0022-3565/00/2952-0810$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
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JPET 295:810–817, 2000

Vol. 295, No. 2
2784/858773
Printed in U.S.A.

Functional Evidence of a Constitutively Active Population of α_{1D}-Adrenoceptors in Rat Aorta[1]

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Accepted for publication August 1, 2000 This paper is available online at http://www.jpet.org

ABSTRACT

After depletion of intracellular calcium stores sensitive to noradrenaline, a spontaneous increase in the resting tone (IRT) when incubated in Ca^{2+}-containing solution was observed in isolated rat aorta, but not in tail artery. This IRT does not depend on agonist activation of α_1-adrenoceptors but it is inhibited by prazosin. A close relationship was found between the inhibitory potencies of prazosin (pIC_{50} = 9.833), BMY 7378 (pIC_{50} = 8.924), and 5-methylurapidil (pIC_{50} = 7.883) against IRT and their affinities for cloned α_{1D}-adrenoceptors. Chloroethylclonidine (100 $\mu mol \cdot l^{-1}$) did not inhibit the IRT. After depletion of internal calcium stores by noradrenaline in absence of the agonist, loading in Ca^{2+}-containing solution also brings about an increase in the inositol phosphate (IP) levels in rat aorta (not seen in tail artery) that is inhibited by prazosin (1 $\mu mol \cdot l^{-1}$), BMY 7378 (10 $\mu mol \cdot l^{-1}$), and 5-methylurapidil (10 $\mu mol \cdot l^{-1}$), thus confirming the results obtained in contractile studies. Chloroethylclonidine (100 $\mu mol \cdot l^{-1}$) did not inhibit this IP accumulation. The fact that the IRT and the IP accumulation related to it can be selectively inhibited by different α_1-adrenoceptor antagonists suggests the existence of a population of α_{1D}-adrenoceptors that show constitutive activity in rat aorta, not in tail artery.

Recent experimental evidence suggests a two-state receptor activation model in which G protein-coupled receptors are in equilibrium between an inactive and a spontaneously active conformation that couples to the G protein in absence of a ligand (Lefkovitz et al., 1993; Leff et al., 1997; Colquhoun, 1998). The existence of this active conformation has been revealed in artificial models as receptor mutants, systems that show an overexpression of a certain type of receptor or cloned receptors (Bond et al., 1995; Burstein et al., 1997; Gether et al., 1997; Hwa et al., 1997; Scheer et al., 1997; García Sainz and Torres-Padilla, 1999; McCune et al., 2000), but at present little is known about whether constitutively active native receptors have any physiological or pathological significance.

In previous articles (Noguera and D'Ocon, 1993; Noguera et al., 1996) we have suggested the existence of a population of constitutively active α_1-adrenoceptors in rat aorta and that some compounds traditionally used as antagonists, such as prazosin, WB 4101, and benoxathian really act as inverse agonists in this preparation. The experimental procedure that allows us to suggest the existence of this constitutive activity is a simple model in which, after depletion of intracellular calcium stores sensitive to noradrenaline, a spontaneous increase in the resting tone (IRT) of the aorta was obtained by incubation in a Ca^{2+}-containing solution. This IRT does not depend on noradrenaline activation because the presence of the agonist is excluded but is selectively inhibited by the α_1-adrenoceptor antagonists cited above.

The present report deals with the analysis of this α_1-adrenoceptor constitutive activity in rat aorta, examining not only the contractile activity of this vessel but also the phosphoinositide hydrolysis as the intracellular signal linked to α_1-adrenoceptor stimulation. We also extend the study to another vessel, tail artery, to determine more about the physiological implications of the constitutive activity of α_1-adrenoceptors in the functionality of the cardiovascular system.

Materials and Methods

Contractile Studies. Rings of the thoracic aorta or tail artery (approximately 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_2 and 5% CO_2. An initial load of 1 g was applied to each preparation and maintained throughout a 75- to 90-min equilibration period. After this time, contractile responses to noradrenaline in Ca^{2+}-containing or Ca^{2+}-free solution were elicited according to the experimental procedure described in Fig. 1 (under *Results*). The pretension of 1 g was kept constant, but there was a loss of tension (<10–15%) when the preparations were placed in Ca^{2+}-free medium. Tension was recorded isometrically by Grass FTO3 force-displacement transducers, and data were recorded on disc (MacLab). The absence of relaxant response (10%) after acetyl-

Received for publication April 4, 2000.
[1] This work was supported by a research grant from the Spanish Comisión Interministerial de Ciencia y Tecnología (SAF98-0123).

ABBREVIATIONS: IRT, increase in the resting tone; IP, inositol phosphate; NA, noradrenaline; W, washing; A, antagonist.

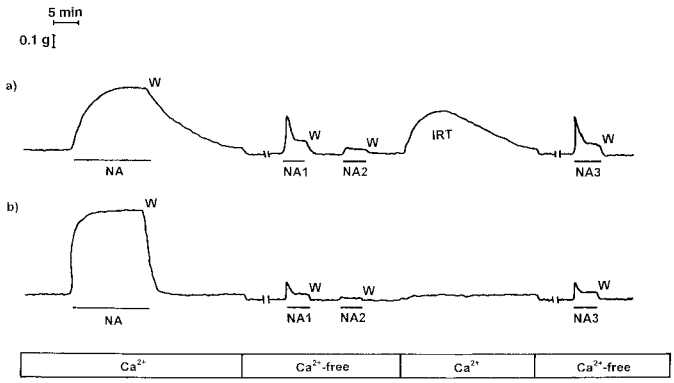

Fig. 1. Experimental procedure designed to study the depletion of intracellular Ca^{2+} stores sensitive to NA in rat aorta (a) and tail artery (b) in Ca^{2+}-free medium, and the IRT obtained in aorta by subsequent exposure to Ca^{2+}-containing solution during the refilling of the noradrenaline-sensitive Ca^{2+} stores. Agonist was added in Ca^{2+}-containing solution (Ca^{2+}) and after W and recovery of the basal tone, the tissue was incubated for 20 min in Ca^{2+}-free, EDTA-containing solution (Ca^{2+}-free). After this time the agonist was applied (NA1, NA2) and washed until no contraction was induced, indicating complete depletion of internal Ca^{2+} stores sensitive to noradrenaline. The tissue was then incubated for 20 min in Krebs' solution and a spontaneous increase in the resting tone of aorta (not tail artery) was observed. After washing and 20 min of loading in Ca^{2+}-free solution, a new addition of noradrenaline (NA3) was made. In the experiments designed to assess the effects of different agents on IRT, the aorta was pretreated with different concentrations of these agents 5 min before and during (20 min) the IRT was induced for second time.

choline (100 $\mu mol \cdot l^{-1}$) addition to preparations precontracted with noradrenaline (1 $\mu mol \cdot l^{-1}$) indicated the absence of a functional endothelium in all the rings.

The composition of the physiological Ca^{2+}-containing solution was as follows: 118 mM NaCl, 4.75 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose. Ca^{2+}-free solution had the same composition except that $CaCl_2$ was omitted and EDTA (0.1 mM) was added.

Contractions in Ca^{2+}-containing solution were expressed in milligrams of developed tension and, when elicited in Ca^{2+}-free medium, as a percentage of the noradrenaline-induced contractions obtained in Ca^{2+}-containing solution. Increases in resting tone were also expressed as a percentage of the noradrenaline-induced contraction in Ca^{2+}-containing solution. The concentration ($-\log$ [M]) needed to produce 50% relaxation or inhibition (pIC_{50}) was obtained from a nonlinear regression plot (GraphPad Software; San Diego, CA). It was impossible to calculate the S.E. of the mean of the pIC_{50} values for antagonists relative to the inhibition of the IRT. The other results are presented as the mean ± S.E. for n determinations obtained from different animals.

Inositol Phosphate (IP) Determination. The determination of total inositol phosphate accumulation was adapted from Berridge et al. (1982). Briefly, rat thoracic aortae or tail arteries (four or five animals were sacrificed) were exposed to Ca^{2+}-containing solution containing 1 $\mu mol \cdot l^{-1}$ of myo-[^{3}H]inositol (specific activity 70.0 $Ci \cdot mmol^{-1}$) for 2 h at 37°C and gassed with 95% O_2 plus a 5% CO_2 mixture. After this incubation, the tissue was washed twice with physiological solution. The vessels were cut into rings (1 mm for aorta, 2 mm for tail artery) and pooled. Two pieces of tail artery or four rings of aorta were placed in individual tubes that were incubated at 37°C. Different experimental conditions were applied in each determination (performed in triplicate), as are detailed under *Results*. In control experiments, tissues were incubated for 10 min with saline or prazosin (1 $\mu mol \cdot l^{-1}$) in Ca^{2+}-containing solution and stimulated with noradrenaline (1 $\mu mol \cdot l^{-1}$ or 10 $\mu mol \cdot l^{-1}$ in aorta or tail samples, respectively) for 30 min. LiCl (10 $mmol \cdot l^{-1}$) was added 30 s before treatment to inhibit the metabolism of inositol monophosphates. Incubation was stopped by placing the samples in a cold water bath (4°C) and adding 2 ml of a cold mixture of methanol/chloroform/HCl (40:20:1, v/v/v). The samples were sonicated for 35 min at 2–3°C and, after addition of 0.63 ml of chloroform and 1.26 ml of distilled water, were centrifuged at 2500g for 10 min to facilitate phase separation. The aqueous layer was removed from the tubes to assay the IP formation. Each sample was neutralized and run through an AG1-X8 column, formate form, 100 to 200 mesh (Bio-Rad, Hercules, CA). The resin was washed successively with 6 ml of water and 6 ml of 60 $mmol \cdot l^{-1}$ ammonium formate-5 $mmol \cdot l^{-1}$ sodium tetraborate to eliminate free myo-[^{3}H]inositol and glycerophosphoinositol, respectively. Total IPs were eluted with 3 ml of 1 $mol \cdot l^{-1}$ ammonium formate-0.1 $mol \cdot l^{-1}$ formic acid. The eluent fractions were collected and counted in a scintillation counter. We normalized IP radioactivities in terms of free myo-[^{3}H]inositol in void volume fractions in each experiment to correct for differences in the amount of tissue and myo-[^{3}H]inositol labeling in different rings. The IP accumulation was expressed as percentage of basal release in each case and where ANOVA showed significant differences ($P < .05$), the results were further analyzed using the Student-Newman-Keuls test.

Chemicals. The following drugs were obtained from Sigma (St. Louis, MO): acetylcholine, (−)-noradrenaline, prazosin, chloroethylclonidine, and lithium chloride, or Research Biochemicals International (Natick MA): BMY 7378 and 5-methylurapidil. myo-[^{3}H]Inositol was from Amersham (Buckinghamshire, England). Other reagents were of analytical grade. All compounds were dissolved in distilled water.

Results

Contractile Studies in Rat Aorta or Tail Artery. Table 1 summarizes the results and Fig. 1 shows the experimental procedure designed to study the depletion of intracellular Ca^{2+} stores sensitive to noradrenaline and the IRT obtained by subsequent exposure to Ca^{2+}-containing physiological solution during the refilling of these stores. Noradrenaline at 1 or 10 $\mu mol \cdot l^{-1}$ evoked a sustained contraction in rat aorta or tail artery, respectively, that was used as a control of the maximal response obtained with this agonist in each preparation. After careful washing, the return to the baseline was slower in aorta than in tail artery. Aorta takes 1191 ± 65 s (n = 15) to recovery the basal tone, whereas tail artery only takes 283 ± 16 s (n = 15) (Fig. 2).

TABLE 1

Contractile responses in thoracic aorta or tail artery

Contractile response to maximal concentrations of noradrenaline in thoracic aorta (1 μM) and tail artery (10 μM) loaded in Ca^{2+}-containing (NA expressed in milligrams of contraction ± S.E.M.) or Ca^{2+}-free solution (NA1) and the IRT of rat aorta elicited after depletion of intracellular Ca^{2+} stores by noradrenaline and loading in Ca^{2+}-containing solution. Values ± S.E.M. of NA1 and IRT are expressed as a percentage of the contractile response to noradrenaline in Ca^{2+}-containing solution (NA). n = number of data. (See experimental procedure in Fig. 1.)

	n	Ca^{2+} (+) NA	Ca^{2+} (−) NA1	Ca^{2+} (+) IRT
		mg	%	
Aorta	10	959.9 ± 168.4	32.9 ± 3.65	53.3 ± 4.9
Tail artery	8	704.1 ± 45.7	23.2 ± 5.6	8.8 ± 2.1

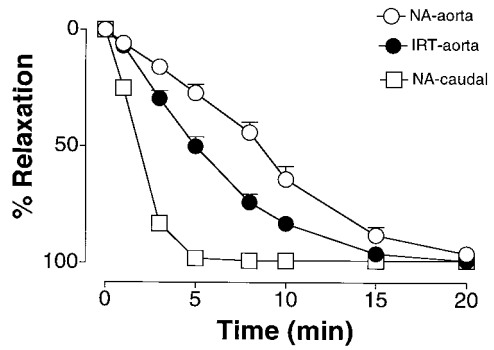

Fig. 2. Time course of the decay in the maximal contractile response to noradrenaline after removal of the agonist in thoracic aorta and tail artery. The resting tone was measured 1, 3, 5, 8, 10, 15, and 20 min after noradrenaline removal. The decay of the IRT observed in aorta after depletion of intracellular calcium stores sensitive to noradrenaline and posterior loading in Ca^{2+}-free medium was also measured at the times given above.

We then changed to a Ca^{2+}-free solution and after 20 min in this medium, the addition of noradrenaline also induced a contraction (NA1, Table 1; Fig. 1) that was used as an index for the content of agonist-sensitive intracellular stores. No contraction was evoked upon a second application of the agonist (NA2, Fig. 1) in the same solution, which indicates complete depletion of internal Ca^{2+} stores sensitive to noradrenaline. The tissue was then incubated for 20 min in Ca^{2+}-containing solution to refill the intracellular Ca^{2+} stores, and a spontaneous increase in the resting tone (IRT = 53.3 ± 4.9% of noradrenaline control) was observed in rat aorta but not in tail artery, whereas only a slight increase in the baseline was obtained (8.8 ± 2.1% of the control response to noradrenaline).

The IRT observed in aorta is not sustained, and takes 1057 ± 54 s to reach the baseline (n = 15). It decreases as slowly as the control response to noradrenaline in Ca^{2+}-containing solution disappears after washing (Fig. 2). Returning the tissues to a Ca^{2+}-free solution reduced the tension to baseline, and further application of noradrenaline (NA3) 20 min later reproduced the contractile response elicited first in Ca^{2+}-free solution, which indicates a complete refilling of internal stores.

Concentration-response curves of relaxation to prazosin (0.001 nmol · l^{-1}–1 μmol · l^{-1}), BMY 7378 (0.001 nmol · l^{-1}–1 μmol · l^{-1}), 5-methylurapidil (0.001 nmol · l^{-1}–10 μmol · l^{-1}), or chloroethylclonidine (0.001 μmol · l^{-1}–100 μmol · l^{-1}) were obtained by addition of cumulative concentrations of the compounds to tissues in which sustained contractions had been induced by maximal concentrations of noradrenaline (1 μmol · l^{-1} in rat aorta and 10 μmol · l^{-1} in tail artery). Relaxations were expressed as a percentage of the maximum increment of tension obtained by agonist addition and the pIC_{50} of relaxation obtained for each antagonist on aorta or tail artery are summarized in Table 2. Concentration-response curves of inhibition to the same compounds were obtained by addition of concentrations of antagonist 15 min before and during the loading period in Ca^{2+}-containing solution that permits the refilling of internal Ca^{2+} stores previously depleted by noradrenaline (Fig. 1). The magnitude of the IRT observed in rat aorta during this period in presence of each concentration of antagonist (Fig. 3) was expressed as a percentage of the reference IRT obtained in absence of any agent and the pIC_{50} calculated was also summarized in Table 2. In this case, chloretylclonidine at the higher concentration assayed (100 μmol · l^{-1}), which completely relaxed noradrenaline-induced contraction of rat aorta, had no effect on IRT. Moreover, when rings of rat aorta were exposed to chloroethylclonidine (100 μmol · l^{-1}) for 30 min and then washed for 20 min to remove the antagonist an IRT was observed of similar magnitude (n = 5) with respect to the reference IRT obtained in absence of any agent. When we compare the pIC_{50} obtained for each antagonist on IRT or noradrenaline-induced contractile response in aorta and tail artery with the pK_i obtained in competition experiments on cloned α_1-adrenoceptors (Kenny et al., 1995; Schwinn et al., 1995), we can observe a close relationship between the results obtained on IRT in aorta and cloned α_{1D}-adrenoceptors, or between tail artery and cloned α_{1A}-adrenoceptors (Table 2). The low affinity of BMY 7378 excludes participation of α_{1D}-adrenoceptors in the functional response of the tail artery to noradrenaline.

IP Determination. To find out whether the IRT observed in functional studies is really due to an activated state of α_{1D}-adrenoceptors we tested the second messenger production, or IP formation, linked to activation of these receptors (Graham et al., 1996). In aorta and tail artery, noradrenaline concentration-dependently increased IP accumulation and the maximal response in both tissues was obtained with 10 μmol · l^{-1} noradrenaline (291.8 ± 23.2% related to basal release, n = 11 in aorta and 1318.2 ± 6.3%, n = 3 in tail artery). Prazosin at 1 μmol · l^{-1} inhibited the maximal accumulation of IP induced by noradrenaline in both tissues. When similar experiments were performed in absence of $CaCl_2$ in the incubating medium, the results obtained were identical (Fig. 4). When LiCl was not present during the

TABLE 2

Comparison of pIC_{50} values of the agents tested with their published pK_i values for α_1-adrenoceptor subtypes

Values are expressed as mean ± S.E.M. pIC_{50} values of the agents tested on the IRT of rat aorta and on noradrenaline-induced contraction in aorta and tail artery. pK_i values for cloned and expressed α_1-adrenoceptor subtypes.

	Aorta		Tail Artery	Cloned α_1-Adrenoceptors[a]		
	Noradrenaline	IRT	Noradrenaline	α_{1A}	α_{1B}	α_{1D}
Prazosin	9.702 ± 0.093 n = 5	9.833 n = 5–7	9.532 ± 0.071 n = 6	9.9 ± 0.16	9.5 ± 0.15	10.400 ± 0.09
BMY 7378	8.114 ± 0.073 n = 5	8.924 n = 4	7.295 ± 0.152 n = 7	6.28 ± 0.1	6.51 ± 0.2	8.44 ± 0.2
5-Methyl urapidil	6.883 ± 0.019 n = 6	7.883 n = 5–6	8.104 ± 0.154 n = 7	8.66 ± 0.1	6.87 ± 0.1	7.46 ± 0.2
Chloroethylclonidine	4.472 ± 0.154 n = 6	NI n = 4	5.286 ± 0.153 n = 6			

NI, no inhibition was observed; n, number of experiments.
[a] Data from Schwinn et al. (1995) and Kenny et al. (1995).

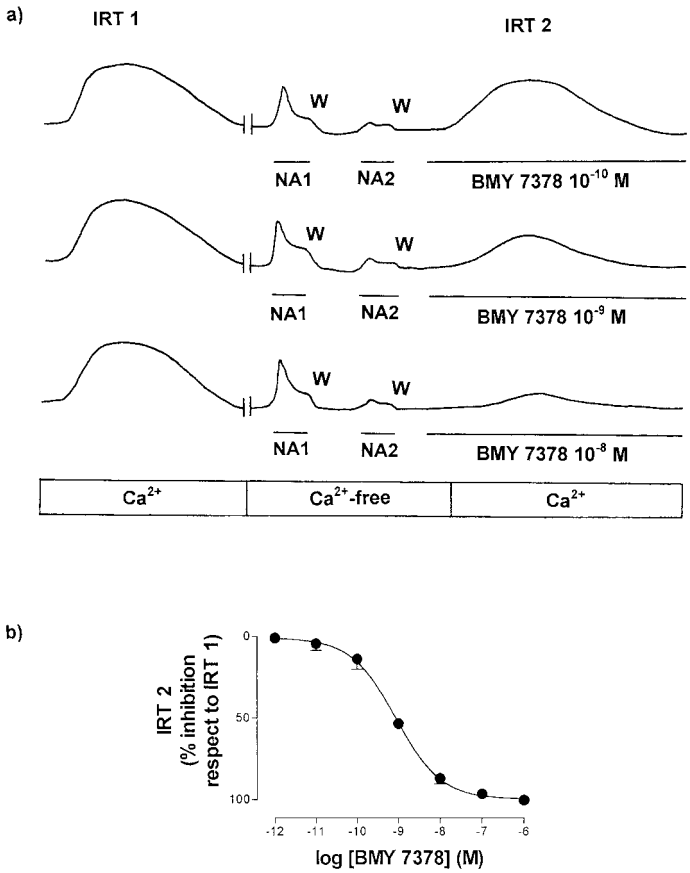

Fig. 3. Modification induced by different concentrations of BMY 7378 in the IRT obtained in aorta after the experimental procedure described in Fig. 1. a, representative tracings showing the IRT observed in absence (IRT 1) or presence (IRT 2) of different concentrations of BMY 7378. NA1 and NA2, 1 μmol $\cdot$ l^{-1}. b, concentration-response curve of inhibition by BMY 7378 of the IRT observed in rat aorta.

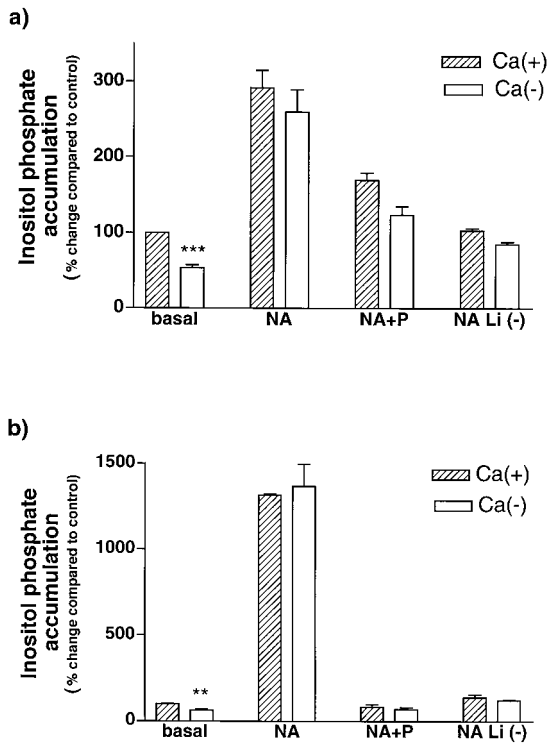

Fig. 4. IP accumulation in Ca^{2+}-containing (▨) and Ca^{2+}-free medium (□), obtained in thoracic aorta (a) and tail artery (b) after incubation with NA (10 μmol $\cdot$ l^{-1}), NA (10 μmol $\cdot$ l^{-1}) + prazosin (10 μmol $\cdot$ l^{-1}) (NA + P), or NA (10 μmol $\cdot$ l^{-1}) in absence of LiCl in the incubating medium [NA Li (−)]. Results were expressed as a percentage with regard to the basal level in Ca^{2+}-containing medium. Basal = 3573 ± 393 dpm $\cdot$ mg of protein^{-1} in aorta (n = 5) and 5200 ± 295 dpm $\cdot$ mg of protein^{-1} in tail artery (n = 6). ***$P < .001$, **$P < .01$ versus basal level in Ca^{2+}-containing medium, Student-Newman-Keuls test.

incubation time in presence of noradrenaline, the accumulation of IP due to this agonist was not detectable (Fig. 4).

A new experimental procedure designed by us attempts to reproduce the conditions developed in contractile studies. After incubation for 2 h in physiological solution containing myo-[^{3}H]inositol, tissues were placed in individual tubes with physiological solution free of $CaCl_2$ for 20 min. We prepared 10 different samples, each of which was subjected to the different experimental conditions that are summarized in Fig. 5.

The samples numbered 1 and 2 in Fig. 5 were used as controls to determine the influence on the IP accumulation of the incubation time in Ca^{2+}-free medium (90 min total, sample 1) or successive washings (W) during this incubation time (sample 2). The results obtained showed that the level of IP was slightly decreased by these parameters (Fig. 6).

The samples numbered 3 to 5 (Fig. 5) were used as controls of the IP formation induced by noradrenaline after two successive additions and washings of noradrenaline in Ca^{2+}-free medium. For this purpose, the samples were incubated for 5 min in presence of noradrenaline (NA1) and washed for 10 min before a new incubation period (5 min), also in presence of noradrenaline (NA2). This was followed by a second washing (10 min). Finally, LiCl was added to samples 3 and 4 but not to 5. An antagonist (A), prazosin at 1 μmol $\cdot$ l^{-1}, was also added to sample 4. Ten minutes later, noradrenaline was added again to the three samples for 30 min. The incubation was then stopped and the IP formation determined. The results obtained were similar to the control response to noradrenaline in Ca^{2+}-containing medium (Fig. 6). Prazosin also inhibits the IP formation elicited by noradrenaline in these conditions and, as has been previously shown in Ca^{2+}-containing medium, IP accumulation cannot be detected in the sample that does not include LiCl.

Samples 6 to 7 represent an attempt to reproduce the experimental procedure used in contraction studies in which IRT was observed. Sample 6 was incubated in presence of noradrenaline two times (5 min each with 10-min washing) before LiCl was added and incubation prolonged 10 min more. Finally, $CaCl_2$ was added during the last 30 min as in the contraction studies. The results obtained are summarized in Fig. 6 and show that after depletion of the intracellular Ca^{2+} stores by addition of noradrenaline in a Ca^{2+}-free medium, when $CaCl_2$ was included in the incubating solution, a significant increase in the IP accumulation was detected (sample 6 versus 2) in aorta, but not in tail artery, that reproduced the IRT observed in the contraction studies. This accumulation was inhibited by addition of 1 μmol $\cdot$ l^{-1} prazosin (sample 7 versus 6), as also occurs in the contraction studies.

To clarify the role of the depletion of intracellular Ca^{2+} stores and/or Ca^{2+} entry in this process, samples 8 and 9 included $CaCl_2$ in the last 30 min but without previous depletion of intracellular Ca^{2+} stores sensitive to noradrena-

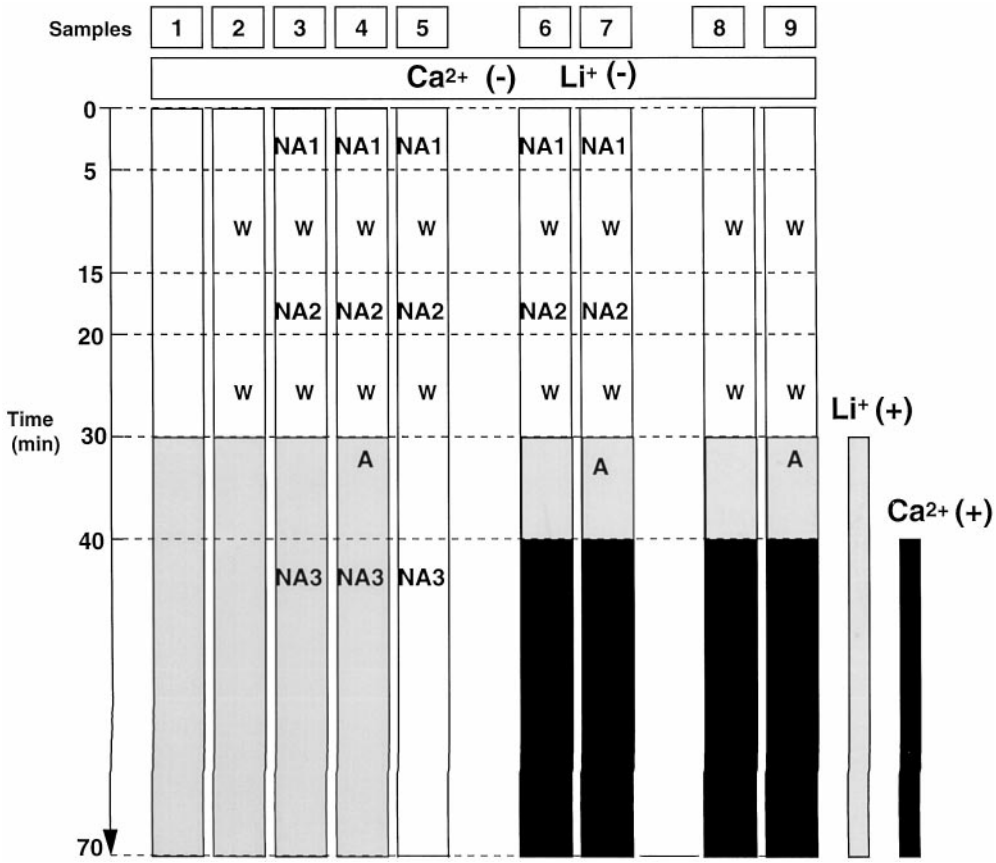

Fig. 5. Description of the experimental procedure designed to determine IP accumulation under different experimental conditions in Ca^{2+}-free medium: samples 1 to 9. NA1, first addition of noradrenaline (10 $\mu mol \cdot l^{-1}$) in Ca^{2+}- and Li^{+}-free solution (5 min). W (10 min). NA2, second addition of noradrenaline (10 $\mu mol \cdot l^{-1}$) in Ca^{2+}- and Li^{+}-free solution (5 min). A represents the addition of a concentration of antagonist in Ca^{2+}-free, Li^{+}-containing solution (10 min). NA3, addition of noradrenaline (10 $\mu mol \cdot l^{-1}$) in Ca^{2+}- and Li^{+}-containing solution (30 min). All samples were incubated in Ca^{2+}- and Li^{+}-free solution (□), and in samples 3 to 7 noradrenaline was added twice (NA1 and NA2) followed by Ws to promote depletion of calcium from internal stores. The exclusion of LiCl from the incubating medium permits IP formation due to noradrenaline activation but prevents it from accumulating. LiCl (10 $mmol \cdot l^{-1}$) was then added to all samples (light gray bar) except sample 5, and A was added to samples 4, 7, and 9 for 10 min. Finally, 1.8 mM $CaCl_2$ was added to samples 6 to 9 (■), and noradrenaline (NA3) was included in samples 3 to 5 for 30 min.

line. The results obtained indicate that on changing the tissues from an incubating medium free of Ca^{2+} to a Ca^{2+}-containing one, a slight increase in the basal formation of IP is observed, but this increase is not inhibited by prazosin (Fig. 6). The magnitude of the increase when Ca^{2+} was added correlates well with the slight decrease previously observed in IP formation when Ca^{2+} was eliminated (Fig. 6, samples 1 and 8 versus control 100%).

To analyze the activity of the selective α_1-adrenoceptor antagonists on this IP accumulation observed in absence of agonist, similar experiments were performed to test BMY 7378 (10 $\mu mol \cdot l^{-1}$), 5-methylurapidil (10 $\mu mol \cdot l^{-1}$), and chloroethylclonidine (100 $\mu mol \cdot l^{-1}$) on this accumulation as well as the activity of these compounds on noradrenaline-induced IP formation in Ca^{2+}-free medium. The results obtained indicate that BMY 7378 and 5-methylurapidil inhibit both noradrenaline-induced IP accumulation (Fig. 7a) and the increase in the IP levels observed in absence of the agonist (Fig. 7b). Chloroethylclonidine, which inhibits the noradrenaline-induced IP signal, did not modify the IP accumulation observed in absence of agonist (Fig. 7).

Discussion

The present results show that in rat aorta, noradrenaline, through activation of α_1-adrenoceptors, induces an IP accumulation that releases Ca^{2+} from internal stores (Berridge, 1992; Graham et al., 1996), and that these stores are depleted by successive additions of this agonist in a Ca^{2+}-free medium. When emptied, the stores can be rapidly replenished by Ca^{2+} influx during the incubation in Ca^{2+}-containing solution in the absence of the agonist (Putney, 1990; Noguera and D'Ocon, 1993; Noguera et al., 1996, 1997, 1998), and this process manifests itself not only by the recovery of the response to noradrenaline in Ca^{2+} free medium but also by the increase in the resting tone observed (IRT in Fig. 1).

That this IRT is closely related to α_1-adrenoceptors and not just to the emptying of intracellular Ca^{2+} pools is demonstrated by the fact that depletion of internal Ca^{2+} stores by methoxamine and phenylephrine also elicits an IRT, whereas clonidine, 5-hydroxytryptamine, caffeine, ryanodine, thapsigargine, and cyclopiazonic acid, which also depleted internal Ca^{2+} stores, did not elicit any IRT (Noguera and D'Ocon, 1993; Noguera et al., 1996, 1998).

If we assume that endogenous agonists are not present, the fact that this IRT was selectively inhibited by prazosin suggests the existence of a population of α_1-adrenoceptors in a constitutively active state, as we previously proposed (Noguera et al., 1996, 1998). The questions that arise from these results are as follows: Is this a general model that can be shown in different vascular smooth muscles? Which subtype of α_1-adrenoceptor is involved? Is the inositol phosphate accumulation implicated in this process? Can we say that we are dealing with constitutive α_1-adrenoceptor activity? and What is the role of this process in the functionality of a vessel?

To answer the first question, we have analyzed this model in another vascular tissue, rat tail artery. The experimental procedure was the same as the one used in aorta, but the results were not similar. After depletion of internal Ca^{2+} stores sensitive to noradrenaline, no increase in the resting tone was observed (Fig. 1), which means that the IRT found

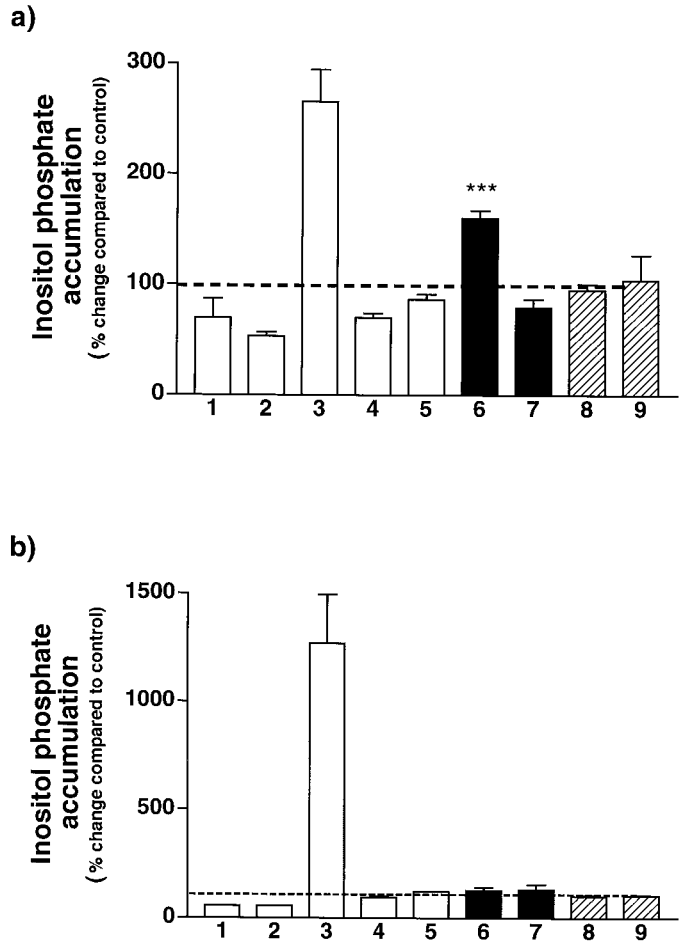

Fig. 6. Determination of the inositol phosphate accumulation obtained in thoracic aorta (a) and tail artery (b) incubated in Ca^{2+}-free medium. Numbers 1 to 9 indicate different samples that receive different treatments according to the experimental procedure described in Fig. 4. Sample 3 represents noradrenaline-induced accumulation of IP. Sample 6 represents IP accumulation observed in absence of the agonist after depletion of internal calcium stores sensitive to noradrenaline and posterior loading in Ca^{2+}-containing solution. This accumulation correlates with the IRT observed in contractile studies in thoracic aorta but not in tail artery (Fig. 1). In samples 4, 7, and 9 the antagonist tested was prazosin (10 μmol · l^{-1}). Values are expressed as percentages of the basal level obtained in Ca^{2+}-containing solution. The discontinuous line represents this value. ***$P < .001$ versus basal level in Ca^{2+}-containing medium, Student-Newman-Keuls test.

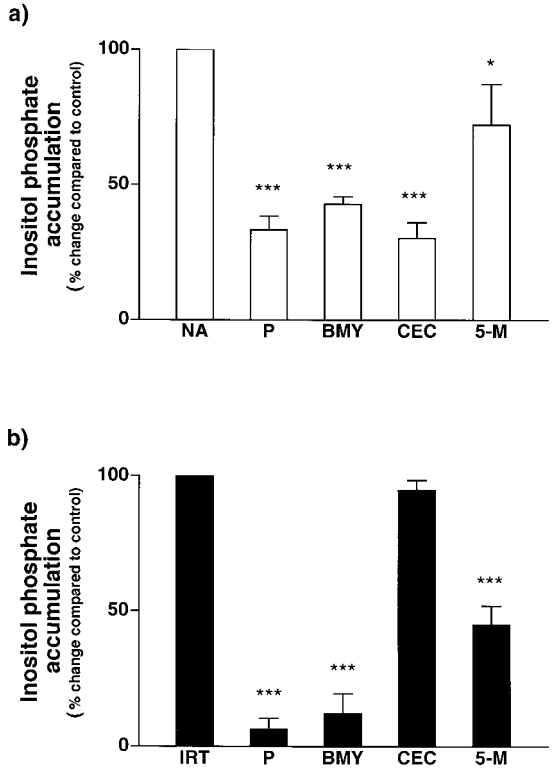

Fig. 7. Modification of the IP accumulation elicited by NA (10 μmol · l^{-1}) (a), and related to the IRT (b) observed in contraction studies after treatment for 40 min with prazosin (1 μmol · l^{-1}) (P), BMY 7378 (10 μmol · l^{-1}) (BMY), chloroethylclonidine (100 μmol · l^{-1}) (CEC), and 5-methylurapidil (10 μmol · l^{-1}) (5-M). Results are represented as a percentage of activation with regard to stimulation of NA or IRT. NA-induced accumulation of IP = 11619 ± 1272 dpm · mg of $protein^{-1}$. IRT-induced accumulation of IP = 7020 ± 312 dpm · mg of $protein^{-1}$. ***$P < .001$, *$P < .05$ versus noradrenaline-induced accumulation (a) or IRT (b), Student-Newman-Keuls test.

in aorta is specifically related to the α_1-adrenoceptors present in this tissue.

Rat tail artery contracts in response to noradrenaline via activation of at least two adrenoceptor subtypes, one of which displays the pharmacology of the α_{1A}-adrenoceptor (Villalobos-Molina and Ibarra, 1996; Lachnit et al., 1997; Mita and Walsh, 1997). Our results confirm participation of α_{1A}-adrenoceptors in the contractile response to noradrenaline in this vessel but also show that α_{1D}-adrenoceptor is not involved in the response. In any case, the α_{1A}-adrenoceptor or the undefined one has not shown constitutive activity in our experimental conditions.

The subtype(s) of α_1-adrenoceptors present in the rat thoracic aorta has been the subject of extensive research (Hieble et al., 1995). Finally, a selective α_{1D}-antagonist BMY 7378 was described, and it was demonstrated that in the rat aorta, the functional activity of this antagonist correlates well with binding affinities for cloned α_{1D}-adrenoceptors (Kenny et al., 1995; Saussy et al., 1996; Hussain and Marshall, 1997). This suggests that the α_{1D}-adrenoceptor plays a functional role in this tissue without excluding participation of the other subtypes. Therefore, the subtype of α_1-adrenoceptor that shows constitutive activity in our experimental model could be the α_{1D}-adrenoceptor.

To confirm this hypothesis, we assayed the activity of three antagonists acting selectively on α_1-adrenoceptor subtypes: BMY 7378, which, as has been mentioned before, acts on the α_{1D}-subtype; 5-methylurapydil, which acts on the α_{1A}-subtype; and chloroethylclonidine, an irreversible antagonist of the α_{1B}- and α_{1D}-subtypes (Hieble et al., 1995; Schwinn et al., 1995).

The results obtained with the three compounds assayed confirm that the population of α_1-adrenoceptors that intervenes in the functional response of rat aorta to noradrenaline belongs, at least in part, to the α_{1D}-subtype. Moreover, the present results show that the IRT that we attribute to the constitutive activity of α_1-adrenoceptors is selectively blocked by BMY 7378, and this compound's potency as an inhibitor of the IRT correlates well with its affinity estimated at the cloned α_{1D}-subtype. This confirms the hypothesis that this subtype of α_1-adrenoceptor can show constitutive activity in our model and makes it clear that BMY 7378 acts as an inverse agonist. 5-Methylurapydil also acts as an inverse

agonist, with an inhibitory potency on IRT that correlates well with its affinity estimated at the cloned α_{1D}-subtype and that is lower than the potency shown at the α_{1A}-adrenoceptor subtype. Chloroethylclonidine lacks activity on IRT but inhibits in a concentration-dependent manner the noradrenaline-induced contraction, thus suggesting that it does not act on α_{1D}-adrenoceptors as an inverse agonist but as a neutral antagonist.

In response to the second question, the present results demonstrate the existence of a mechanical response of rat aorta after depletion of internal Ca^{2+} stores sensitive to α_1-adrenoceptor activation that can be interpreted as the first functional evidence of the constitutive activity of native α_{1D}-adrenoceptors in vascular tissues. Recently, in rat-1 fibroblasts stably expressing α_{1D}-adrenoceptors spontaneous ligand-independent activity has been shown (García-Sainz and Torres-Padilla, 1999; McCune et al., 2000), confirming our previous and present results in native receptors (Noguera et al., 1996, 1998).

The next question to analyze is the intervention of IP accumulation in this process as an intracellular signal of receptor activation. Experiments were performed to mimic the procedure used in the organ bath experiments, and the results obtained give clear evidence of the existence of IP accumulation after depletion of intracellular calcium stores sensitive to noradrenaline, in absence of the agonist and when calcium was added again to the incubation medium (Fig. 5, sample 6). This IP accumulation can be inhibited by prazosin, BMY 7378, and 5-methylurapidil, but chloroethylclonidine does not inhibit it. This is consistent with the observations in organ bath experiments and shows the dependence of the signal on α_1-adrenoceptor activation. Moreover, if internal calcium stores sensitive to noradrenaline were not previously depleted, addition of calcium to the incubation medium only slightly increased the level of IP, and this slight increase, which corresponds in magnitude to the decrease observed when calcium was removed from the medium, is not inhibited by prazosin. The fact that BMY 7378 and 5-methylurapidil, which inhibit IRT in contractile studies, also inhibit this IP response, and that chloroethylclonidine does not inhibit IRT or IP formation indicates a close relationship between the two signals.

In conclusion, the IRT observed in contractile experiments and the IP accumulation related to it can be inhibited by prazosin, BMY 7378, and 5-methylurapidil in conditions in which the presence of exogenous noradrenaline can be ruled out. This observation strongly suggests the existence of a population of α_{1D}-adrenoceptors with constitutive activity. Prazosin, BMY 7378, and 5-methylurapidil behave as "inverse agonists", as has been previously proposed for prazosin (Noguera et al., 1996) and 5-methylurapidil (Lee et al., 1997). Chloroethylclonidine, which inhibits the noradrenaline-induced functional response in both systems (contraction and IP formation), did not affect the IRT or the correlated IP accumulation. This compound did not show inverse agonist activity but these results then demonstrate that endogenous noradrenaline is also not involved in this process. Excluding the participation of an agonist, the model could be considered representative of the functional behavior of a population of constitutively active α_{1D}-adrenoceptors.

Moreover, the data provided in the present study suggest a possible answer to our final question about the physiological role of this constitutive activity of α_{1D}-adrenoceptors. Figure 1 and the results summarized in Fig. 2 show that if we compare the noradrenaline-induced contractile responses in aorta and tail artery in Ca^{2+}-containing solution, we can observe that after removing the agonist, contraction disappears in aorta as slowly as IRT decreases, but that in tail artery the decay of the response to noradrenaline is faster. These results can be interpreted as be due to differences in histology or lipid content between the two vessels but, from our observation about IRT we could also extrapolate that in physiological conditions, after noradrenaline activity and removal, a population of α_{1D}-adrenoceptors could remain temporally in a constitutively active state and could be responsible for the slow disappearance of the contractile response to the agonist. This mechanism is not observed in tail artery, where α_{1D}-adrenoceptors do not seem to play a functional role. Therefore, the presence of a population of α_{1D}-adrenoceptors in a vessel can signify that the contractile responses of this tissue can be sustained even when the agonist is removed, and this would in turn modulate the contractile activity in this vessel, thus preventing abrupt changes in caliber when the agonist disappears. In contrast, an imbalance in this modulating mechanism could give rise to pathologies as hypertension or age-related vascular diseases, in which a possible role of α_{1D}-adrenoceptors in their pathogenesis and/or maintenance has been postulated (Villalobos-Molina and Ibarra, 1996; Villalobos-Molina et al., 1999; Ibarra et al., 1997, 1998; Xu et al., 1998). We are currently investigating this exciting hypothesis.

Further studies are needed to determine the importance of this phenomenon in the contractile response to agonists in physiological or pathological situations and in different vascular beds, but our observations could explain why different α_1-adrenoceptor subtypes are present in different vessels and indicate that they are involved in the different tissue functions.

References

Berridge MJ (1992) Inositol triphosphate and calcium oscillations, in Advances in Second Messenger and Phosphoprotein Research (Putney JW ed) pp 211–224, Raven Press, New York.

Berridge MT, Dawson RMC, Downes ES, Heslop JP and Irvine RF (1982) Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem J* **212:**473–482.

Bond RA, Leff P, Johnson TD, Milano CA, Rockman HA, Mcminn TR, Apparsundaram S, Hyek MF, Kenakin TP, Allen LF and Lefkovitz RJ (1995) Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the β_2-adrenoceptor. *Nature (Lond)* **374:**272–276.

Burstein ES, Spalding TA and Brann MR (1997) Pharmacology of muscarinic receptor subtypes constitutively activated by G proteins. *Mol Pharmacol* **51:**312–319.

Colquhoun D (1998) Binding, gating, affinity and efficacy: The interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. *Br J Pharmacol* **125:**923–947.

García-Sainz J and Torres-Padilla ME (1999) Modulation of basal intracellular calcium by inverse agonists and phorbol myristate acetate in rat-1 fibroblasts stably expressing α_{1D}-adrenoceptors. *FEBS Lett* **443:**277–281.

Gether U, Ballesteros JA, Seifert R, Sanders-Bush E, Weinstein H and Kobilka BK (1997) Structural instability of a constitutively active G protein-coupled receptor. *J Biol Chem* **272:**2587–2590.

Graham RM, Perez DM, Hwa J and Piascik MT (1996) α_1-Adrenergic receptor subtypes. Molecular structure, function and signaling. *Circ Res* **78:**737–749.

Hieble JP, Bylund DB, Clarke DE, Eikenburg DC, Langer SZ, Lefkovitz RJ, Minneman KP and Ruffolo RR Jr (1995) International Union of Pharmacology X. Recommendation for nomenclature of α_1-adrenoceptors: Consensus update. *Pharmacol Rev* **47:**267–270.

Hussain MB and Marshall I (1997) Characterization of α_1-adrenoceptor subtypes mediating contractions to phenilephrine in rat thoracic aorta, mesenteric artery and pulmonary artery. *Br J Pharmacol* **122:**849–858.

Hwa J, Gaivin R, Porter JE and Perez DM (1997) Synergism of constitutive activity in α_1-adrenergic receptor activation. *Biochemistry* **36:**633–639.

Ibarra M, Lopez-Guerrero JJ and Villalobos-Molina R (1998) Further evidence for the predominance of α_{1D}-adrenoceptors in arteries of normotensive and spontaneously hypertensive rats. *Pharmacol Res Commun* **10:**135–142.

Ibarra M, Terron JA, Lopez-Guerrero JJ and Villalobos-Molina R (1997) Evidence for an age-dependent functional expression of α_{1D}-adrenoceptors in the rat vasculature. *Eur J Pharmacol* **322:**221–224.

Kenny BA, Chalmers DH, Philpott PC and Naylor AM (1995) Characterization of an α_{1D}-adrenoceptor mediating the contractile response of rat aorta to noradrenaline. *Br J Pharmacol* **115:**981–986.

Lachnit WG, Tran AM, Clarke DE and Ford APDW (1997) Pharmacological characterization of an α_{1A}-adrenoceptor mediating contractile responses to noradrenaline in isolated caudal artery of rat. *Br J Pharmacol* **120:**819–826.

Lee TW, Cotecchia S and Milligan G (1997) Up-regulation of the levels of expression and function of a constitutively active mutant of the hamster α_{1B}-adrenoceptor by ligands that act as inverse agonists. *Biochem J* **325:**733–739.

Leff P, Scaramellini CL and Mckechnie K (1997) A three-state receptor model of agonist action. *Trends Pharmacol Sci* **18:**355–362.

Lefkovitz RJ, Cotecchia S, Samana P and Costa T (1993) Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol Sci* **14:**303–307.

McCune DF, Edelmann SE, Olges JR, Post GR, Waldrop BA, Waugh DJJ, Perez DM and Piascik MT (2000) Regulation of the cellular localization and signaling properties of the α_{1B} and α_{1D}-adrenoceptors by agonists and inverse agonists. *Mol Pharmacol* **57:**659–666.

Mita M and Walsh MP (1997) α_1-Adrenoceptor-mediated phosphorylation of myosin in rat-tail arterial smooth muscle. *Biochem J* **327:**669–674.

Noguera MA and D'Ocon MP (1993) Evidence that depletion of internal calcium stores sensitive to noradrenaline elicits a contractile response dependent on extracellular calcium in rat aorta. *Br J Pharmacol* **110:**861–867.

Noguera MA, Ivorra MD, Chulia S and D'Ocon P (1997) Capacitative Ca^{2+} entry associated with α_1-adrenoceptors in rat aorta. *Naunyn-Schmiedeberg's Arch Pharmacol* **356:**83–89.

Noguera MA, Ivorra MD and D'Ocon MP (1996) Functional evidence of inverse agonism in vascular smooth muscle. *Br J Pharmacol* **119:**158–154.

Noguera MA, Madrero Y, Ivorra MD and D'Ocon MP (1998) Characterization of two different Ca^{2+} entry pathways dependent on depletion of internal Ca^{2+} pools in rat aorta. *Naunyn-Schmiedeberg's Arch Pharmacol* **357:**92–99.

Putney JW (1990). Capacitative calcium-entry revisited. *Cell Calcium* **11:**611–634.

Saussy DL Jr, Goetz AS, Queen KL, King HK, Lutz MW and Rimele TJ (1996) Structure activity relationships of a series of buspirone analogs at alpha-1 adrenoceptors: Further evidence that rat aorta alpha-1 adrenoceptors are of the alpha-1D-subtype. *J Pharmacol Exp Ther* **278:**136–144.

Scheer A, Fanelli F, Costa T, De Benedetti PG and Cotecchia S (1997) The activation process of the α_{1B}-adrenergic receptor: Potential role of protonation and hydrophobicity of a highly conserved aspartate. *Biochemistry* **94:**808–813.

Schwinn DA, Johnston GI, Page EO, Mosley MJ, Wilson KH, Worman NP, Campbell S, Fidock MD, Furness LM, Parry-Smith DJ, Peter B and Bailey DS (1995) Cloning and pharmacological characterization of human alpha-1 adrenergic receptors: Sequence corrections and direct comparison with other species homologues. *J Pharmacol Exp Ther* **272:**134–142.

Villalobos-Molina R and Ibarra M (1996) α_1-Adrenoceptors mediating contraction in arteries of normotensive and spontaneously hypertensive rats are of the α_{1D} or α_{1A} subtypes. *Eur J Pharmacol* **298:**257–263.

Villalobos-Molina R, Lopez-Guerrero JJ and Ibarra M (1999) Functional evidence of α_{1D}-adrenoceptors in the vasculature of young and adult spontaneously hypertensive rats. *Br J Pharmacol* **126:**1534–1536.

Xu K, Lu Z, Wei H, Zhang Y and Han C (1998) Alteration of α_1-adrenoceptor subtypes in aortas of 12-month-old spontaneously hypertensive rats. *Eur J Pharmacol* **344:**31–36.

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