

# Functional characterization of $\alpha_1$ -adrenoceptor subtypes in vascular tissues using different experimental approaches: a comparative study

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**1** The  $\alpha_1$ -adrenergic responses of rat aorta and tail artery have been analysed measuring the contractility and the inositol phosphate (IP) formation induced by noradrenaline. Three antagonists, prazosin, 5-methylurapidil ( $\alpha_{1A}$  selective) and BMY 7378 ( $\alpha_{1D}$  selective) have been used in different experimental procedures.

**2** Noradrenaline possesses a greater potency inducing contraction and IP accumulation in aorta (pEC<sub>50</sub>-contraction =  $7.32 \pm 0.04$ ; pEC<sub>50</sub>-IPs =  $6.03 \pm 0.08$ ) than in the tail artery (pEC<sub>50</sub>-contraction =  $5.71 \pm 0.07$ ; pEC<sub>50</sub>-IPs =  $5.51 \pm 0.10$ ). Although the maximum contraction was similar in both tissues (E<sub>max</sub>-tail =  $619.1 \pm 55.6$  mg; E<sub>max</sub>-aorta =  $698.2 \pm 40.8$  mg), there were marked differences in the ability of these tissues to generate intracellular second messengers the tail artery being more efficient (E<sub>max</sub>-tail =  $1060 \pm 147\%$ ; E<sub>max</sub>-aorta =  $108.1 \pm 16.9\%$ ).

**3** Concentration response curves of noradrenaline in presence of antagonist together with concentration inhibition curves for antagonists added before (CICb) or after (CICa) noradrenaline-induced maximal response in Ca<sup>2+</sup>-containing or Ca<sup>2+</sup>-free medium have been performed. A comparative analysis of the different procedures as well as the mathematical approaches used in each case to calculate the antagonist potencies, were completed.

**4** The CICa was the simplest method to characterize the predominant  $\alpha_1$ -adrenoceptor subtype involved in the functional response of a tissue.

**5** In aorta, where constitutively active  $\alpha_{1D}$ -adrenoceptors are present, the use of different experimental procedures evidenced a complex equilibrium between  $\alpha_{1D}$ - and  $\alpha_{1A}$ -adrenoceptor subtypes.

**6** The appropriate management of LiCl in IP accumulation studies allowed us to reproduce the different experimental procedures performed in contractile experiments giving more technical possibilities to this methodology.

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**Keywords:**  $\alpha_1$ -Adrenoceptor subtypes; vascular smooth muscle; noradrenaline; BMY 7378; 5-methylurapidil; contraction; inositol phosphates; antagonist potencies

**Abbreviations:** AR, adrenoceptor; BMY 7378, 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl-8-azaspiro[4,5]decane-7,9-dione dihydrochloride; Ca (+) or Ca (–) = Ca<sup>2+</sup> containing or Ca<sup>2+</sup>-free solution; CICb or CICa = concentration-inhibition curves for the antagonists added 'before' or 'after' noradrenaline-induced maximal response, respectively; IP, inositol phosphate; NA, noradrenaline

## Introduction

In functional studies, in which an antagonist affects the response produced by an agonist on a tissue, the relation between antagonist, agonist and response observed is complex, even when analysing competitive antagonists. In fact, the binding may follow the law of mass action needed to apply the Cheng & Prusoff (1973) equation, but the functional response curve can be altered by non-linear amplification of the signal. This is specially true if different subtypes of receptors, with different subcellular localizations and a different degree of coupling to internal signals, are

involved in the response, as occurs with  $\alpha_1$ -adrenoceptors (Schwinn *et al.*, 1995; Theroux *et al.*, 1996; McCune *et al.*, 2000; Piasecki & Perez, 2001) or, as has recently been shown, if a constitutively active receptor, such as  $\alpha_{1D}$ -adrenoceptor, intervenes in it (Noguera *et al.*, 1996; García-Sáinz & Torres-Padilla, 1999; Gisbert *et al.*, 2000; 2002; McCune *et al.*, 2000; Ziani *et al.*, 2002).

Until now, the Schild analysis is considered the most reliable method to calculate the equilibrium dissociation constant for a competitive antagonist in functional studies (Arunlakshana & Schild, 1959). This analysis needs the construction of full concentration-response curves for an agonist in the absence and in the presence of fixed antagonist concentrations and this makes the Schild analysis

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awkward but useful in contractile studies in isolated organ baths. However, some functional responses, notably those where a desensitization appears or those where second messenger generation was measured, are not susceptible to this kind of experimental design. The most common methodology in these situations is an inhibition curve design that involves antagonist titration in the presence of a fixed concentration of agonist. In this case, the equation of Cheng & Prusoff (1973) and its modifications for functional studies (Craig, 1993; Leff & Douglas, 1993) are the methods chosen to calculate the estimated  $K_i$ , although they are not considered a theoretically valid alternative to the Schild analysis (Craig, 1993; Leff & Douglas, 1993; Lazareno & Birdsall, 1993).

The aim of this study was to make a comparative analysis between the two different methodological approaches cited above and commonly used to calculate the antagonist potency in functional studies, and a new experimental procedure which we validate as the simplest alternative method when ethical reasons or difficulties to obtain tissue samples are considered. In order to do so, we have chosen the family of  $\alpha_1$ -adrenoceptors ( $\alpha_1$ -ARs) integrated by three different subtypes ( $\alpha_{1A}$ ,  $\alpha_{1B}$  and  $\alpha_{1D}$ ), with different cellular localization and different degrees of coupling to intracellular signals (Piascik & Perez, 2001). We studied the contractility of vascular smooth muscle (aorta and tail artery) as an indicator of its functional activity, and the inositol phosphate (IP) accumulation as the biochemical signal linked to its activation. We have chosen aorta and tail artery as tissues representative of the functionality of different subtypes of  $\alpha_1$ -ARs since it is well established that the  $\alpha_{1D}$ - and the  $\alpha_{1A}$ -ARs are mainly responsible for the contractile response to adrenergic stimulus in aorta and tail artery respectively (Kenny *et al.*, 1995; Lachnit *et al.*, 1997; Saussy *et al.*, 1996; Hussain & Marshall, 1997; Gisbert *et al.*, 2000). The evidence that  $\alpha_{1D}$ -ARs in native tissues such as aorta, exhibit constitutive activity (Noguera *et al.*, 1996; Gisbert *et al.*, 2000; 2002; Ziani *et al.*, 2002), was also an important reason to choose the aorta in this study.

## Methods

### *Functional studies in isolated organ bath*

Rings of the aorta and tail artery (approx 3–5 mm in length) of female Wistar rats (200–220 g) were denuded of endothelium by gentle rubbing and suspended in a 10 ml organ bath containing physiological solution, maintained at 37°C and gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. An initial load of 1 g was applied and maintained throughout a 75–90 min equilibration period. Tension was recorded isometrically by Grass FT03 force displacement transducers, and data were recorded on disc (McLab). The composition of the physiological Ca<sup>2+</sup>-containing solution was (mM): NaCl 118, KCl 4.75, CaCl<sub>2</sub> 1.8, MgCl<sub>2</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25 and glucose 11. The Ca<sup>2+</sup>-free solution had the same composition, except that CaCl<sub>2</sub> was omitted and EDTA (0.1 mM) was added.

The absence of relaxant response (10%) after acetylcholine (100  $\mu$ M) addition to preparations precontracted with noradrenaline (10 or 1  $\mu$ M in tail artery or aorta, respectively)

indicated the absence of a functional endothelium in all the rings.

Three different experimental procedures were used:

- (1) *Concentration-response curves to noradrenaline*. These were performed by the addition of cumulative concentrations of the agonist (0.1 nM–100  $\mu$ M) to tissues in the absence or presence of antagonist (incubated for 15 min). Contractions were expressed in mg of developed tension or as a percentage of the maximal contraction to the agonist in normal physiological solution ( $E_{max}$ ). The concentration ( $-\log [M]$ ) of agonist required to produce 50% of the maximal response ( $pEC_{50}$ ) was obtained from a non-linear regression plot (Graph Pad Software; San Diego, CA, U.S.A.). Estimates of the antagonist affinity were determined from the negative logarithm of the antagonist dissociation constant ( $K_B$ ), determined from the equation  $K_B = [A]/(DR-1)$  where the dose ratio (DR) was produced by a single concentration of antagonist [A].
- (2) *Concentration-inhibition curves to selective  $\alpha_1$ -adrenoceptor antagonists added 'before' agonist stimulation (CICb)*. CICb to Prazosin (0.001 nM–10  $\mu$ M, BMY 7378 (0.001 nM–30  $\mu$ M) and 5-methylurapidil (0.01 nM–10  $\mu$ M) were obtained by the addition of one concentration of a compound 15 min before and during noradrenaline-induced contraction in Ca<sup>2+</sup> containing, or Ca<sup>2+</sup> free medium.

In physiological Ca<sup>2+</sup>-containing solution, two successive additions of maximal concentrations of NA (10  $\mu$ M in rat tail artery or 1  $\mu$ M in rat aorta) give similar sustained contractile responses. After washing, a third addition of the agonist was made in the presence of a concentration of antagonist.

The experimental procedure designed to study the actions of compounds on the contractile response to noradrenaline in Ca<sup>2+</sup>-free medium was as follows: noradrenaline (NA<sub>1</sub> 10  $\mu$ M in rat tail artery or 1  $\mu$ M in rat aorta) was added in physiological Ca<sup>2+</sup>-containing solution at 37°C and then the tissue was treated with Ca<sup>2+</sup>-free, EDTA-containing solution for 20 min. After this time, NA (NA<sub>2</sub>) was applied and the amplitude of the resulting contraction was monitored as a reference. A new addition of agonist did not reproduce this response because intracellular Ca<sup>2+</sup> stores were depleted, then the tissue was incubated for 20 min in Krebs solution to refill the intracellular Ca<sup>2+</sup> stores. After washing and 20 min of incubation in Ca<sup>2+</sup>-free solution, NA (NA<sub>3</sub>) was added again in the absence of Ca<sup>2+</sup> and a response, similar to the first one obtained in Ca<sup>2+</sup>-free solution (NA<sub>2</sub>), was observed. The effect of different concentrations of each compound added 15 min before was tested on this last agonist-induced contraction in Ca<sup>2+</sup>-free medium.

The magnitude of the noradrenaline-induced contraction in the presence of the different concentrations of the antagonists was expressed as a percentage of noradrenaline-induced response obtained in the absence of any agent.

- (3) *Concentration-inhibition curves to selective  $\alpha_1$ -adrenoceptor antagonists added 'after' agonist stimulation (CICa)*. CICa to selective  $\alpha_1$ -adrenoceptor antagonists were performed by addition of cumulative concentrations of pra-

zozin (0.001 nM–1  $\mu$ M), BMY 7378 (0.001 nM–30  $\mu$ M) and 5-methylurapidil (0.01 nM–10  $\mu$ M) to tail artery and aortic rings in which sustained contractions had been induced by a maximal concentration of noradrenaline (10 or 1  $\mu$ M respectively). Relaxations were expressed as a percentage of the maximum increment in tension obtained by agonist addition.

The concentration of antagonist ( $-\log [M]$ ) needed to produce 50% relaxation (procedure CICa) or inhibition (procedure CICb) was obtained ( $pIC_{50}$ ) from a non-linear regression plot (Graph Pad Software; San Diego, CA, U.S.A.).  $K_i$  value was calculated from the  $IC_{50}$  estimates according to the Cheng & Prusoff (1973) equation:

$$K_i = IC_{50}/1 + (A/EC_{50})$$

where A is the concentration of noradrenaline present in the assay and  $EC_{50}$  is the concentration of noradrenaline required for half-maximal stimulation in each tissue.

### Accumulation of [ $^3H$ ]-inositol phosphates

The determination of the accumulation of inositol phosphates was adapted from Berridge *et al.* (1982). Briefly, rat tail arteries or rat thoracic aortas (four or five female Wistar rats 200–220 g were sacrificed) were cut into rings (2 mm for tail artery, 1 mm for aorta), pooled and incubated at 37°C for 30 min in physiological solution (composition in mM): NaCl 118, KCl 4.7, CaCl<sub>2</sub> 1.8, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, and glucose 11, aerated with 95% O<sub>2</sub>/5% CO<sub>2</sub>. The physiological solution was changed twice. Subsequently, both tissues were labelled in the presence of 10  $\mu$ Ci ml<sup>-1</sup> of *myo* [ $^3H$ ]-inositol (specific activity 81.0 Ci mmol<sup>-1</sup>) in physiological solution for 2 h at 37°C with vigorous shaking, aerating the mixture every 15 min. After incubation, the samples were washed twice with physiological solution and two pieces of tail artery or four rings of aorta (0.1–0.2 mg of protein) were placed in individual tubes which were incubated at 37°C under an atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub> in a final volume of 500  $\mu$ l of physiological solution. The tissues were incubated for 30 min with increasing concentrations of noradrenaline (0.01  $\mu$ M–1 mM) in the presence of LiCl (10 mM) in order to inhibit the metabolism of inositol monophosphates.

The antagonist action of prazosin (0.1 nM–10  $\mu$ M), BMY 7378 (1 nM–100  $\mu$ M) and 5-methylurapidil (0.1 nM–100  $\mu$ M) on noradrenaline-induced inositol phosphate accumulation was tested in two experimental conditions in order to reproduce the experiments carried out in organ bath studies: (i) In the CICb protocol, the tissues were incubated for 15 min with different concentrations of the antagonist and subsequently stimulated for 30 min with a maximal concentration of noradrenaline (10  $\mu$ M), LiCl was added 30 s before agonist addition. (ii) In the CICa protocol, the tissues were stimulated with 10  $\mu$ M for 30 min and allowed to incubate for an additional period of 15 min with different concentrations of the antagonists. LiCl (10  $\mu$ M) was then added to the tubes during an additional incubation period of 30 min.

In all cases, unstimulated (basal) as well as noradrenaline-stimulated (maximal response) [ $^3H$ ]-IP formation was determined simultaneously. In the CICa procedure, a sample was included to determine the [ $^3H$ ]-IP accumulation induced by

noradrenaline in the absence of LiCl. Each sample was performed in triplicate.

The reaction was stopped by the addition of 2 ml cold CH<sub>3</sub>OH/CHCl<sub>3</sub>/HCl 40:20:1 (v/v/v) mixture and the samples were sonicated for 45 min at 2–5°C in an ultrasonic water bath. After the addition of 0.63 ml of CHCl<sub>3</sub> and 1.26 ml of distilled water, the samples were centrifuged at 1500  $\times$  g for 10 min to facilitate phase separation. The aqueous layer was removed from the tubes for the [ $^3H$ ]-IP assay. Each sample was neutralized to pH 7–7.5 and run through a column containing Dowex AG 1X8 formate (100–200 mesh,  $\sim$ 0.5 ml bed volume, Bio-Rad) ion exchange resin, previously equilibrated with 30 ml 10 mM Tris-formate, pH 7.4. The columns were washed with 6 ml distilled water and 6 ml 60 mM sodium formate/5 mM sodium tetraborate to eliminate free *myo*[ $^3H$ ]-inositol and glycerophosphoinositol, respectively. Total [ $^3H$ ]-IPs were eluted with 3 ml of 0.1 M formic acid in 1 M ammonium formate according to the method of Berridge *et al.* (1982), and counted for radioactivity. The lipid layer remaining after removal of the aqueous phase was used for measurement of [ $^3H$ ]-phosphatidylinositols. Aliquots of the lipid phase (200  $\mu$ l) were removed and placed in scintillation vials, allowed to evaporate overnight, and counted for radioactivity in order to calculate total [ $^3H$ ]-inositol incorporated in each sample.

Accumulation of [ $^3H$ ]-IPs was routinely calculated as a percentage (d.p.m.%) of total [ $^3H$ ]-inositol labelled lipids in each individual sample to correct interexperimental variations in label incorporation and sample sizes or was expressed as a percentage over the unstimulated [ $^3H$ ]-IP accumulation (basal). The [ $^3H$ ]-IP accumulation in the presence of the different concentrations of the antagonists was expressed as percentages of the maximum increase obtained in presence of the agonist after subtracting the unstimulated [ $^3H$ ]-IP accumulation (basal).

Concentration-response data for both noradrenaline-induced [ $^3H$ ]-IP accumulation and antagonist inhibition were fitted by non-linear regression plot (Graph Pad Software; San Diego, CA, U.S.A.) and the  $pEC_{50}$  and  $pIC_{50}$  was obtained. The  $K_i$  value was calculated from the  $IC_{50}$  estimates according to the Cheng & Prusoff (1973) equation as described above.

**Chemicals** The following drugs were obtained from SIGMA (St. Louis MO, U.S.A.): acetylcholine chloride, (–)-noradrenaline bitartrate, prazosin, lithium chloride or Research Biochemicals International (Natick, MA, U.S.A.): BMY 7378 (8-[2-[4-(2-Methoxyphenyl)-1-piperazynil-8-azaspiro[4,5]decane-7,9-dione dihydrochloride), 5-methylurapidil. *Myo*-[ $^3H$ ]-inositol with PT6 was from Amersham (Buckinghamshire, U.K.). Other reagents were of analytical grade. All compounds were dissolved in distilled water.

### Statistical analysis

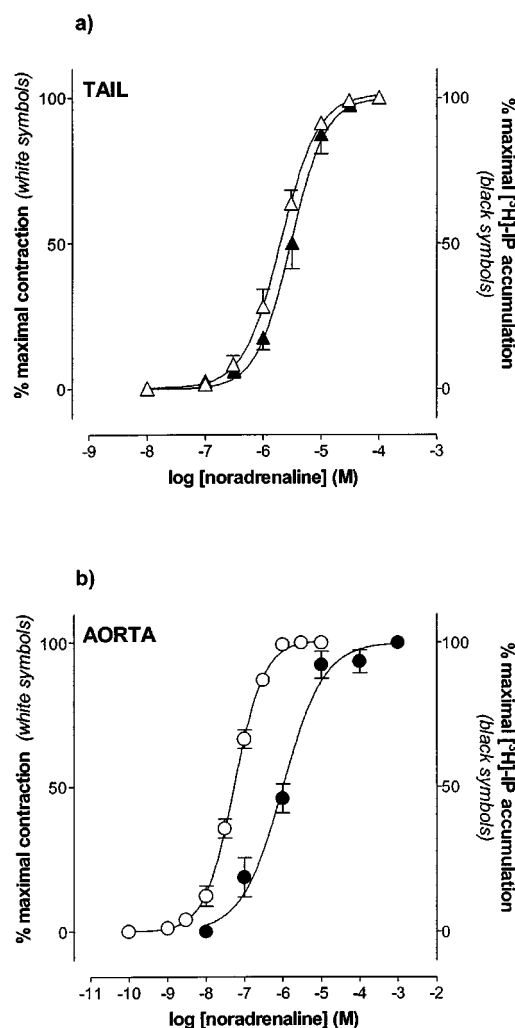
The results are presented as the mean  $\pm$  s.e.mean or 95% confidence intervals for  $n$  determinations obtained from different animals. Where ANOVA showed significant differences ( $P < 0.05$ ), the results were further analysed using the Student–Newman–Keuls test and differences were considered significant when  $P < 0.05$ .

## Results

### *Effects of noradrenaline on contractile force and [<sup>3</sup>H]-inositol phosphate accumulation in rat tail artery and rat aorta*

Noradrenaline (0.1 nM–1 mM) elicited concentration-dependent contractions and [<sup>3</sup>H]-IP accumulation in both aorta and tail artery (Figure 1). The pEC<sub>50</sub> and E<sub>max</sub> obtained are summarized in Table 1. The maximal contraction to noradrenaline is similar in both tissues and similar also to the maximal contractile response induced by a depolarizing solution (KCl 80 mM) (Tail: 610.7 ± 33.3 mg, *n* = 4; Aorta: 600.8 ± 48.8 mg, *n* = 6). However, the potency of noradrenaline was significantly higher in aorta than in tail artery (Table 1).

When we analysed the [<sup>3</sup>H]-IP accumulation as the intracellular signal linked to  $\alpha_1$ -AR activation, we found that the potency of noradrenaline was also significantly higher in aorta than in tail artery (Table 1) but, this increase in



**Figure 1** Comparative analysis of concentration-response curves of contraction and [<sup>3</sup>H]-inositol phosphate accumulation to noradrenaline in tail artery (a) and aorta (b). Data are means (expressed as a percentage of the maximal response to the agonist) ± s.e. mean of 5–6 experiments.

potency was accompanied by a lower efficiency of IP formation because the maximum [<sup>3</sup>H]-IP accumulation induced by noradrenaline was up to 10 times lower in aorta than in tail artery (Table 1).

These results evidence a close relationship between the contractile force and the [<sup>3</sup>H]-IP formation induced by noradrenaline in tail artery (Figure 1a). This relationship was not found in rat aorta where the pEC<sub>50</sub> value for noradrenaline induced contraction was significantly higher than the pEC<sub>50</sub> value for this agonist in stimulating [<sup>3</sup>H]-IPs formation (Figure 1b, Table 1). In fact, the maximum contractile response was obtained with noradrenaline 1  $\mu$ M whereas the maximum [<sup>3</sup>H]-IP accumulation was obtained with noradrenaline 10  $\mu$ M (Figure 1b).

### *Effects of $\alpha_1$ -adrenoceptor antagonists on noradrenaline-induced contractile response*

The pK<sub>B</sub> values obtained for prazosin (non selective antagonist between the three subtypes), BMY 7378 ( $\alpha_{1D}$ -selective) and 5-methylurapidil ( $\alpha_{1A}$ -selective) on rat tail artery and rat aorta are summarized in Tables 2 and 3, respectively, and are in the same range of the pA<sub>2</sub>/pK<sub>B</sub> values previously obtained by other authors in these tissues (see Table 5).

The CICb protocol was performed in two experimental conditions: in Ca<sup>2+</sup>-containing or Ca<sup>2+</sup>-free solution and the results were different depending on the presence or not of this ion in the incubating medium (Tables 2 and 3, Figure 2). The pIC<sub>50</sub> value of each antagonist determined in Ca<sup>2+</sup>-free medium was similar to the pK<sub>B</sub> value previously obtained (Tables 2 and 3). However, the pIC<sub>50</sub> values determined in Ca<sup>2+</sup>-containing solution were lower than pK<sub>B</sub>, but similar values were obtained when the pIC<sub>50</sub> was transformed to pK<sub>i</sub> according to the Cheng & Prusoff (1973) equation. An exception is the case of 5-methylurapidil in rat aorta, since this calculated pK<sub>i</sub> value from the CICb Ca (+) protocol was approximately one order of magnitude higher than the pK<sub>B</sub> (Table 3).

CICa to the antagonists were obtained and shown in Figure 2. The estimate of the antagonist affinity expressed as pIC<sub>50</sub> value in this case, was not significantly different from the pK<sub>B</sub> obtained previously (Tables 2 and 3).

In general, the results obtained evidence that the CICa and CICb obtained in Ca<sup>2+</sup>-free medium were similar but the CICb performed in Ca<sup>2+</sup>-containing solution was significantly shifted to the right (Figure 2).

### *Effects of $\alpha_1$ -adrenoceptor antagonists on noradrenaline-induced [<sup>3</sup>H]-inositol phosphate accumulation*

As described in Methods, different concentrations of each antagonist were added for 15 min before (CICb) or after (CICa) an incubation period (30 min) with the agonist as we performed in contraction studies.

A control response to the agonist demonstrates that there were no differences in the noradrenaline-induced [<sup>3</sup>H]-IP accumulation in both experimental conditions (tail artery: CICa = 859.7 ± 105.4% over basal, *n* = 12; CICb = 860.4 ± 75.3% over basal, *n* = 15. Aorta: CICa = 136.0 ± 16.5% over basal, *n* = 9; CICb = 126.1 ± 8.5% over basal, *n* = 16). When LiCl was not present during the

**Table 1** pEC<sub>50</sub> and E<sub>max</sub> values for noradrenaline-induced contraction and [<sup>3</sup>H]-inositol phosphate accumulation in rat aorta and tail artery

	<i>E</i> <sub>max</sub> (mg)	<i>Contraction</i>			<i>[<sup>3</sup>H]-inositol phosphate accumulation</i>			<i>n</i>
		<i>pEC</i> <sub>50</sub>	<i>Slope</i>	<i>n</i>	<i>E</i> <sub>max</sub> (%)	<i>pEC</i> <sub>50</sub>	<i>Slope</i>	
Tail	619.1 ± 55.6	5.71 ± 0.07***	1.42 ± 0.10	6	1060 ± 147***	5.51 ± 0.10**	1.54 ± 0.09	5
Aorta	698.2 ± 40.8	7.32 ± 0.04	1.11 ± 0.06	6	108.1 ± 16.9	6.03 ± 0.08***	0.97 ± 0.27	5

Values are expressed as mean ± s.e.mean, *n* = number of experiments, taking into account that for [<sup>3</sup>H]-IP accumulation each experiment was performed in triplicate. The E<sub>max</sub> for noradrenaline-induced [<sup>3</sup>H]-IP accumulation is expressed as a percentage over unstimulated [<sup>3</sup>H]-IP activity (basal). \*\**P* < 0.01, \*\*\**P* < 0.001 vs aorta. \*\*\**P* < 0.001 vs contraction.

**Table 2** Functional potencies (pIC<sub>50</sub> or pK<sub>B</sub>) of antagonists against noradrenaline-induced contraction in rat tail artery determined in different experimental conditions

	<i>pK</i> <sub>B</sub>	<i>CICa</i>	<i>CICb Ca (+)</i>	<i>CICb Ca (-)</i>
		<i>pIC</i> <sub>50</sub>	<i>pIC</i> <sub>50</sub> / <i>pK</i> <sub>i</sub>	<i>pIC</i> <sub>50</sub>
Prazosin	8.36 ± 0.02	8.46 ± 0.07	7.30 (7.17–7.41)/ <b>8.08</b>	8.84 (8.71–8.97)
BMY 7378	5.82 ± 0.08	6.14 ± 0.08	5.38 (5.31–5.44)/ <b>6.16</b>	6.20 (6.06–6.34)
5-methylurapidil	7.99 ± 0.06	8.13 ± 0.09	7.10 (6.95–7.27)/ <b>7.88</b>	8.22 (8.04–8.40)

CICa or CICb = concentration-inhibition curves for the antagonists added 'after' or 'before' noradrenaline-induced maximal response, respectively. Ca (+) or Ca (-) = Ca<sup>2+</sup> containing or Ca<sup>2+</sup>-free solution. Values are expressed as mean ± s.e.mean. of *n* = 4–9 experiments, except the values of pIC<sub>50</sub> on CICb experimental procedures, which are presented as mean with 95% confidence intervals. pK<sub>i</sub> was calculated from IC<sub>50</sub> values according to the Cheng & Prusoff (1973) equation.

**Table 3** Functional potencies (pIC<sub>50</sub> or pK<sub>B</sub>) of antagonists against noradrenaline-induced contraction in rat aorta determined in different experimental conditions

	<i>pK</i> <sub>B</sub>	<i>CICa</i>	<i>CICb Ca (+)</i>	<i>CICb Ca (-)</i>
		<i>pIC</i> <sub>50</sub>	<i>pIC</i> <sub>50</sub> / <i>pK</i> <sub>i</sub>	<i>pIC</i> <sub>50</sub>
Prazosin	9.37 ± 0.01	9.68 ± 0.15	7.96 (7.80–8.11)/ <b>9.28</b>	9.45 (9.24–9.66)
BMY 7378	8.28 ± 0.07	8.09 ± 0.06	6.59 (6.47–6.70)/ <b>7.90</b>	7.87 (7.70–8.03)
5-methylurapidil	6.82 ± 0.10	6.78 ± 0.02	6.22 (6.12–6.32)/ <b>7.54</b>	6.61 (6.44–6.78)

CICa or CICb = concentration-inhibition curves for the antagonists added 'after' or 'before' noradrenaline-induced maximal response, respectively. Ca (+) or Ca (-) = Ca<sup>2+</sup> containing or Ca<sup>2+</sup>-free solution. Values are expressed as mean ± s.e.mean of *n* = 4–9 experiments, except the values of pIC<sub>50</sub> on CICb experimental procedures, which are presented as mean with 95% confidence intervals. pK<sub>i</sub> was calculated from IC<sub>50</sub> values according to the Cheng & Prusoff (1973) equation.

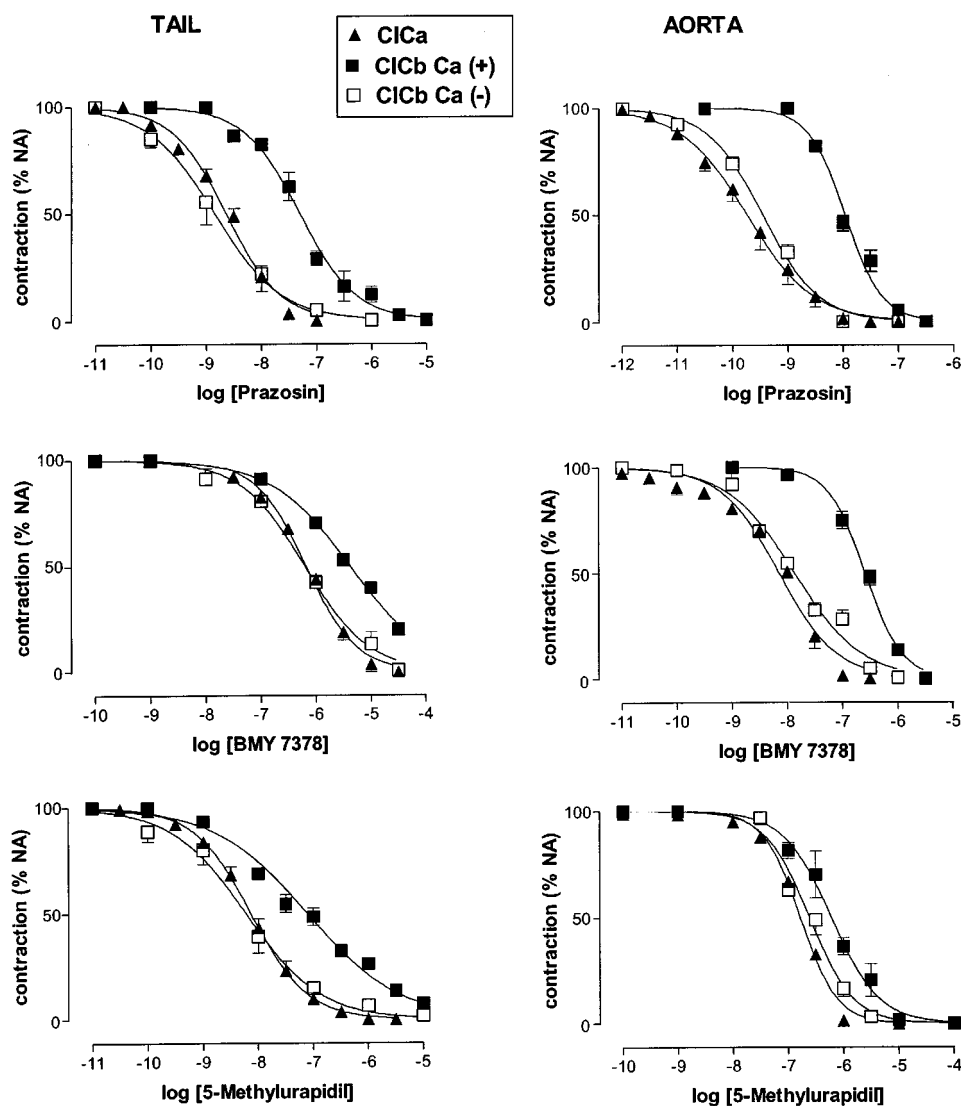
incubation time in presence of noradrenaline, the accumulation of [<sup>3</sup>H]-IP due to this agonist was not detectable (*n* = 9–12). The appropriate management of LiCl allowed us to reproduce in [<sup>3</sup>H]-IP accumulation studies the experimental conditions performed in contractile studies.

Figure 3 and Table 4 show the inhibitory effect of the tested compounds on noradrenaline-induced [<sup>3</sup>H]-IP formation and, as has been found in contractile studies, there was also a shift to the right in the CICb with respect to the CICa and subsequently, the pIC<sub>50</sub> values obtained for the different antagonists were higher in the CICa protocol than in the CICb protocol, except for 5-methylurapidil in the aorta. As happens in contractile studies, application of the Cheng & Prusoff (1973) equation using the pIC<sub>50</sub> values determined from the CICb gives pK<sub>i</sub> values that were similar to the pIC<sub>50</sub> determined from CICa (except for 5-methylurapidil in the aorta).

In rat aorta, the estimated affinities for the three antagonists obtained in the different experimental procedures are lower when IPs accumulation was measured but as previously pointed out, the inhibition curves of the antagonists were performed against a concentration of noradrenaline that elicits the maximal response and this concentration is 10 times higher in IPs determinations (10 μM) than in contractile studies (1 μM).

## Discussion

The results obtained show that the contractile response induced by noradrenaline was similar in tail artery and aorta, whereas there were marked differences in the ability of the tissues to generate intracellular second messengers, the  $\alpha_1$ -ARs present in tail artery being more efficient in increasing IP accumulation. This difference could be explained by the different subtypes implicated in the functional response of each vessel,  $\alpha_{1A}$ -AR in tail artery and  $\alpha_{1D}$ -AR in aorta (Kenny *et al.*, 1995; Piascik *et al.*, 1995; Buckner *et al.*, 1996; Saussy *et al.*, 1996; Lachnit *et al.*, 1997; Hussain & Marshall, 1997; Hrometz *et al.*, 1999; Gisbert *et al.*, 2000; Ziani *et al.*, 2002). Previous reports with cloned  $\alpha_{1A}$ -,  $\alpha_{1B}$  and  $\alpha_{1D}$ -ARs showed that all of them couple to phospholipase C but there were marked differences in the ability of each subtype to generate intracellular second messengers: the  $\alpha_{1A}$  was the most efficiently coupled to calcium release and IP production whereas the  $\alpha_{1D}$  was poorly coupled to intracellular signalling cascades (Schwinn *et al.*, 1995; Theroux *et al.*, 1996; Taguchi *et al.*, 1998). Moreover, the experimental evidence that the  $\alpha_{1D}$ -ARs present in aorta induced a weak response in second messenger generation (IP accumulation and increase in intracellular Ca<sup>2+</sup>) compared to that produced by the  $\alpha_{1A}$ -ARs present in tail artery (Gisbert *et al.*, 2000; submitted),



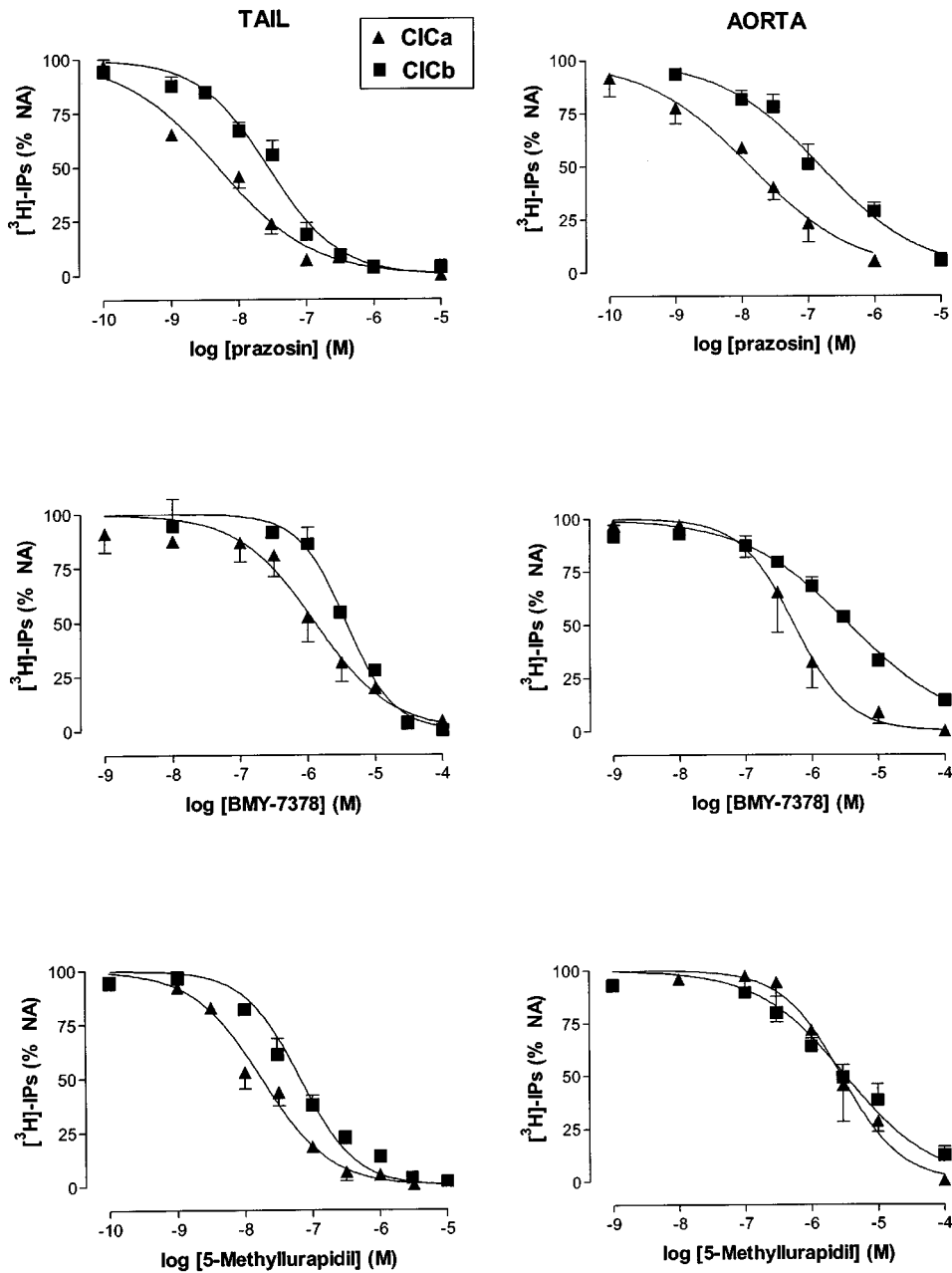
**Figure 2** Concentration inhibition curves for prazosin, BMY 7378 and 5-methylurapidil, added after (CICa) or before (CICb) the maximal noradrenaline-induced contraction in tail artery and aorta, in  $\text{Ca}^{2+}$ -containing (CICb Ca (+)) or  $\text{Ca}^{2+}$ -free solution (CICb Ca (-)). Data are mean  $\pm$  s.e. mean of 5–9 experiments. The  $\text{pIC}_{50}$  values are shown in Tables 2 and 3.

but the contractile response was similar in the two tissues, suggests the existence of a sensitization process associated with  $\alpha_{1D}$ -ARs.

The results also showed that noradrenaline possesses a greater potency in producing contractile response and IP accumulation in aorta although as has been described the maximal response is similar (contraction) or even smaller (IP accumulation) than that obtained in tail artery. Another time this difference could be explained considering the subtype mainly involved in the response, since previous reports showed a higher affinity of noradrenaline for cloned  $\alpha_{1D}$ -ARs (see Table 5; Schwinn *et al.*, 1995; Buckner *et al.*, 1996). The constitutive activity of  $\alpha_{1D}$ -ARs (Noguera *et al.*, 1996; García-Sáinz & Torres-Padilla, 1999; Gisbert *et al.*, 2000; 2002; McCune *et al.*, 2000; Ziani *et al.*, 2002) would also explain the higher affinity of agonists for this subtype (Piasick & Perez, 2001).

However, it is also interesting to point out that in aorta, the potency of noradrenaline in stimulating IP formation was

lower than in producing a contractile response. If we consider the theoretical possibility that, together with the  $\alpha_{1D}$ -AR, another  $\alpha_1$ -AR subtype would intervene in a small proportion of the response of aorta to noradrenaline, for example, the  $\alpha_{1A}$  subtype, we can suppose that, according to the above, the different efficiency of coupling between the  $\alpha_{1A}$ - and  $\alpha_{1D}$ -ARs to IP cascade can 'apparently increase' the participation of the  $\alpha_{1A}$ -ARs with respect to the  $\alpha_{1D}$ -AR subtype in the IP signal but not in the contractile response, then, non parallelism must be expected between the concentration-response curves of contraction and IP accumulation with a lower potency of noradrenaline on the latter compared with the former. Following the same reasoning, in tail artery, where the  $\alpha_{1A}$ -AR is the subtype mainly responsible for the functional responses, and is also the subtype most efficiently coupled to the IP formation, a little participation of another subtype poorly coupled to the intracellular signal cascades can be uncertain, then the noradrenaline-induced concentration-response curves of contraction or IP accumulation must



**Figure 3** Concentration inhibition curves for prazosin, BMY 7378 and 5-methylurapidil added after (CICa) or before (CICb) the maximal noradrenaline-induced [ $^3$ H]-inositol phosphate accumulation in tail artery and aorta. Data are mean  $\pm$  s.e. mean of 3–8 separate experiments performed in triplicate. The  $pIC_{50}$  values are shown in Table 4.

**Table 4** Functional potencies ( $pIC_{50}$ ) of antagonists against noradrenaline-induced [ $^3$ H]-inositol phosphate accumulation in rat tail artery and aorta determined in different experimental conditions

	Tail		Aorta	
	CICa $pIC_{50}$	CICb $pIC_{50}/pK_i$	CICa $pIC_{50}$	CICb $pIC_{50}/pK_i$
Prazosin	$8.27 \pm 0.04$	$7.76 \pm 0.15/8.35$	$7.86 \pm 0.18$	$6.93 \pm 0.09/7.97$
BMY 7378	$5.76 \pm 0.18$	$5.38 \pm 0.05/5.97$	$6.35 \pm 0.13$	$5.46 \pm 0.05/6.51$
5-methylurapidil	$7.87 \pm 0.14$	$7.25 \pm 0.10/7.84$	$5.50 \pm 0.16$	$5.41 \pm 0.16/6.45$

CICa or CICb = concentration-inhibition curves for the antagonists added 'after' or 'before' noradrenaline-induced [ $^3$ H]-inositol phosphate formation, respectively. Values are expressed as mean  $\pm$  s.e. mean. of  $n=3-8$  experiments.  $pK_i$  was calculated from  $IC_{50}$  values according to the Cheng & Prusoff (1973) equation.

**Table 5** Comparison of published agonist pD<sub>2</sub> or antagonist pA<sub>2</sub> or pK<sub>B</sub>\* values on rat blood vessels (tail or aorta) with their published pK<sub>i</sub> values calculated by use of measurements with cloned receptor subtypes ( $\alpha_{1A}$ -,  $\alpha_{1B}$ - and  $\alpha_{1D}$ -adrenoceptors)

	Noradrenaline	Prazosin	BMY 7378	5-Methylurapidil
Tail	5.56 <sup>(10)</sup>	8.9* <sup>(10)</sup> ; 9.1* <sup>a(10)</sup> ; 9.0 <sup>(15)</sup>	6.3* <sup>a(10)</sup> ; 6.5 <sup>(15)</sup>	8.9* <sup>a(10)</sup>
Aorta	7.88 <sup>(1)</sup> ; 7.74 <sup>(6)</sup> ; 8.5 <sup>(14)</sup> ; 7.92 <sup>(8)</sup>	9.8 <sup>(1)</sup> ; 9.5 <sup>(5)</sup> ; 9.46 <sup>(6)</sup> ; 9.9 <sup>(11)</sup> ; 9.66* <sup>(14)</sup> ; 9.7 <sup>(15)</sup>	8.3 <sup>(1)</sup> ; 8.88* <sup>(5)</sup> ; 9.0 <sup>(11)</sup> ; 9.08 <sup>(12)</sup> ; 8.35* <sup>(14)</sup> ; 8.5 <sup>(15)</sup>	7.8 <sup>(1)</sup> ; 7.8 <sup>(5)</sup> ; 7.27 <sup>(6)</sup> ; 8.1 <sup>(11)</sup> ; 7.95 <sup>(12)</sup> ; 7.87* <sup>(14)</sup>
$\alpha_{1A}$	5.0/5.1 <sup>(4)</sup> ; 6.38 <sup>(8)</sup>	9.7 <sup>(1)</sup> ; 9.5 <sup>(2)</sup> ; 9.9/9.3 <sup>(4)</sup> ; 9.72/9.88 <sup>(5)</sup> ; 9.14 <sup>(7)</sup> ; 9.9 <sup>(13)</sup> ; 9.37* <sup>(14)</sup> ; 9.82 <sup>(15)</sup>	6.2 <sup>(1)</sup> ; 6.1 <sup>(3)</sup> ; 6.54/6.8 <sup>(5)</sup> ; 6.57 <sup>(9)</sup> ; 6.92* <sup>(14)</sup> ; 6.96 <sup>(15)</sup>	8.5 <sup>(1)</sup> ; 8.6 <sup>(2)</sup> ; 8.5/8.7 <sup>(4)</sup> ; 9.12/9 <sup>(5)</sup> ; 8.69* <sup>(7)</sup> ; 9.2 <sup>(13)</sup> ; 8.65* <sup>(14)</sup>
$\alpha_{1B}$	5.5/5.8 <sup>(4)</sup> ; 6.54 <sup>(8)</sup>	9.6 <sup>(1)</sup> ; 9.8 <sup>(2)</sup> ; 9.5/9.9 <sup>(4)</sup> ; 9.42/10.08 <sup>(5)</sup> ; 9.34 <sup>(7)</sup> ; 9.9 <sup>(13)</sup> ; 10.44* <sup>(14)</sup> ; 10.6 <sup>(15)</sup>	6.7 <sup>(1)</sup> ; 6.2 <sup>(3)</sup> ; 6.24/7.25 <sup>(5)</sup> ; 6.77 <sup>(9)</sup> ; 7.36* <sup>(14)</sup> ; 7.32 <sup>(15)</sup>	6.8 <sup>(1)</sup> ; 6.9 <sup>(2)</sup> ; 6.6/6.5 <sup>(4)</sup> ; 7/7.47 <sup>(5)</sup> ; 5.98 <sup>(7)</sup> ; 7.7 <sup>(13)</sup> ; 7.22* <sup>(14)</sup>
$\alpha_{1D}$	6.7/6.9 <sup>(4)</sup> ; 7.69 <sup>(8)</sup>	9.5 <sup>(1)</sup> ; 9.6 <sup>(2)</sup> ; 10.4/10.1 <sup>(4)</sup> ; 9.46/10.04 <sup>(5)</sup> ; 8.71 <sup>(7)</sup> ; 9.9 <sup>(13)</sup> ; 10.03* <sup>(14)</sup> ; 10.1 <sup>(15)</sup>	8.2 <sup>(1)</sup> ; 8.2 <sup>(3)</sup> ; 8.16/9.39 <sup>(5)</sup> ; 8.94 <sup>(9)</sup> ; 9.00* <sup>(14)</sup> ; 9.09 <sup>(15)</sup>	7.8 <sup>(1)</sup> ; 7.3 <sup>(2)</sup> ; 7.2/7.1 <sup>(4)</sup> ; 7.3/7.92 <sup>(5)</sup> ; 6.30* <sup>(7)</sup> ; 8.0 <sup>(13)</sup> ; 7.59* <sup>(14)</sup>

Data values are from <sup>(1)</sup>Kenny *et al.*, 1995; <sup>(2)</sup>Michel *et al.*, 1995; <sup>(3)</sup>Goetz *et al.*, 1995; <sup>(4)</sup>Schwinn *et al.*, 1995; <sup>(5)</sup>Saussy *et al.*, 1996; <sup>(6)</sup>Van der Graaf *et al.*, 1996; <sup>(7)</sup>Testa *et al.*, 1996; <sup>(8)</sup>Buckner *et al.*, 1996; <sup>(9)</sup>Yang *et al.*, 1997; <sup>(10)</sup>Lachnit *et al.*, 1997 ('with A-61603 as agonist'); <sup>(11)</sup>Hussain & Marshall, 1997; <sup>(12)</sup>Figura *et al.*, 1997; <sup>(13)</sup>Ford *et al.*, 1997; <sup>(14)</sup>Muramatsu *et al.*, 1998; <sup>(15)</sup>Murata *et al.*, 1999.

be identical. The results summarized in Figure 1 validate these theoretical considerations.

According to these results, if different subtypes with different efficiencies of coupling to signals coexist in a tissue, confusing results about participation of each subtype in a given response can be obtained and, in these circumstances, a complete analysis of different functional responses must be done in order to get decisive results.

The experiments performed using selective antagonists show that all of them were able to inhibit the contractile response and the IP production induced by noradrenaline in aorta and tail artery, but essential differences in the antagonist potencies were found depending on the procedure used and on the vessel studied. Another time, a more complex picture appears in aorta than in tail artery. In both tissues, the pK<sub>B</sub> values obtained (Tables 2 and 3) were similar to that described in the literature (see Table 5). If we compare these values with the pK<sub>i</sub> obtained in competition binding experiments on cloned  $\alpha_1$ -ARs (see Table 5), a main role of  $\alpha_{1A}$  or  $\alpha_{1D}$ -ARs in the contractile response of tail artery (lower pK<sub>B</sub> values for BMY 7378 and higher for 5-methylurapidil, see Table 2) or rat aorta (higher pK<sub>B</sub> values for BMY 7378 and lower for 5-methylurapidil, see Table 3) respectively, can be evidenced as has previously been published (Kenny *et al.*, 1995; Lachnit *et al.*, 1997; Saussy *et al.*, 1996; Hussain & Marshall, 1997; Gisbert *et al.*, 2000).

We also performed two other experimental approaches: concentration-inhibition curves obtained using different concentrations of antagonists added before (CICb protocol, see Methods) or after (CICa protocol, see Methods) the maximal noradrenaline-induced contractile response or IP accumulation.

When the contractile response in tail artery and aorta was analysed, the pIC<sub>50</sub> values obtained for the different antagonist in the CICa protocol were similar to the pK<sub>B</sub> obtained in the same tissue. Moreover, the pIC<sub>50</sub> obtained from these CICa experiments were similar to the pA<sub>2</sub> obtained by other authors (see Table 5). These results are the experimental evidence of a good relationship between the antagonist potencies determined using the CICa procedure and the Schild plot analysis. Previous data obtained in our laboratory where we applied the CICa protocol to study the potencies of  $\alpha_1$ -AR antagonists (Noguera & D'Ocon, 1993;

Noguera *et al.*, 1996; Gisbert *et al.*, 2000; 2002; Ziani *et al.*, 2002) but also other compounds such as serotonergic antagonists (ketanserin; Catret *et al.*, 1998), Ca<sup>2+</sup> channel blockers (nifedipine, nimodipine, diltiazem, verapamil; Ivorra *et al.*, 1992; Noguera & D'Ocon, 1993; Noguera *et al.*, 1997), papaverine and other related benzylisoquinolines and aporphines (Ivorra *et al.*, 1992; 1998; Chuliá *et al.*, 1994; Valiente *et al.*, 1998), confirm this point.

When we performed the CRCb protocol lower pIC<sub>50</sub> values were obtained (contractile studies and IPs accumulation), but if we transform these pIC<sub>50</sub> to the pK<sub>i</sub> according to the Cheng & Prusoff (1973) equation the resulting data were similar to the pK<sub>B</sub> or to the pIC<sub>50</sub> values obtained using the CICa protocol. One exception to this was the behaviour of 5-methylurapidil in aorta, where a higher pK<sub>i</sub> value (approximately an order of magnitude) was obtained in the presence of calcium.

In order to know if the lower pIC<sub>50</sub> obtained in the CICb as opposed to CICa experiments was due to just the methodological approach (antagonist addition 'before' or 'after' agonist addition), or responded to a more complex scenario in the interaction agonist-antagonist-receptor, we reproduced the CICb procedure in different experimental conditions, excluding Ca<sup>2+</sup> from the physiological medium. In this case, the pIC<sub>50</sub> values were similar to the pK<sub>B</sub> or to the pIC<sub>50</sub> values obtained using the CICa protocol and a transformation of pIC<sub>50</sub> to pK<sub>i</sub> values is not required.

Three main observations can be made from these results: (1) Similar results can be obtained by different experimental procedures if the mathematical method chosen to calculate the antagonist potency is appropriate. (2) The application of the Cheng & Prusoff (1973) equation when an 'inhibition curve design' was performed is not a general rule because the pIC<sub>50</sub> obtained depends not only on the protocol but also on the experimental conditions used as CICb protocol in presence or absence of calcium show. Why did the Cheng & Prusoff transformation give only rational results when CICb procedure was performed in presence of Ca<sup>2+</sup> and is not necessary in the others? This is an open question that needs to be considered at least when drug-receptor interaction analysis of  $\alpha_1$ -ARs are performed. (3) The CICa experimental procedure offers the simplest alternative method to pharmacologically characterize the main receptor subtype/



s implicated in a functional response of a given tissue offering clear advantages with respect to the classical Schild plot analysis, specially when ethical reasons or difficulties in obtaining tissue samples must be considered.

Special attention needs to be given to the behaviour of 5-methylurapidil in rat aorta. This antagonist exhibits a greater potency in the CICb procedure performed in the presence of calcium than in the other experimental methods. This anomalous behaviour, observed in contractile studies as well as in IP accumulation analysis, may reflect changes in the adrenoceptor subtype responsible for the response to noradrenaline in aorta, depending on the experimental procedure used. Taking account of the different localization of  $\alpha_1$ -AR subtypes (the  $\alpha_{1A}$  in the cell membrane and the constitutive  $\alpha_{1D}$  in a perinuclear orientation: McCune *et al.*, 2000; Piascik & Perez, 2001; Chalothorn *et al.*, 2002), the greater potency showed by 5-methylurapidil indicates a higher participation of the  $\alpha_{1A}$  subtype in the initial response elicited by a maximal concentration of noradrenaline in  $\text{Ca}^{2+}$ -containing solution, participation that becomes less

evident when time permits reaching an equilibrium between subtypes.

In conclusion, a complex interaction between agonists, antagonists and the  $\alpha_1$ -AR subtypes is evidenced using different experimental procedures, but in order to obtain more rational and simple knowledge of the predominant subtype involved in the functional response of a tissue, the CICa procedure seems to be the best, especially when ethical reasons are invoked. The use of this procedure in contractile studies has previously been established by us and other authors but is new in IP accumulation studies. As present results show, the appropriate management of LiCl allows us to reproduce the experimental conditions performed in the contractile studies increasing the possibilities of this methodology.

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