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Functional evidence of inverse agonism in vascular smooth muscle

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1 In the present study, depletion of internal Ca^{2+} stores sensitive to noradrenaline (1 μM) in rat aorta, is the signal for the entry of extracellular Ca^{2+}, not only to refill the stores but also, in our experimental conditions, to activate the contractile proteins. This induces an increase in the resting tone that constitutes, the first functional evidence of this Ca^{2+} entry.

2 The fact that methoxamine (100 μM) reproduces the same processes as noradrenaline but clonidine (1 μM) does not, indicates that α_1-adrenoceptor activation is related to the increase in the resting tone observed after depletion of adrenoceptor-sensitive internal Ca^{2+}-stores.

3 Benoxathian and WB 4101 (α_{1A}- and α_{1D}-adrenoceptor antagonists) selectively inhibit, in a concentration-dependent manner, this mechanical response observed in absence of the agonist, which suggests that these agents can act as inverse agonists and provide a functional model for studying this phenomenon. Since chloroethylclonidine (100 μM) has no effect on this response, the participation of α_{1B}-adrenoceptors can be ruled out.

4 Contractile responses to noradrenaline (1 μM) in Ca^{2+}-free medium were selectively blocked by chloroethylclonidine. This suggests that the response to noradrenaline in Ca^{2+}-free medium mainly depends on the activation of the α_{1B}-adrenoceptor subtype.

Keywords: Inverse agonism; intracellular calcium stores; α_1-adrenoceptors; vascular smooth muscle

Introduction

The 'capacitative Ca^{2+} entry' hypothesis in non-excitable cells (Putney, 1986; 1990) postulates that depletion of the intracellular Ca^{2+} pools, even in the absence of receptor activation or increases in inositol polyphosphates, is sufficient to activate a Ca^{2+} entry mechanism so long as the intracellular Ca^{2+} pools are not permitted to refill.

We have found that in rat aorta, noradrenaline induces a biphasic contractile response in Ca^{2+}-free medium, which appears to be associated with two different intracellular pools, one of them common to caffeine (Noguera & D'Ocon, 1992). These intracellular Ca^{2+} pools, when emptied, could be rapidly repleted from the extracellular space by incubation in Ca^{2+}-containing solution, and the refilling does not depend on continued receptor activation (Noguera & D'Ocon, 1993a, b; D'Ocon *et al.*, 1995). Moreover, our previous results have shown that during this refilling process, there occurred a spontaneous increase in the resting tone of aorta (IRT) when the tissue was incubated in the presence of Ca^{2+} but in the absence of noradrenaline. On this basis, we may assume, as has been proposed in non-excitable (Parekh *et al.*, 1993; Vaca & Kunze, 1993; 1994) and excitable cells (Fasolato *et al.*, 1994; Koike *et al.*, 1994, Ohta *et al.*, 1995), that the depleted Ca^{2+}-stores sensitive to noradrenaline are the signal for the entry of extracellular Ca^{2+} not only to refill the stores, but, in our experimental conditions, also to activate the contractile proteins, thus giving the first evidence of the functional role of this Ca^{2+} entry.

Very little is known about the signal mechanism relating store emptying to plasma membrane Ca^{2+} entry. The aim, therefore, of the present work was to analyze the mechanisms involved in this phenomenon and in the repletion of intracellular Ca^{2+} pools sensitive to noradrenaline in rat aorta.

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Methods

Helically cut strips of the thoracic aorta of male Wistar rats (200-220 g) were prepared and mounted as described by Furchgott & Zawadzki (1980). In some experiments, thoracic aortic strips were bisected and the halves were used to perform parallel experiments. Each preparation was 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 75-90 min equilibration period. This tension 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 on a polygraph (Grass M7) via force-displacement transducers (Grass FT03).

Endothelium-denuded aortic strips were prepared by rubbing the entire intimal surface. The absence of relaxant response (100%) after acetylcholine (100 μM) addition to preparations contracted with noradrenaline (1 μM) indicated the absence of a functional endothelium in all strips (Furchgott & Zawadzki, 1980).

Experimental procedures

Figure 1 shows the experimental procedure designed to study the depletion of intracellular Ca^{2+}-stores sensitive to noradrenaline, methoxamine or clonidine in Ca^{2+} free medium, and the increase in the resting tone obtained by subsequent exposure to Ca^{2+}-containing solution (Krebs) during the refilling of these stores. Agonist was added in Ca^{2+}-containing solution at 37°C and then the tissue was treated with Ca^{2+}-free, EDTA-containing solution for 15 min. After this time, agonist was applied and washed until no contraction was induced, indicating complete depletion of internal Ca^{2+} stores sensitive to the agonist. The tissue was incubated for 20 min in Krebs to refill the intracellular Ca^{2+} stores and a spontaneous increase in the resting tone of the aorta (IRT) was observed. After washing and 15 min of loading in Ca^{2+}-free solution, a new addition of agonist was made. Subsequent incubation in Krebs for 20 min and addition of agonist permitted us to check the state of the preparation.

Concentration-response curves of relaxation (CRCR) to benoxathian WB 4101 and clorethylclonidine, were obtained by addition of cumulative concentrations of the compounds to tissues in which sustained contractions had been induced by 1 μM noradrenaline or by exposure to a solution containing 80 mM KCl (prepared by equimolar substitution of KCl for NaCl in the physiological solution). Relaxations were expressed as a percentage of the maximum increment of tension obtained by agonist addition. Concentration-response curves of inhibition (CRCI) to the same compounds were obtained by addition of one concentration of a compound 15 min before and during 1 μM noradrenaline- or 80 mM KCl-induced contraction. A separate series of experiments assessed the effects of the testing agents on the increase in tension (IRT). In this case, the experimental procedure was similar to that described in Figure 1, but 15 min before and during the loading period in Krebs solution that permits the refilling of internal Ca^{2+}-stores previously depleted by noradrenaline, different concentrations of the compounds assayed were added. The magnitude of the IRT in the presence of each concentration of each compound was expressed as a percentage of the reference IRT obtained in the absence of any agent.

E_{max} represents the maximal relaxation (CRCR) or inhibition (CRCI) obtained after addition of the highest concentration of each compound. The concentration needed to produce 50% relaxation or inhibition (IC_{50}) was calculated from a linear regression analysis of all the points between 20% and 80% of the maximal response (Graph Pad Software; San Diego, California, U.S.A.) but it was impossible to calculate s.e.mean in the concentration-response curves of inhibition.

Drugs and solutions

The following drugs were used: acetylcholine, (−)-noradrenaline, clonidine (all from Sigma St. Louis MO, U.S.A.) RBI (Natick, MA, U.S.A.); methoxamine, chloroethylclonidine, benoxathian, WB 4101 (2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxanehydrochloride). Other reagents were of analytical grade. All compounds were dissolved in distilled water. Composition of the physiological solution was (mM): NaCl 118, KCl 4.75, $CaCl_2$ 1.8, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. Ca^{2+}-free solution had the same composition except that $CaCl_2$ was omitted and EDTA (0.1 mM) was added.

Analysis of results

Contractions in physiological solution were expressed in mg of developed tension and, when elicited in Ca^{2+}-free medium, as a percentage of the noradrenaline-induced contractions obtained in normal physiological solution. Increases in resting tone were also expressed as a percentage of the noradrenaline-induced contraction in normal physiological solution.

Results are presented as the mean ± s.e.mean for n determinations obtained from different animals. Statistical sig-

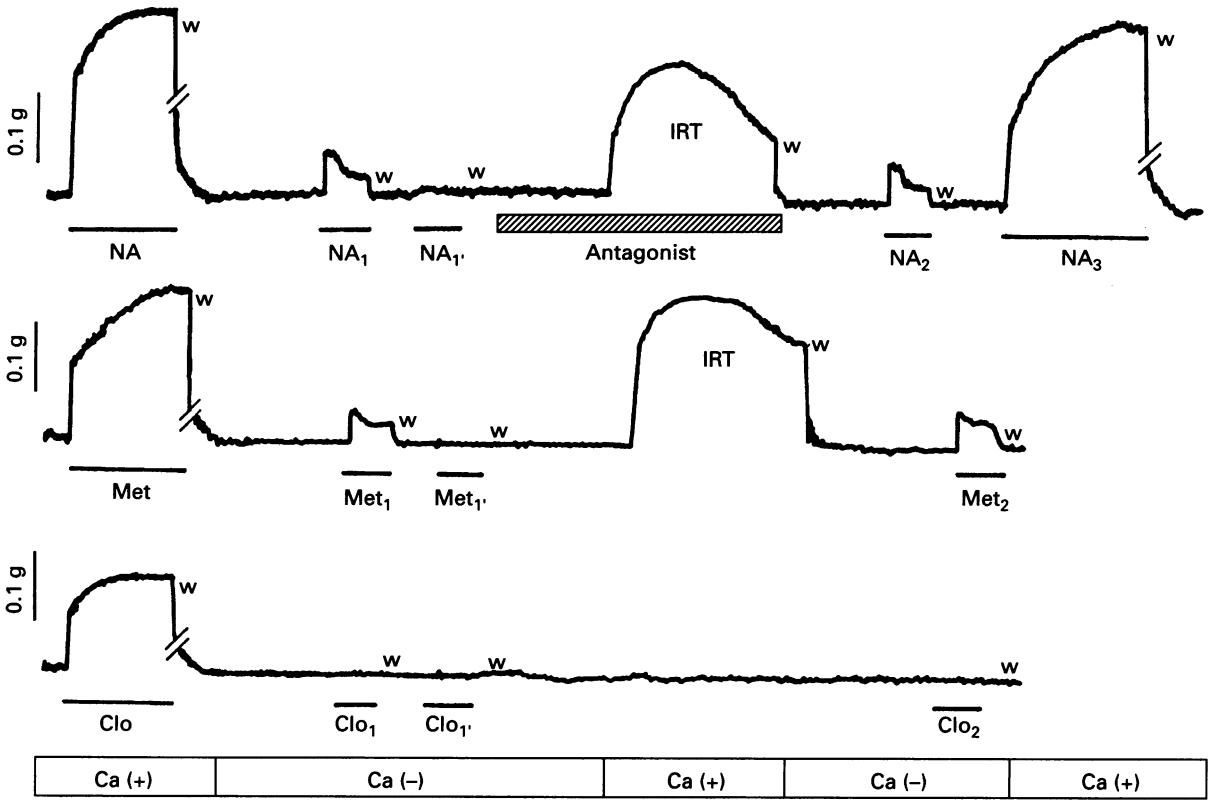

Figure 1 Schematic representation of the experimental procedure designed to study the depletion of intracellular Ca^{2+}-stores sensitive to noradrenaline (NA), methoxamine (Met) or clonidine (Clo) in Ca^{2+}-free medium, and the increase in the resting tone obtained by subsequent exposure to Ca^{2+}-containing solution during the refilling of these stores. Agonist was added in Ca^{2+}-containing solution and then the tissue was treated with Ca^{2+}-free, EDTA-containing solution for 15 min. After this time the agonist was applied (NA_1', NA_1',...) and washed (W) until no contraction was induced, indicating complete depletion of internal Ca^{2+} stores sensitive to the agonist. The tissue was incubated for 20 min in Krebs to refill the intracellular Ca^{2+} stores and a spontaneous increase in the resting tone of aorta (IRT) was observed. After washing and 15 min of loading in Ca^{2+}-free solution, a new addition of agonist (NA_2) was made. Subsequent incubation in Krebs during 20 min and addition of agonist (NA_3) permit us to check the state of the preparation. In the experiments designed to assess the effects of different agents on IRT, the aorta was pretreated with different concentrations of these agents 15 min before and during IRT was induced.

nificance was evaluated by Student's t test for paired or unpaired data. Differences were considered significant when $P<0.05$.

Results

Increase in the resting tone of rat aorta after depletion of intracellular stores sensitive to noradrenaline and methoxamine

The experimental procedure is shown in Figure 1 and the results are summarized in Table 1. Noradrenaline, 1 μM, evoked a maximal contraction in rat aorta that reached a magnitude of 776.1 ± 67.3 mg ($n=16$). This response consisted of two phases, phasic (1 min) and tonic (30 min). A new addition of the agonist reproduced this contractile response. After 15 min in Ca^{2+}-free solution the addition of noradrenaline (1 μM) also induced a biphasic contraction (NA_1), the magnitude of which is described in Table 1. A contraction was not evoked upon a second application of noradrenaline in Ca^{2+}-free solution ($NA_{1'}$). Upon re-exposure of the tissues to a Ca^{2+}-containing solution for 20 min, an increase in the resting tone was observed (IRT). Returning the tissues to a Ca^{2+}-free solution reduced the tension to baseline and further application of noradrenaline (NA_2) 15 min later induced a contraction similar to that of the first contraction elicited in Ca^{2+}-free solution.

Methoxamine (1 – 100 μM) elicited a concentration-dependent contraction in Krebs solution and the maximal response (phasic and tonic) obtained at the concentration 100 μM was similar to that of noradrenaline 1 μM. After an incubation period of 15 min in Ca^{2+}-free medium, methoxamine 100 μM induced a biphasic response (Met_1) similar to noradrenaline. During the 20 min exposure to physiological solution, refilling of the intracellular Ca^{2+} stores was observed (since a contraction was obtained at Met_2), and this refilling was also accompanied by an increase in the resting tone similar to that observed after depletion of intracellular calcium stores sensitive to noradrenaline.

Clonidine, 1 μM, produced a biphasic response in Krebs solution, but it was significantly smaller than the noradrenaline-induced one. After 15 min in Ca^{2+}-free medium, no contractile response was observed after addition of clonidine (Clo_1) and subsequent loading for 20 min in Ca^{2+}-containing solution produced no increase in the resting tone of the aorta. Further application of clonidine (Clo_2) in the absence of Ca^{2+} did not evoke any contractile response.

Modification of the increase in the resting tone of aorta by preincubation with different concentrations of α1-adrenoceptor antagonist

To investigate the possible relation between the increase in the resting tone of aorta and α_1-adrenoceptor subtypes, we studied the action of benoxathian, WB 4101 and chloroethylclonidine, on this tension. Concentration-response curves of relaxation (CRCR) to the testing agents were obtained by addition of cumulative concentration of the compounds to tissues precontracted by KCl (80 mM) or noradrenaline (1 μM). Concentration-response curves of inhibition (CRCI) to the same compounds, of the increase in the resting tone, noradrenaline-induced contraction in Ca^{2+}-free or Ca^{2+}-containing solution and KCl-induced contractions, were also obtained. E_{max} and IC_{50} values of the agents tested are described in Table 2 and Figure 2.

Benoxathian and WB 4101 selectively relaxed noradrenaline-induced contractions in Ca^{2+}-containing or Ca^{2+}-free medium, but their potency was significantly lower than that shown when they antagonized the increase in the resting tone (IRT) promoted by depletion of noradrenaline-sensitive internal stores. Benoxathian showed the same inhibitory potency in the Ca^{2+}-containing solution when it was cumulatively added to tissue contracted by noradrenaline (CRCR) as when added before the agonist (CRCI). However, the results with WB 4101 are different in the two experimental procedures with noradrenaline in Ca^{2+}-containing medium because in CRCI the phase reponse of noradrenaline was not as inhibited as the tonic one.

Table 1 Increase in the resting tone (IRT) of rat aorta elicited after depletion of intracellular Ca^{2+}- stores by 1 μM noradrenaline (NA), 100 μM methoxamine (Me) and 1 μM clonidine (Clo)

		Ca (+) Agonist (%)		*Ca (−) Agonist$_1$* (%)		*Ca (+) IRT* (%)	*Ca (−) Agonist$_2$* (%)	
	n	*Phasic*	*Tonic*	*Phasic*	*Tonic*		*Phasic*	*Tonic*
NA	5	57.2 ± 2.2	100	24.6 ± 1.2	9.0 ± 1.4	51.5 ± 5.5	25.1 ± 1.4	10 ± 1.2
Me	4	43.6 ± 7.8	87.8 ± 11.1	19.3 ± 2.5	10.1 ± 2.9	52.3 ± 8.4	19.1 ± 1.4	10.0 ± 2.3
Clo	7	27.9 ± 1.8	56.7 ± 2.5	0	0	0	0	0

Increases in the resting tone and contractions in Ca^{2+}-containing or Ca^{2+}-free medium are expressed as a percentage of noradrenaline-induced contractions in Krebs solution. All values represent mean ± s.e.mean, n = number of experiments.

Table 2 IC_{50} values of the agents tested on the increase in the resting tone of rat aorta (IRT), noradrenaline (NA)-induced contractions in Ca^{2+}-containing solution and noradrenaline-induced contraction in Ca^{2+}-free medium

		NA (1 μM) Ca^{2+} (+) *CRCR*	*CRCI*	Ca^{2+} (−) *CRCI*	*IRT* Ca^{2+} (+) *CRCI*
Benoxathian	−log IC_{50}	6.82 ± 0.01	6.92	6.90	8.52
	n	4	4–5	5–6	5–6
WB 4101	−log IC_{50}	8.17 ± 0.09	7.22	8.62	9.77
	n	4	4–5	4–5	5–6
CEC	−log IC_{50}	NC	4.70	5.77	NC
	n	8	3–4	4–6	4

Ca^{2+} (+) = Ca^{2+}-containing solution; Ca^{2+} (−) = Ca^{2+}-free medium; CRCR = concentration-response curves of relaxation; CRCI = concentration-response curves of inhibition (see methods); NC = not calculated. All values represents mean ± s.e.mean, except the values of IC_{50} on CRCI experimental procedures, which are presented as mean. n = number of experiments.

Table 3 Contractile response to noradrenaline in Ca^{2+}-free medium before (NA_1) and after (NA_2) a refilling period in the presence of the testing agents in Ca^{2+}-containing solution (see experimental procedure in Figure 1)

			NA_1 (%) Ca (−)		*IRT* (%) Ca (+)	*NA_2* (%) Ca (−)	
Agent	(μM)	n	*Phasic*	*Tonic*		*Phasic*	*Tonic*
Control	–	7	29.4±0.4	9.3±0.2	53.8±1.9	30.2±1.2	8.9±0.9
WB 4101	0.01	4	33.2±2.1	8.2±0.5	0	0	0
Benoxathian	0.1	4	34.0±5.4	7.9±1.6	0	13.2±1.7*	2.3±0.3*
CEC	100	5	35.1±3.0	7.3±0.4	51.8±2.3	0	0

IRT = increase in the resting tone observed during the refilling of noradrenaline-sensitive internal Ca^{2+}-stores previously depleted by the addition of the agonist in Ca^{2+}-free medium. Contractions are expressed as a percentage of the noradrenaline-induced contraction in Krebs solution. All values represent mean±s.e.mean, n = number of experiments. *$P<0.05$ with respect to NA_1.

Chloroethylclonidine (CEC) had no effect on IRT and relaxed noradrenaline-induced contraction preferentially when the response to this agonist was elicited in Ca^{2+}-free medium. The inhibitory action of CEC on sustained responses elicited by noradrenaline was increased in a time-dependent manner, and after 30 min of exposure to the higher concentration of CEC tested (100 μM), a complete relaxation was observed.

As shown Figure 2, none of the tested agents significantly relaxed KCl-induced contractile response of rat aorta, and only slight relaxation was observed with the highest concentration assayed of chloroethylclonidine or benoxathian. This relaxation could be related to inhibition of the contractile action of endogenous noradrenaline released by depolarization (Ivorra *et al.*, 1993; Chuliá *et al.*, 1994).

Influence of agents on the refilling of intracellular Ca^{2+} stores sensitive to noradrenaline

In order to clarify the possible action of benoxathian, WB 4101, and chloroethylclonidine on the refilling of the intracellular Ca^{2+} stores sensitive to noradrenaline, the magnitude of the contractile response obtained by the subsequent addition of noradrenaline in Ca^{2+}-free medium was assumed to be related to the content of the agonist-sensitive Ca^{2+}-pools and the values of this response obtained after refilling the stores in presence of the highest concentration of each agent tested are summarized in Table 3. Experimental procedure is shown in Figure 1.

Benoxathian and WB 4101, which inhibited the increase in the resting tone (IRT), also inhibited the refilling of internal stores previously depleted by noradrenaline as demonstrated by the lower response or the lack of response to noradrenaline in Ca^{2+}-free medium after treatment with these agents (NA_2, Table 3). However, a complete recovery of noradrenaline-induced contraction in Ca^{2+}-containing solution was observed (NA_3, Figure 1), which means that this inhibition cannot be due to a residual presence of the antagonist in the medium. Treatment with chloroethylclonidine (CEC), which did not affect the IRT, inhibited the response of noradrenaline in Ca^{2+}-free medium (NA_2, Figure 1), but this may be attributed to an irreversible inactivation of α_1-adrenoceptors rather than to a blocking of the refilling of internal Ca^{2+} stores sensitive to the agonist, since in Ca^{2+} containing solution there was again no response to noradrenaline.

Discussion

The present results show that in rat aorta, addition of noradrenaline in Ca^{2+}-free medium induced a biphasic contraction that was used as an index for the content of agonist-sensitive intracellular stores, but a contraction was not evoked upon a second application of the agonist in Ca^{2+}-free solution, which shows that noradrenaline-sensitive intracellular stores (Noguera & D'Ocon, 1992; 1993a) had been depleted. Upon re-

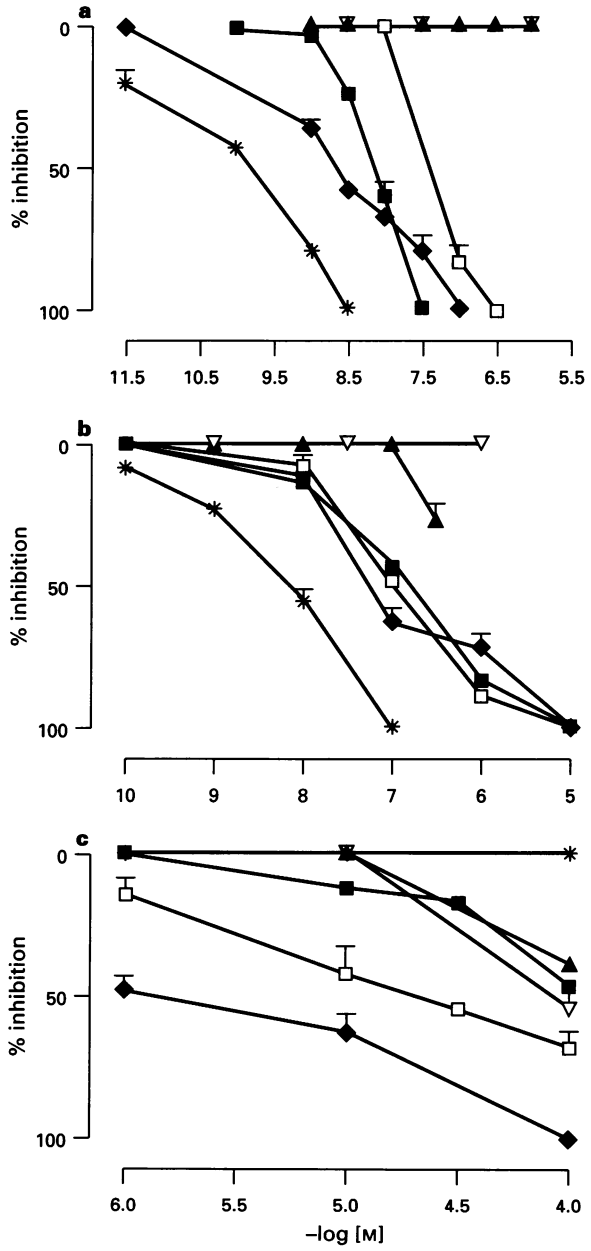

Figure 2 Concentration-response curves for relaxation (CRCR) or inhibition (CRCI) induced by (a) WB 4101, (b) benoxathian and (c) chloroethylclonidine against KCl, noradrenaline in Ca^{2+}-containing solution (NA), noradrenaline in Ca^{2+}-free solution (NA Ca(−)) and against the increase in the resting tone (IRT) observed during the refilling of the internal Ca^{2+}-stores sensitive to α_1-adrenoceptors: (■) CRCR NA; (□) CRCI NA; (◆) CRCI NA Ca(−); (*) IRT; (▲) CRCR KCl; (▽) CRCI KCl.

exposure of the tissues to a Ca^{2+}-containing solution, an increase in the resting tone was observed. The magnitude of the mechanical response decreases proportionally with the time of exposure to Ca^{2+}-containing medium, which means that the accelerated entry of Ca^{2+} to replenish the intracellular pools inactivates the entry mechanism. In our experimental conditions, the removal of the endothelium rules out possible involvement of endothelium-derived factors in this mechanical response. Returning the tissues to a Ca^{2+}-free solution reduced the tension to the baseline, and further application of noradrenaline reproduced a contractile response similar to the first one elicited in Ca^{2+}-free solution thus indicating a complete refilling of internal stores.

Methoxamine elicited a concentration-dependent contraction in Krebs solution and the maximal response was similar to that of noradrenaline. In Ca^{2+}-free medium, methoxamine also induced a biphasic response similar to noradrenaline. During the refilling of the intracellular Ca^{2+} stores there was an increase in the resting tone, similar to that observed after depletion of intracellular calcium stores sensitive to noradrenaline.

Different results were obtained when clonidine was added to the organ bath at a concentration sufficient to evoke a maximal contraction (Noguera *et al.*, 1993). In Ca^{2+}-free medium, no contractile response was observed after addition of clonidine, and subsequent loading in Ca^{2+}-containing solution produced no increase in the resting tone of the aorta. These results exclude the participation of α_2-adrenoceptors in this process.

We can assume that activation of α_1-adrenoceptors promotes Ca^{2+} release from internal stores that lose their Ca^{2+} content. These stores, when emptied, can be rapidly replenished by incubation in Ca^{2+} containing solution in the absence of the agonist, 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. Previous studies have shown the existence of a Ca^{2+} current dependent on the emptying of internal Ca^{2+} stores by means of direct measurements of membrane currents (Parekh *et al.*, 1993; Vaca & Kunze, 1994; Pacaud *et al.*, 1993) or cytosolic free Ca^{2+} levels (Randriamampita & Tsien, 1993). In our experimental conditions, a mechanical response was observed and this makes it easier to analyze the functional implications of this Ca^{2+} current as well as the mechanisms related to it.

This increase in the resting tone of aorta therefore seems to be strictly related to adrenoceptors and not just to the emptying of intracellular Ca^{2+} pools sensitive to an agonist since after emptying of internal Ca^{2+}-stores by exposure to caffeine or 5-hydroxytryptamine (Noguera & D'Ocon, 1993a) or to thapsigargin and cyclopiazonic acid (two selective Ca^{2+}-ATPase inhibitors) and to ryanodine (which interacts specifically with the sarcoplasmic reticulum Ca^{2+}-release) did not result in any contractile response during the subsequent refilling of these stores by loading in Ca^{2+}-containing solution (results not shown).

The first question that arises has to do with the results obtained in the previous study (Noguera & D'Ocon, 1993a), which showed that the increase in the resting tone observed upon adding Ca^{2+} after depletion of internal Ca^{2+}-stores sensitive to noradrenaline, is selectively blocked by prazosin. The fact that this α_1-adrenoceptor antagonist inhibited this mechanical response in the absence of the agonist relates to the recent concept of *inverse agonist* (Black & Shankley, 1995; Milligan *et al.*, 1995), and provides a basis for analyzing in this experimental model, the action of selective α_1-adrenoceptor antagonists.

The receptor that mediates contractions of the rat aorta produced by noradrenaline belongs to the α_1 type (Noguera & D'Ocon, 1993a; Noguera *et al.*, 1993), but the fact that these responses were sensitive to α_{1A} and α_{1B} antagonists suggests either the presence of both receptor subtypes (Piascik *et al.*, 1991; Oriowo & Ruffolo, 1992), or that this receptor is a non α_{1A} non α_{1B} receptor that possesses some of the characteristics of each of them: the α_{1D}-adrenoceptor (Aboud *et al.*, 1993; Ko *et al.*, 1994; Kenny *et al.*, 1994; Hieble *et al.*, 1995). However, functional data still suggest a mixed receptor population in this tissue (Van der Graaf *et al.*, 1993; Kenny 1995; Hieble *et al.*, 1995). In order to determine what subtype of α_1-adrenoceptor is involved in the mechanical response dependent on the previous depletion of intracellular stores, we analyzed the influence of different antagonist: benoxathian ($\alpha_{1A/D}$ WB 4101 ($\alpha_{1A/D}$) and chloroethylclonidine (α_{1B}).

Treatment with benoxathian and WB 4101, induced a concentration-dependent inhibition of the increase in the resting tone, whereas chloroethylclonidine did not affect this process. These results exclude the participation of α_{1B}-adrenoceptors in this mechanical response and relate it to α_{1A}- or α_{1D}-adrenoceptors. Moreover, the close correlation between the potency shown by benoxathian and WB 4101 against the increase in the resting tone in the present work and α_{1A}- or α_{1D}-adrenoceptor binding affinity in other tissues (Kenny *et al.*, 1994; Ford *et al.*, 1994) supports the involvement of one of these adrenoceptor subtypes in the development of the increase in the resting tone. It has been suggested that the α_{1A}-adrenoceptor subtype gates Ca^{2+} influx through dihydropyridine-sensitive channels in smooth muscle, while the α_{1B}-adrenoceptor has been associated with the formation of inositol 1,4,5-trisphosphate and the mobilization of intracellular Ca^{2+} (Minneman 1988; Nelson *et al.*, 1988; Pacaud *et al.*, 1991; Testa *et al.*, 1995). The functional role of the α_{1D} receptor is not well established but in rat aorta it could be related to Ca^{2+} entry through dihydropyridine-sensitive channels. Our results lend support to this suggestion, for they show that the response to noradrenaline in Ca^{2+}-free medium was selectively blocked by chloroethylclonidine, which does not affect the increase in the resting tone promoted by Ca^{2+} entry from the extracellular space. Thus, according to the present results, the response to noradrenaline in Ca^{2+}-free medium involves depletion of internal Ca^{2+} stores sensitive to this agonist and depends on the activation of the α_{1B} receptor subtype since chloroethylclonidine selectively inhibits it. The increase in the resting tone observed during the refilling of these stores is sensitive to α_{1A}-α_{1D}-adrenoceptor antagonists, and therefore must be related to this adrenoceptor subtype. The fact that the calcium channel blockers, nifedipine (Noguera & D'Ocon, 1993a), nimodipine (results not shown), verapamil and dilitiazem (Noguera & D'Ocon, 1993b), also block the increase in the resting tone corroborates the involvement of a voltage-dependent Ca^{2+} channel in this process. Our results are consistent with the hypotheses that in rat aorta, contraction to noradrenaline consists of an initial response due to the release of intracellular Ca^{2+} (mediated by α_{1B}-adrenoceptors) and a larger tonic contraction due to extracellular Ca^{2+} influx. This influx may involve activation of an α_{1A} or α_{1B}-adrenoceptor. Similar results, but not identical, were observed by Burt *et al.* (1995) in rat spleen.

It is interesting to note that benoxathian and WB 4101 block the mechanical response observed in the absence of the agonist as does prazosin (Noguera & D'Ocon, 1993a). An explanation suggested by recent evidence about the inverse agonism concept (Lefkowitz *et al.*, 1993; Black & Shankley, 1995; Bond *et al.*, 1995; Milligan *et al.*, 1995) is that mobilization of an intracellular Ca^{2+} pool sensitive to noradrenaline could determine the increase in the number of receptors that are spontaneously active in absence of agonist. The antagonists bind to the receptor and change it from the activated state to an inactivated one, or better yet, bind to inactive receptors and disrupt the equilibrium between the two states (active and inactive) in favour of the inactive state. The refilling of the internal stores sensitive to noradrenaline displaces again the equilibrium to the inactive state in absence of the agonist. Benoxathian and WB 4101 then act as inverse agonists, decreasing the proportion of the active form of the α_{1A}- or α_{1D} adrenoceptors that have been increased previously by the emptying of intracellular Ca^{2+}-stores. This hypothesis requires further investigation, but Schütz & Freissmuth (1992) have suggested that antagonists not only bind to G-protein coupled

receptors, but also induce a conformational change unfavourable to the coupling of the receptor to its G protein. Moreover, new evidence suggests a two-state model, in which receptors are in equilibrium between the inactive conformation and a spontaneously active conformation that couples to G protein in the absence of ligand. The first state can be fixed by inverse agonists (Bond *et al.*, 1995). The present work shows that in our experimental conditions, benoxathian and WB 4101 do not act as antagonists but as inverse agonists, for both of them inhibit the increase in the resting tone in absence of the agonist, and provides a simple model for analyzing this concept in functional studies. It is known that the characteristics of inverse agonists tend to be more apparent in systems that express relatively high receptor levels with associated higher basal effector activity, but the lack of suitable *in vivo* models has restricted the evidence of the existence of inverse agonists mainly to computer simulations and *in vitro* systems (Bond *et al.*, 1995). Our model could prove very useful in studying this phenomenon for we have a large number of receptors in the active state but in the absence of the agonist.

The exact nature of the signal that relates the emptying of intracellular Ca^{2+} stores and the increase in constitutive activity shown by α_{1A}- or α_{1D}-adrenoceptors remains elusive and further research on this topic is needed.

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