdivided into two subgroups ($n=6$ each) that received an i.v. continuous infusion of, respectively, 15 and 30 $\mu g\,kg^{-1}\,min^{-1}$ methoxamine; whereas the second group was subdivided into two subgroups ($n=6$ each) that received an i.v. continuous infusion of, respectively, 10 and 30 $\mu g\,kg^{-1}\,min^{-1}$ clonidine. Thirty minutes later, baseline values of diastolic blood pressure and heart rate were determined as described above in both groups; subsequently, the vasodepressor responses elicited by i.v. bolus injections of exogenous α-CGRP (0.1, 0.18, 0.31, 0.56 and 1 $\mu g\,kg^{-1}$) were examined during the infusions of methoxamine or clonidine.

Other procedures applying to protocols 1 and/or 2
The doses of methoxamine, clonidine and hexamethonium were continuously infused at a rate of 0.02 $ml\,min^{-1}$ by a WPI model sp100i pump (World Precision Instruments Inc., Sarasota, FL, USA). The electrical stimuli and the doses of methoxamine and clonidine were selected from preliminary experiments. Moreover, the interval between the different frequencies of stimulation/doses of α-CGRP were dependent on the duration of the resulting vasodepressor responses (between 5 and 15 min), as we waited until diastolic blood pressure had returned to baseline values.

Data presentation and statistical evaluation
All data in the text, tables and figures, unless otherwise stated, are presented as mean ± s.e.mean. The peak changes in diastolic blood pressure by electrical stimulation or exogenous α-CGRP were expressed as percent change from baseline. The difference in the absolute values of diastolic blood pressure and heart rate within one subgroup of animals before and during the continuous infusions of methoxamine (15 and 30 $\mu g\,kg^{-1}\,min^{-1}$) or clonidine (3, 10 and 30 $\mu g\,kg^{-1}\,min^{-1}$) (after 30 min) was evaluated with paired Student's *t*-test. Moreover, a one-way ANOVA was used to compare the absolute values of diastolic blood pressure and heart rate obtained: (i) before and during the continuous infusions of methoxamine (15 and 30 $\mu g\,kg^{-1}\,min^{-1}$) and clonidine (3, 10 and 30 $\mu g\,kg^{-1}\,min^{-1}$) (after 30 min) in the different subgroups; or (ii) during the continuous infusions of methoxamine (15 $\mu g\,kg^{-1}\,min^{-1}$) and clonidine (10 $\mu g\,kg^{-1}\,min^{-1}$) before, immediately after and 10 min after administration of saline, BRL44408, imiloxan or MK912 within one subgroup. Finally, the vasodepressor responses induced by electrical stimulation or exogenous α-CGRP in the different subgroups of animals were compared with a two-way ANOVA (randomized block design). The one- and two-way ANOVAs were followed, if applicable, by the Student–Newman–Keuls' *post hoc* test (Steel and Torrie, 1980). Statistical significance was accepted at $P<0.05$.

Drugs
Apart from the anaesthetic (diethyl ether), the compounds used in this study (obtained from the sources indicated) were gallamine triethiodide, hexamethonium chloride, rat α-CGRP, methoxamine hydrochloride, clonidine hydrochloride, rauwolscine hydrochloride, imiloxan hydrochloride and MK912, ((2S, 12bs)1′,3′ dimethylspiro(1,3,4,5′,6,6′,7,12b-octahydro-2H-benzo[b]furo [2,3-a] quinazoline)-2,4′-pyrimidin-2′one hydrochloride) (Sigma Chemical Co., St Louis, MO, USA); and BRL44408 (2-[2H-(1-methyl-1,3-dihydroisoindole) methyl]-4,5-dihydroimidazole maleate) (Tocris Cookson Inc., Ellisville, MO, USA). All compounds were dissolved in saline. When needed, some drops of 10% ($v\,v^{-1}$) dimethylsulphoxide were used to dissolve BRL44408 and the resulting solution was finally diluted with saline. This vehicle had no effect on baseline diastolic blood pressure or heart rate (data not shown). Fresh solutions were prepared for each experiment. The doses of the antagonists refer to their respective salts, whereas those of the agonists refer to their free base.

Table 2 Baseline values of diastolic blood pressure and heart rate before and 30 min after administration of the i.v. continuous infusions of methoxamine or clonidine in the animals receiving electrical stimulation

Treatment	*Dose ($\mu g\,kg^{-1}\,min^{-1}$)*	n	*Diastolic blood pressure (mm Hg)*		*Heart rate (beats per min)*	
			Before	*After*	*Before*	*After*
Methoxamine	15	6	54 ± 2	130 ± 4*	248 ± 9	301 ± 12*
Methoxamine	30	6	53 ± 2	144 ± 6*	247 ± 4	287 ± 9*
Clonidine	3	6	48 ± 2	129 ± 4*	232 ± 10	264 ± 11*
Clonidine	10	6	51 ± 3	142 ± 6*	253 ± 11	277 ± 12*
Clonidine	30	6	52 ± 5	141 ± 8*	233 ± 17	283 ± 11*

*$P<0.05$, after *vs* before from the corresponding baseline value (paired *t*-test). It is noteworthy that the absolute values of diastolic blood pressure and heart rate obtained in the different subgroups, before and 30 min after, the continuous infusions of methoxamine and clonidine, were not significantly different ($P>0.05$).

Table 3 Values of diastolic blood pressure and heart rate during the infusion of methoxamine (15 $\mu g\,kg^{-1}\,min^{-1}$): (i) before; (ii) immediately after; and (iii) 10 min after i.v. administration of saline, rauwolscine, BRL44408, imiloxan or MK912

Treatment	*Dose ($\mu g\,kg^{-1}$)*	n	*Diastolic blood pressure (mm Hg)*			*Heart rate (beats per min)*		
			Before	*Immediately after*	*10 min after*	*Before*	*Immediately after*	*10 min after*
Saline	1[a]	6	112 ± 5	119 ± 3	120 ± 3	268 ± 9	271 ± 11	276 ± 15
Rauwolscine	300	6	133 ± 6	80 ± 11*	133 ± 8	245 ± 13	233 ± 15	266 ± 25
BRL44408	300	6	110 ± 2	78 ± 3*	113 ± 3	280 ± 7	268 ± 6	280 ± 9
Imiloxan	3000	6	100 ± 3	65 ± 3*	110 ± 3	270 ± 18	235 ± 18	268 ± 12
MK912	30	6	125 ± 5	96 ± 10*	134 ± 7	329 ± 13	304 ± 25	334 ± 16

*$P<0.05$, significantly different from before.
[a]Saline was given at a dose of 1 ml kg^{-1}.

Table 4 Values of diastolic blood pressure and heart rate during the infusion of clonidine (10 $\mu g\,kg^{-1}\,min^{-1}$): (i) before; (ii) immediately after; and (iii) 10 min after i.v. administration of saline, rauwolscine, BRL44408, imiloxan or MK912

Treatment	*Dose ($\mu g\,kg^{-1}$)*	n	*Diastolic blood pressure (mm Hg)*			*Heart rate (beats per min)*		
			Before	*Immediately after*	*10 min after*	*Before*	*Immediately after*	*10 min after*
Saline	1[a]	6	155 ± 7	158 ± 7	157 ± 8	238 ± 13	240 ± 12	245 ± 12
Rauwolscine	300	6	141 ± 7	111 ± 8*	135 ± 7	257 ± 12	240 ± 15	256 ± 8
BRL44408	300	6	141 ± 10	83 ± 5*	135 ± 9	242 ± 10	228 ± 7	240 ± 13
Imiloxan	3000	6	135 ± 10	87 ± 8*	135 ± 8	289 ± 14	241 ± 15*	295 ± 14
MK912	30	6	136 ± 6	105 ± 7*	129 ± 6	259 ± 8	254 ± 12	256 ± 5

*$P<0.05$, significantly different from before.
[a]Saline was given at a dose of 1 ml kg^{-1}.

Results

Systemic haemodynamic effects of the different treatments

The baseline values of diastolic blood pressure and heart rate in the 144 pithed rats were 52 ± 1 mm Hg and 242 ± 5 beats per min, respectively; these variables remained unchanged after gallamine or hexamethonium (data not shown). Table 2 shows that diastolic blood pressure and heart rate were significantly increased 30 min after the infusions of methoxamine or clonidine had commenced in the animals receiving electrical stimulation. Similar effects were obtained in the animals receiving i.v. bolus injections of α-CGRP (data not shown). It is noteworthy that the absolute values of diastolic blood pressure and heart rate obtained in the different subgroups before and 30 min after the continuous infusions of methoxamine and clonidine were not significantly different ($P>0.05$) (Table 2). Except when constructing the stimulus–response curves (see below), these effects were sustained throughout the experiments. Likewise, 10 min after administration of saline, rauwolscine, BRL44408, imiloxan or MK912 (during the infusion of methoxamine or clonidine), these haemodynamic variables remained without significant changes (see Tables 3 and 4, respectively); however, immediately after administration of these compounds (except saline), a transient—but significant—decrease in diastolic blood pressure was produced during the continuous infusion of methoxamine (see Table 3) or clonidine (see Table 4).

Vasodepressor responses produced by electrical stimulation

Overall, as shown in Figures 1a and b as well as in Figures 2a and b, during the continuous infusions of methoxamine or clonidine, electrical stimulation of the perivascular sensory outflow resulted in frequency-dependent vasodepressor responses, but the magnitude of these responses were

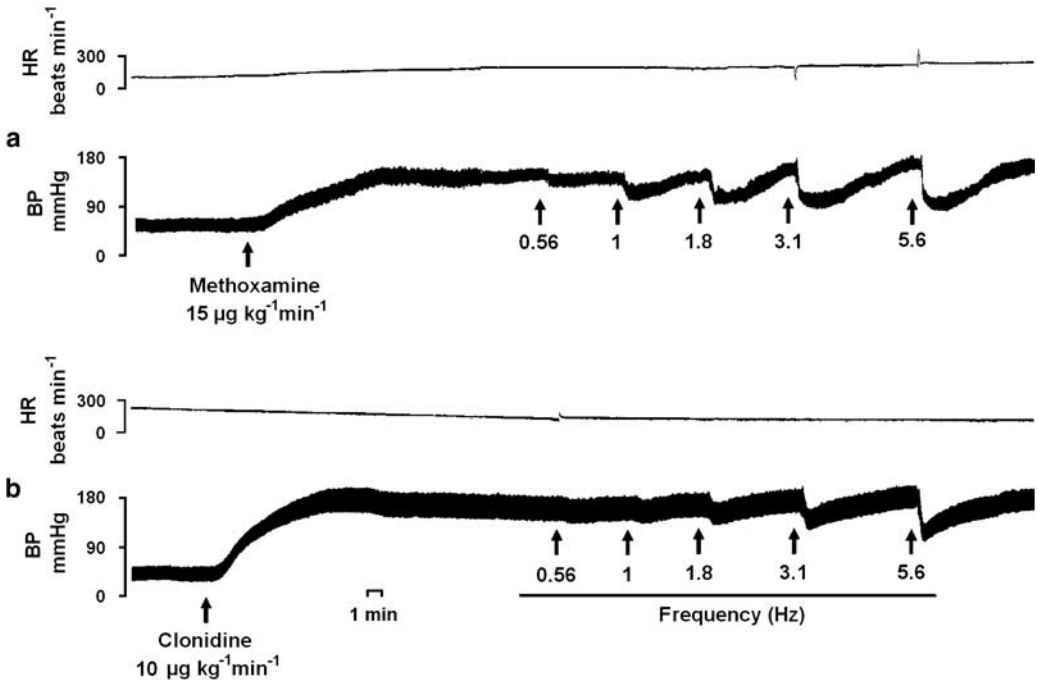

Figure 1 Original experimental tracings illustrating the vasodepressor responses produced by electrical stimulation of the perivascular sensory outflow during the continuous infusions of: (**a**) methoxamine (15 μg kg^{-1} min^{-1}) or (**b**) clonidine (10 μg kg^{-1} min^{-1}) in pithed rats. Note that the vasodepressor responses obtained during the clonidine infusion are smaller than those obtained during the methoxamine infusion. Similar results were obtained with the total number of experiments ($n=6$ for methoxamine and clonidine).

dependent on the agonist infused. In this respect, Figure 1 shows some representative experimental tracings illustrating that the electrically induced vasodepressor responses obtained during the infusion of clonidine (10 μg kg^{-1} min^{-1}; Figure 1b) were smaller (and even lesser when expressed as percent change from baseline) than those during the infusion of methoxamine (15 μg kg^{-1} min^{-1}; Figure 1a). In both cases, the vasodepressor responses appeared about 10 s after starting each electrical stimulus and reached a maximum 1 min after the stimulus had ended (see Figure 1). Moreover, Figure 2a shows that during the infusions of 15 and 30 μg kg^{-1} min^{-1} methoxamine, electrical stimulation of the perivascular sensory outflow produced similar ($P>0.05$) frequency-dependent vasodepressor responses. Although equivalent vasodepressor responses were also produced during the infusion of 3 μg kg^{-1} min^{-1} clonidine ($P>0.05$ *vs* those during 15 μg kg^{-1} min^{-1} methoxamine; Figure 2b), these responses were significantly inhibited during 10 and 30 μg kg^{-1} min^{-1} clonidine, particularly at higher frequencies of stimulation (Figure 2b); there was no significant difference between the inhibition produced by 10 and 30 μg kg^{-1} min^{-1} clonidine.

Vasodepressor responses produced by i.v. bolus injections of exogenous α-CGRP

Figure 2c shows that during infusions of 15 and 30 μg kg^{-1} min^{-1} methoxamine the dose-dependent vasodepressor responses to exogenous α-CGRP were not significantly different ($P>0.05$). Comparable vasodepressor responses to α-CGRP were also produced during infusions of 3 and 10 μg kg^{-1} min^{-1} clonidine ($P>0.05$ *vs* those during 15 μg kg^{-1} min^{-1} methoxamine; Figure 2d), but these responses were significantly attenuated during 30 μg kg^{-1} min^{-1} clonidine, particularly at higher doses of α-CGRP (Figure 2d). Irrespective of methoxamine or clonidine being infused, the vasodepressor responses to α-CGRP were immediate and reached a maximum 1 min after the corresponding i.v. injection had been given (data not shown).

Note that heart rate remained unaffected after electrical stimulation (Figure 1) or i.v. bolus injections of α-CGRP (data not shown). In contrast, the decreases in diastolic blood pressure returned to baseline levels within 5–10 min after electrical stimulation (Figure 1) or α-CGRP (data not shown), as reported by Taguchi *et al*., 1992.

As infusion of 10 μg kg^{-1} min^{-1} clonidine selectively inhibited the electrically induced vasodepressor responses (Figure 2b), without significantly affecting those by exogenous α-CGRP (Figure 2d), this dose of clonidine was chosen for further pharmacological analysis.

Effect of saline, rauwolscine, BRL44408, imiloxan or MK912 per se *on the electrically induced vasodepressor responses during an infusion of methoxamine*

As shown in Figure 3, during the i.v. continuous infusion of methoxamine (15 μg kg^{-1} min^{-1}), the vasodepressor responses produced by electrical stimulation in control animals remained practically unaltered ($P>0.05$) when compared to those elicited in animals pretreated with an i.v. bolus injection of: (i) saline (1 ml kg^{-1}; Figure 3a) or (ii) the α_2-adrenoceptor antagonists rauwolscine (300 μg kg^{-1}; Figure 3b), BRL44408 (300 μg kg^{-1}; Figure 3c),

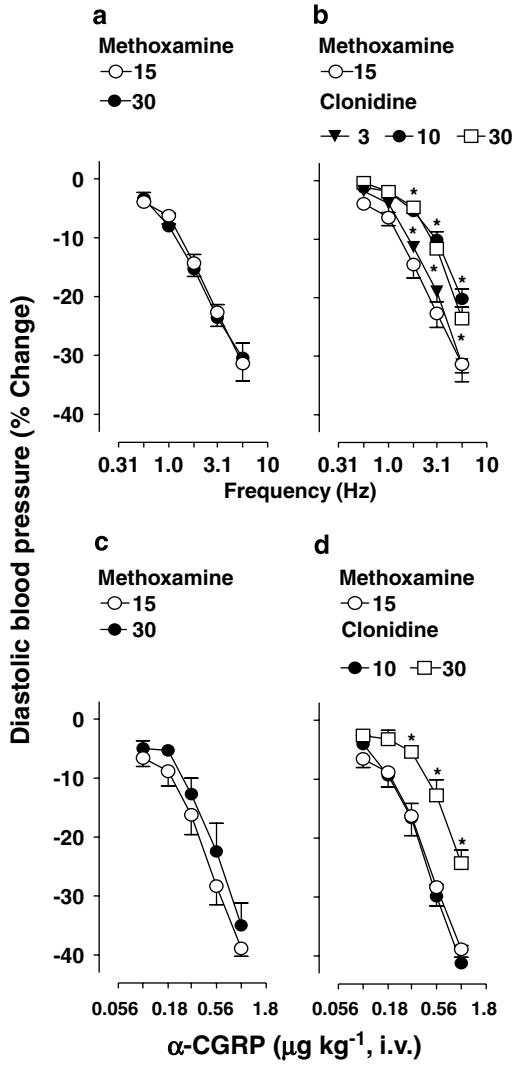

Figure 2 Vasodepressor responses induced by electrical stimulation (**a** and **b**) or i.v. bolus injections of exogenous α-CGRP (**c** and **d**) induced during i.v. continuous infusions of 15 and 30 $\mu g\,kg^{-1}\,min^{-1}$ methoxamine (left panel) or 3, 10 and 30 $\mu g\,kg^{-1}\,min^{-1}$ clonidine (right panel) ($n=6$ each dose). *$P<0.05$ *vs* the corresponding control response.

imiloxan (3000 $\mu g\,kg^{-1}$; Figure 3d) or MK912 (30 $\mu g\,kg^{-1}$; Figure 3e). These results indicate that these compounds, at the doses used and under the present experimental conditions, were essentially devoid of any effect *per se* on the electrically induced vasodepressor responses.

Effect of saline, rauwolscine, BRL44408, imiloxan or MK912 on clonidine-induced inhibition of the electrically induced vasodepressor responses

Figure 4 shows that clonidine- (10 $\mu g\,kg^{-1}\,min^{-1}$) induced inhibition of electrically induced vasodepressor responses (as compared to those during 15 $\mu g\,kg^{-1}\,min^{-1}$ methoxamine; control responses), which remained unaltered in animals pretreated with 1 $ml\,kg^{-1}$ saline (Figure 4a), was: (i) antagonized in animals pretreated with 300 $\mu g\,kg^{-1}$ rauwolscine (Figure 4b), 300 and 1000 $\mu g\,kg^{-1}$ BRL44408 (Figures 4d and e) or 10 and 30 $\mu g\,kg^{-1}$ MK912 (Figures 4i and j); and (ii) resistant to blockade in animals pretreated with 100 $\mu g\,kg^{-1}$ BRL44408 (Figure 4c), 3000 and 10000 $\mu g\,kg^{-1}$ imiloxan (Figures 4f and g) or 3 $\mu g\,kg^{-1}$ MK912 (Figure 4h). It must be emphasized that the doses of the above antagonists were high enough to completely block their respective α_2-adrenoceptor subtypes in the cardiovascular system (Bulloch *et al*., 1987; Gavin and Docherty, 1996; Willems *et al*., 2001; Cobos-Puc *et al*., 2007).

Discussion and conclusions

General

Several lines of evidence have shown the role of prejunctional α_2-adrenoceptors inhibiting noradrenaline release from sympathetic nerves (see Boehm and Kubista, 2002). Our study in pithed rats has investigated the role of specific α_2-adrenoceptor subtypes inhibiting the vasodepressor sensory outflow. To set up the appropriate experimental conditions for this purpose, in preliminary experiments we observed that only one stimulus–response curve could be carried out per animal since tachyphylaxis was observed when eliciting a second stimulus–response curve. This phenomenon may involve depletion of neuronal CGRP, uncoupling from the G protein, sequestration and/or receptor downregulation of CGRP receptors (see Buxton, 2006). However, our experimental model did not allow us to assess the extent to which each of these mechanisms is involved.

Apart from the implications discussed below, our study shows: (i) stimulation of the vasodepressor sensory outflow or i.v. injections of α-CGRP produced vasodepressor responses; (ii) infusions of 10 $\mu g\,kg^{-1}\,min^{-1}$ clonidine inhibited the electrically induced vasodepressor responses without affecting those by exogenous α-CGRP, a finding that may suggest a prejunctional inhibitory action; and (iii) infusions of 30 $\mu g\,kg^{-1}\,min^{-1}$ clonidine attenuated both responses (possibly due to both prejunctional and non-selective postjunctional inhibitory interactions). Admittedly, we have no clear-cut explanation for 30 $\mu g\,kg^{-1}\,min^{-1}$ clonidine producing the same inhibition as 10 $\mu g\,kg^{-1}\,min^{-1}$ clonidine on the electrically induced vasodepressor responses. Thus, the inhibition by 10 $\mu g\,kg^{-1}\,min^{-1}$ clonidine, being antagonized by rauwolscine ($\alpha_{2A/2B/2C}$), BRL44408 (α_{2A}) or MK912 (α_{2C}), but not by imiloxan (α_{2B}), may be mainly mediated by prejunctional α_{2A}/α_{2C}-adrenoceptor subtypes which, upon activation, inhibit the perivascular sensory outflow.

Systemic haemodynamic effects produced by the different treatments

As reported by Taguchi *et al*. (1992), stimulation of the perivascular sensory outflow produces vasodepressor responses only after continuous i.v. infusions of: (i) hexamethonium (to block autonomic outflow) and (ii) methoxamine (for a sustained increase in blood pressure). Our study reproduced these conditions, so that resting diastolic blood pressure was artificially increased and maintained at around 135 mm Hg by continuous i.v. infusions of either

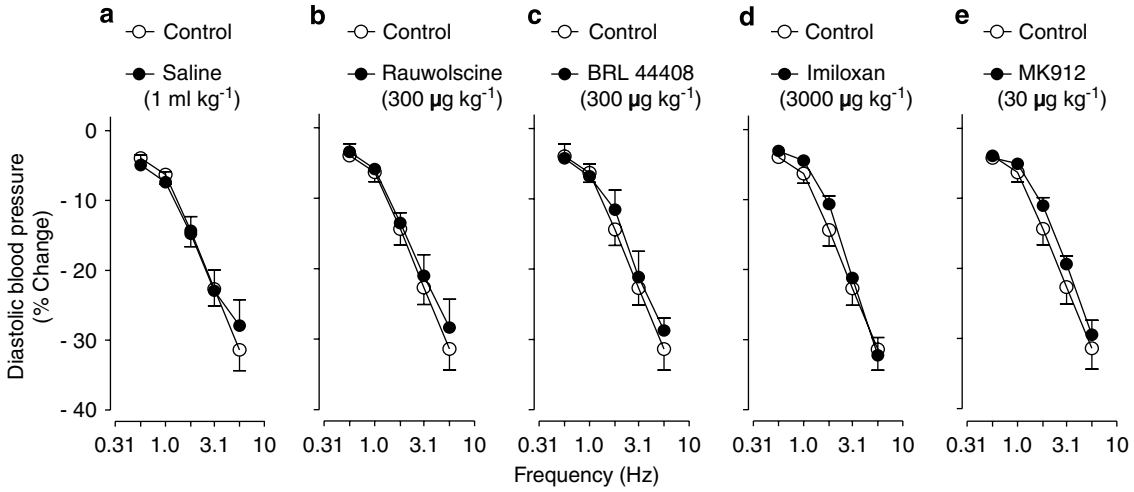

Figure 3 Effect of i.v. bolus injections of saline (**a**; 1 ml kg^{-1}), rauwolscine (**b**; 300 µg kg^{-1}), BRL44408 (**c**; 300 µg kg^{-1}), imiloxan (**d**; 3000 µg kg^{-1}) or MK912 (**e**; 30 µg kg^{-1}) *per se* ($n=6$ each dose) on the electrically induced vasodepressor responses produced during an i.v. continuous infusion of methoxamine (15 µg kg^{-1} min^{-1}) ($n=6$ for each group).

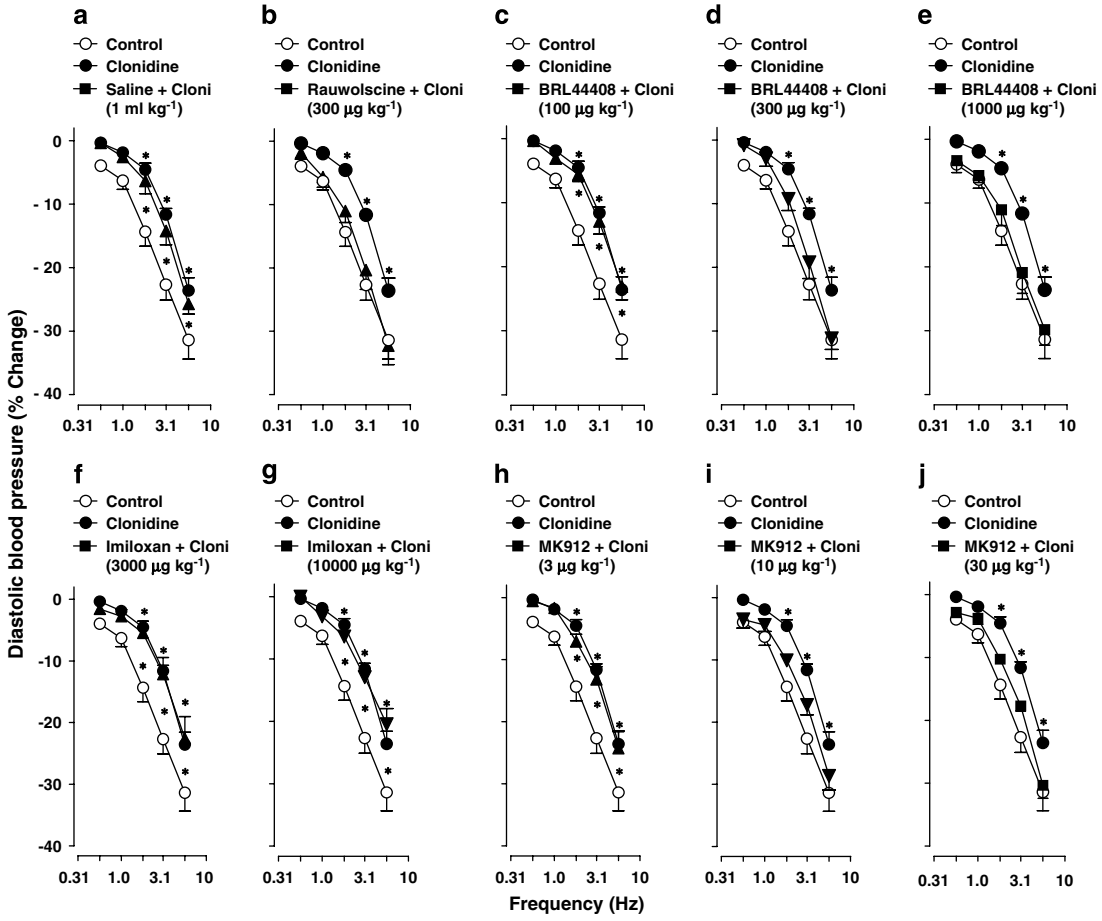

Figure 4 Effect of i.v. bolus injections of saline (**a**; 1 ml kg^{-1}), rauwolscine (**b**; 300 µg kg^{-1}), BRL44408 (**c–e** for, respectively, 100, 300 and 1000 µg kg^{-1}), imiloxan (**f** and **g** for, respectively, 3000 and 10000 µg kg^{-1}) or MK912 (**h–j** for, respectively, 3, 10 and 30 µg kg^{-1}) ($n=6$ each dose) on the inhibition induced by clonidine (cloni.; 10 µg kg^{-1} min^{-1}) of the electrically induced vasodepressor responses. The control group represents that of animals receiving an i.v. continuous infusion of methoxamine (15 µg kg^{-1} min^{-1}; shown for comparison). *$P<0.05$ *vs* the corresponding control response.

methoxamine (used as a control) or clonidine (used as a treatment). This sustained increase in diastolic blood pressure (Table 2), resulting from an increase in peripheral vascular resistance (see Hoffman, 2001), can be mainly attributed to stimulation of vascular α_1-(methoxamine; Decker *et al.*, 1984) and α_2-(clonidine; Kobinger and Pichler,

1978) adrenoceptors; admittedly, clonidine may also interact with α_1-adrenoceptors (Timmermans and Van Zwieten, 1980) and imidazoline-binding sites (Eglen *et al*., 1998).

In contrast, we have no clear-cut explanation for: (i) the slight (although significant) increases in heart rate produced by the infusions of methoxamine or clonidine (Table 2), which do not activate β-adrenoceptors (see Hoffman, 2001); and (ii) the transient decreases in diastolic blood pressure produced immediately after administration of the α_2-adrenoceptor antagonists (Tables 3 and 4). These effects are most likely to be drug-induced as a similar infusion schedule of saline in pithed rats did not significantly change either heart rate (Sánchez-López *et al*., 2003) or diastolic blood pressure (see Tables 3 and 4).

The role of specific α_2-adrenoceptor subtypes inhibiting the vasodepressor sensory outflow

Since clonidine- ($10\,\mu g\,kg^{-1}\,min^{-1}$) induced inhibition of the vasodepressor sensory outflow was abolished by rauwolscine at a dose that completely blocks α_2-adrenoceptors (Cobos-Puc *et al*., 2007), the role of these receptors is clearly proven. Admittedly, clonidine can also interact with imidazoline receptors (Eglen *et al*., 1998), but rauwolscine does not block them at concentrations that completely antagonize α_2-adrenoceptors (Göthert *et al*., 1999). On this basis, we investigated the role of the α_{2A}, α_{2B} and α_{2C} subtypes by using the antagonists BRL44408 (α_{2A}), imiloxan (α_{2B}) and MK912 (α_{2C}) (Table 1) at doses high enough to completely antagonize their respective receptor subtypes (Bulloch *et al*., 1987; Gavin and Docherty, 1996; Willems *et al*., 2001; Cobos-Puc *et al*., 2007). As BRL44408 (300 and $1000\,\mu g\,kg^{-1}$) or MK912 (10 and $30\,\mu g\,kg^{-1}$), but not imiloxan (3000 and $10000\,\mu g\,kg^{-1}$), antagonized clonidine-induced inhibition (Figure 4), the simplest interpretation of these findings is that α_{2A} (blocked by BRL44408) and α_{2C} (blocked by MK912), rather than α_{2B}, adrenoceptors could be involved. This interpretation is consistent with other studies showing the inhibitory role or expression of α_{2A}- and α_{2C}-, but not of α_{2B}-, adrenoceptors in other rat nerve terminals including peptidergic (that is, trigeminal ganglion; Takeda *et al*., 2002) and cardioaccelerator sympathetic (Cobos-Puc *et al*., 2007) neurons.

Nonetheless, the binding properties (Table 1) and profile of blockade displayed by BRL44408 and MK912 (Figure 4) deserve further considerations. First, MK912 has a very high affinity for the α_{2C} subtype ($pK_i = 10.2$), but it cannot selectively discriminate among α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors (Table 1). Second, no information is available on the selectivity of MK912 at rodent α_2-adrenoceptor subtypes. Third, the *in vitro* α_{2A}- *vs* α_{2C}-selectivity of BRL44408 and MK912 is small (Devedjian *et al*., 1994), leaving very little room for *in vivo* selectivity. Fourth, although $3\,\mu g\,kg^{-1}$ MK912 failed to block clonidine-induced inhibition (Figure 4h), we cannot categorically rule out that the similar blockade produced by 10 and $30\,\mu g\,kg^{-1}$ MK912 (Figures 4i and j) could reflect antagonism of α_{2A}- and α_{2C}-adrenoceptors (Table 1). Last, if two receptor subtypes (that is, α_{2A} and α_{2C}) were involved in clonidine-induced inhibition, it could be argued that the corresponding antagonists given together should be additive in their blocking effects and/or that a component of the response could have been resistant to one antagonist, but not to another one. However, our results show that the 'window' of inhibition produced by $10\,\mu g\,kg^{-1}\,min^{-1}$ clonidine was so small that it would be practically impossible to obtain a clear-cut partial blockade when using only one antagonist; in fact, as shown in Figure 4, $300\,\mu g\,kg^{-1}$ BRL44408 or $10\,\mu g\,kg^{-1}$ MK912 seem to produce a partial blockade of the response to clonidine, but this response does not significantly differ ($P > 0.05$) from the control response (that is, it was already blocked).

Certainly, the failure of 3000 and $10\,000\,\mu g\,kg^{-1}$ imiloxan to block clonidine-induced inhibition (Figures 4f and g) does not support the role of α_{2B}-adrenoceptors. Consistent with this view, the inhibitory effect of the α_2-adrenoceptor agonist brimonidine on noradrenaline release was similar in α_{2B}-KO mice and wild-type mice, indicating that the α_{2B}-subtype does not modulate neurotransmitter release (Hein *et al*., 1999).

Regarding the extent to which α_{2A}/α_{2C}-adrenoceptors contribute to clonidine-induced inhibition, there seems to be a great debate on the differential distribution of α_{2A}- and α_{2C}-adrenoceptor immunoreactivity on rat sensory neurons. For example, Stone *et al*. (1998), using immunocolocalization studies in the rat spinal cord, suggest that the main localization of α_{2A}-adrenoceptors is on capsaicin-sensitive (afferent) terminals, whereas the majority of α_{2C}-adrenoceptors was not of primary afferent origin. In contrast, Shi *et al*. (2000), using *in situ* hybridization with riboprobes, demonstrated the presence of the α_{2A} (20%) and α_{2C} (80%), but not of α_{2B}, adrenoceptor mRNAs in rat dorsal root ganglia. Admittedly, the extent to which α_{2A}- and α_{2C}-adrenoceptors contribute to clonidine-induced inhibition would require further experiments, which fall beyond the scope of the present investigation.

In conclusion, the above results, taken together, suggest that the inhibition produced by $10\,\mu g\,kg^{-1}\,min^{-1}$ clonidine on the vasodepressor sensory outflow may be mainly mediated by prejunctional α_{2A}- and α_{2C}-adrenoceptors.

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Conflict of interest

The authors state no conflict of interest.

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