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Evidence that depletion of internal calcium stores sensitive to noradrenaline elicits a contractile response dependent on extracellular calcium in rat aorta

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1 Noradrenaline 1 μM induced a contractile response in rat isolated aorta in the presence or in the absence of extracellular Ca^{2+} with depletion of intracellular Ca^{2+} stores. Thereafter, during incubation in the presence of Ca^{2+}, an increase in the resting tone was observed. Such a contractile response did not occur after exposure to caffeine or 5-hydroxytryptamine.

2 This increase in tension was inhibited in a concentration-dependent manner by α-adrenoceptor antagonists (prazosin, phentolamine and yohimbine), the non-specific relaxing compound, papaverine and by the Ca^{2+}-entry blocker, nifedipine. Therefore, this contractile process is related to depletion of Ca^{2+} stores sensitive to noradrenaline and is linked to Ca^{2+} entry through voltage-operated Ca^{2+} channels and α-adrenoceptors.

3 Phentolamine and yohimbine did not block the Ca^{2+} refill pathway; prazosin and nifedipine inhibited the reuptake of Ca^{2+} by an internal store sensitive only to noradrenaline; papaverine inhibited the refilling of caffeine- and noradrenaline-sensitive Ca^{2+}-stores.

Keywords: Intracellular Ca^{2+}; rat aorta; noradrenaline; receptor-regulated Ca^{2+} entry; papaverine; α-adrenoceptors

Introduction

The characteristics of noradrenaline-induced contractions in smooth muscle have been widely studied and it is currently believed that the increase in tension in Ca^{2+}-containing solution comprises two distinct phases. The initial phasic contraction results from intracellular Ca^{2+} release following an increase in the turnover of phosphatidylinositol and the production of inositol 1,4,5-trisphosphate. In the continuing presence of noradrenaline, contraction is maintained and this phase is associated with Ca^{2+}-influx via a specific pathway (Bolton, 1979; Bray *et al.*, 1991). However, the results of electrophysiological studies of Ca^{2+} currents in smooth muscle are still confusing: noradrenaline increases the L-type Ca^{2+} current (Benham & Tsien, 1988; Fukumitsu *et al.*, 1990), but produces either no effect on voltage-activated inward currents (Yatani *et al.*, 1987) or reduces them (Imaizumi *et al.*, 1991) in different experimental procedures or in different smooth muscles.

In rat aorta, different authors (Morel & Godfraind, 1991; Nishimura *et al.*, 1991) have shown that noradrenaline activates voltage-operated Ca^{2+} channels that contain specific, voltage-sensitive binding sites for Ca^{2+} channel blocking dihydropyridines, but the existence of a fraction of noradrenaline-stimulated Ca^{2+}-entry that is resistant to nisoldipine blockade suggests that another Ca^{2+} entry pathway is also activated by the agonist. Whereas the role of endoplasmic reticulum in agonist-induced Ca^{2+}-release which involves the generation of inositol 1,4,5-trisphosphate is reasonably well established, the route, or routes, of Ca^{2+} entry into the agonist-sensitive internal Ca^{2+} stores remains an interesting dispute. Putney (1986, 1990) has postulated that depletion of the intracellular Ca^{2+} pools, even in the absence of receptor activation or increases in inositol polyphosphates, should activate the same Ca^{2+} entry mechanism that is normally activated, so long as the intracellular Ca^{2+} pools are not permitted to refill. We have found that after depleting noradrenaline releasable Ca^{2+} pools in Ca^{2+}-free medium, exposure to Ca^{2+}-containing solution, in the absence of the agonist, to allow the refilling of these intracellular Ca^{2+} stores, induces a considerable increase in the resting tone (IRT) of rat aorta.

The present work analyzes the pathways for this Ca^{2+} entry into the cell that are responsible for generating an increase in the resting tone of rat aorta during the refilling of Ca^{2+} pools, and their relation to voltage-operated Ca^{2+} channels and α-adrenoceptor activation. In order to do so, we have elicited this increase in tension in the presence of different concentrations of nifedipine, an organic Ca^{2+} entry blocker, and prazosin, phentolamine and yohimbine, α-adrenoceptor antagonists. Papaverine was also tested as a non-specific relaxing compound that prevents contractile responses in different smooth muscles (Lugnier *et al.*, 1986; Iino *et al.*, 1988; Sato *et al.*, 1988).

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. Tension was recorded isometrically on a Philips recorder (PM 8222) coupled to a Hewlett Packard amplifier (8805D) using force-displacement transducers (Gould Statham UC2).

Endothelium-denuded aortic strips were prepared by rubbing the entire intimal surface. The absence of relaxant response (100%) after acetylcholine (10^{-4} M) addition to preparations contracted with noradrenaline (1 μM) indicated the absence of a functional endothelium in all strips (Furchgott & Zawadzki, 1980).

Experimental procedures

Concentration-response curves of relaxation to prazosin, yohimbine, phentolamine, nifedipine and papaverine were

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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. E_{max} represents the maximal relaxation (100%) obtained after addition of the highest concentration of each compound. The concentration needed to produce 50% inhibition (IC_{50}) was obtained from a linear regression plot of all points between 20% and 80% of the maximal response.

Figure 1 shows the different experimental procedures designed to study the increase in the resting tone of aorta obtained at 37°C by exposure to physiological solution during the refilling of the intracellular Ca^{2+} stores sensitive to noradrenaline.

A separate series of experiments assessed the effects of prazosin, yohimbine, phentolamine, nifedipine and papaverine on the increase in tension. Noradrenaline (1 μM) was added in Ca-containing solution at 37°C and then the tissue was treated with Ca^{2+}-free, EDTA-containing solution for 15 min. After this time noradrenaline was applied until no contraction was induced, indicating complete depletion of internal Ca^{2+} stores sensitive to noradrenaline. The aorta was then pretreated with different concentrations of prazosin, yohimbine, phentolamine nifedipine and papaverine 15 min before an increase in the resting tone of aorta was induced (Figure 2). The magnitude of this increase in the presence of different concentrations of each compound was expressed as a percentage of the reference increase in resting tone obtained in the absence of any agent. In order to determine the IC_{50} values for each compound relative to the inhibition of the increase in tension, a linear regression analysis of all points obtained was performed; IC_{50} was calculated from this linear regression plot, but it was impossible to calculate s.e.mean. Each point was the mean of 4–7 experiments.

Another series of experiments was done to clarify the possible action of these compounds on the refilling of the intracellular Ca^{2+} stores. In this experimental procedure noradrenaline (NA2; Figure 2) or caffeine (Caf1; Figure 3) was added in Ca^{2+}-free medium after the incubation period in the presence of Ca^{2+} and of the highest concentrations of the compounds that completely inhibited the increase in resting tone. When caffeine was added to the organ bath, noradrenaline was also applied later (NA2; Figure 3). These experiments were carried out at 25°C because the contractile activity of caffeine in Ca^{2+}-free medium has only been observed at this temperature (Sato *et al.*, 1988; Noguera & D'Ocon, 1992). Subsequently, the tissue was incubated again in the presence of Ca^{2+}, and noradrenaline (NA3; Figure 3) was applied in physiological solution.

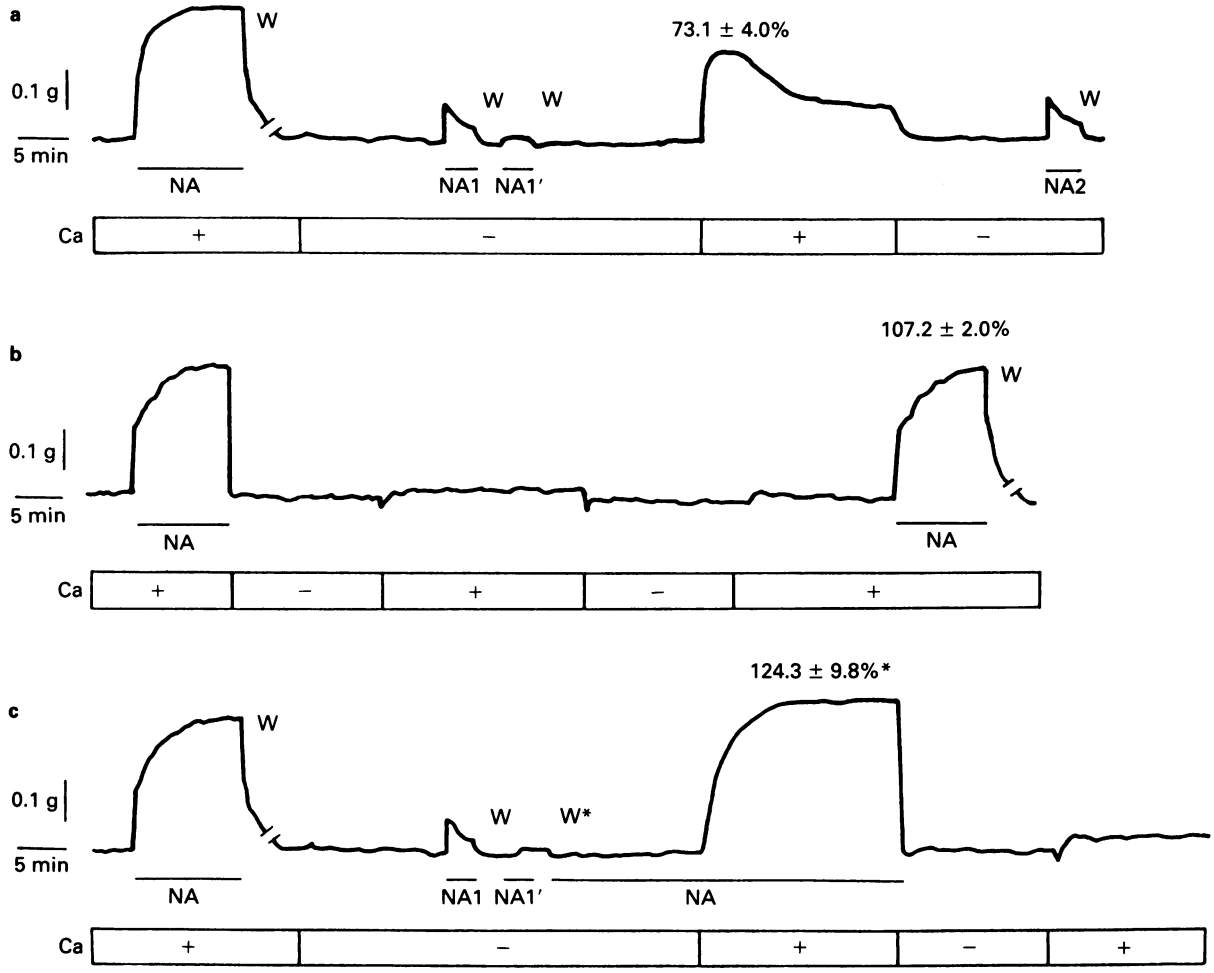

Figure 1 Schematic representation of different experimental procedures used to analyze the increases in tone observed after noradrenaline (NA)-induced depletion of intracellular Ca^{2+} stores. The increase in the resting tone and contractions in Ca^{2+}-free medium are expressed as a percentage of the noradrenaline-induced contraction in physiological solution. All values represent mean ± s.e.mean. Each value is the mean of 5–10 experiments. Preincubation time in Ca^{2+}-free medium: 15 min. W = washing; W* = washing with Ca^{2+} and EDTA-free solution. $^*P < 0.001$, significantly different from the noradrenaline-induced contraction in physiological solution.

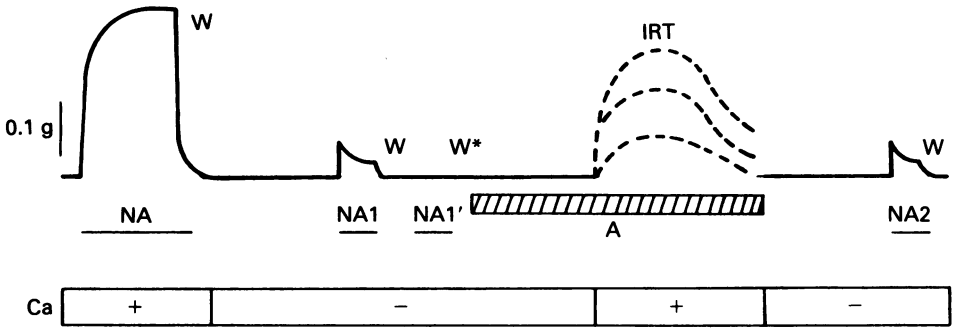

Figure 2 Experimental procedure used to analyze the modification of the increase in the resting tone of aorta obtained in the presence of different concentrations of the testing agents (A). Preincubation time in Ca^{2+}-free medium: 15 min. W = washing, W* = washing with Ca^{2+} and EDTA-free solution. NA1: addition of the agonist after 15 min of incubation in Ca^{2+}-free medium. NA1': addition of the agonist after washing (W) in Ca^{2+}-free medium. NA2: addition of the agonist after a 20 min resting period in physiological solution and 15 min in Ca^{2+}-free medium.

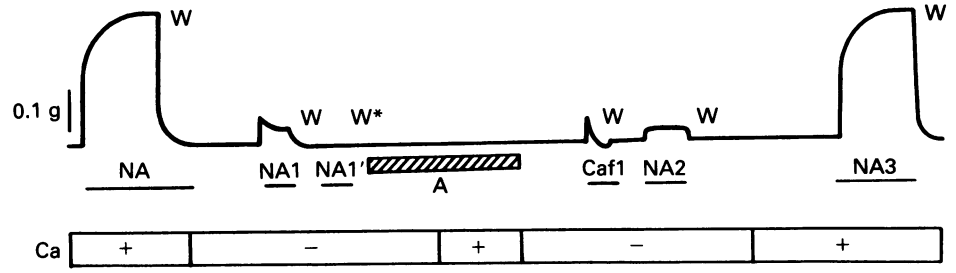

Figure 3 Experimental procedure used to analyze the refilling of internal Ca^{2+} storage sites in the presence of the testing agents (A) after depletion of noradrenaline-sensitive intracellular Ca^{2+} pools. The experiments were carried out at 25°C. Preincubation time in Ca^{2+}-free medium = 15 min. W = washing. W* = washing with Ca^{2+} and EDTA-free solution. NA1': first addition of noradrenaline after 15 min of incubation in Ca^{2+}-free medium. NA1': addition of the agonist after washing (W) in Ca^{2+}-free medium. Caf1: addition of caffeine after an incubation for 20 min in the presence of Ca^{2+} and 15 min in Ca^{2+}-free medium. NA2: addition of noradrenaline in Ca^{2+}-free medium after removing caffeine from the organ bath. NA3: addition of noradrenaline in physiological solution after 20 min exposure to Ca^{2+}-containing solution.

Drugs and solutions

The following drugs were obtained from Sigma (St. Louis MO, U.S.A.): anhydrous caffeine, 5-hydroxytryptamine creatinine sulphate complex, acetylcholine, phentolamine, yohimbine, prazosin, nifedipine and papaverine; (−)-noradrenaline L-tartrate was from Merck (Darmstadt, FRG). Other reagents were of analytical grade. All compounds were dissolved in distilled water with the exception of caffeine, which was dissolved in Ca^{2+}-free physiological solution (prepared by omission of $CaCl_2$). Nifedipine was dissolved in ethanol (10^{-2} M) before being diluted in distilled water and stored in the dark.

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-, caffeine- or 5-hydroxytryptamine-induced contractions obtained in normal physiological solution, respectively. 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 significance was evaluated by Student's t test for unpaired data. Differences were considered significant when $P<0.05$.

To determine IC_{50} values a linear regression analysis was performed (Graph Pad Software; San Diego, California, U.S.A).

Results

Analysis of the increase in the resting tone of rat aorta in different experimental conditions

In the presence of 1 μM noradrenaline, a concentration sufficient to evoke a maximal contraction, the magnitude of the contractile response of rat aortic tissues was 358.0 ± 55.3 mg ($n = 6$). The tissues were then exposed to Ca^{2+}-free solution for 15 min (Figure 4a) when addition of noradrenaline induced a biphasic contraction (NA1). The magnitude of these contractions after 15 min in Ca^{2+}-free medium is described in Table 1a. A contraction was not evoked upon a second application of 1 μM noradrenaline in Ca^{2+}-free solution (NA1'; Figure 4a). Upon re-exposure of the tissues to a Ca^{2+}-containing solution for 20 min, an increase in the resting tone was observed. This increase was 73.1 ± 4.0% ($n = 6$) relative to noradrenaline-induced contractions in physiological solution. Returning the tissues to a Ca^{2+}-free solution reduced the tension to baseline and further application of noradrenaline (NA2) 15 min later induced a contraction similar in size to that of the first contraction elicited in Ca^{2+}-free solution (NA1; Figure 4a; Table 1).

Caffeine (10 mM) elicited a rapid transient contraction in Ca^{2+}-containing solution at 37°C (113.1 ± 12.2 mg; $n = 9$),

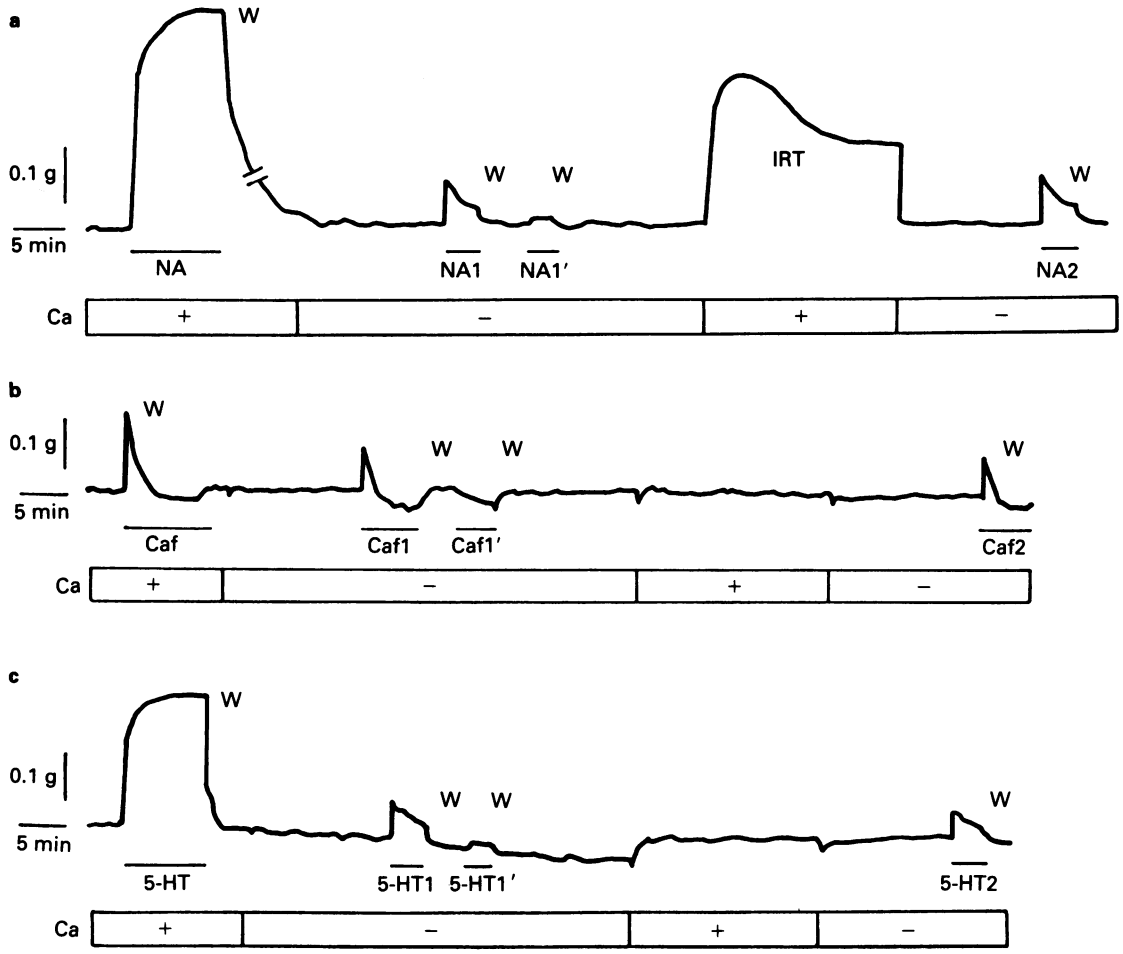

Figure 4 Schematic representation of (a) the increase in the resting tone of aorta obtained after depletion of noradrenaline (NA)-sensitive intracellular Ca^{2+} stores by exposure to noradrenaline in the absence of Ca^{2+}, (b) the effect of caffeine (Caf) and (c) the effect of 5-hydroxytryptamine (5-HT). Preincubation time in Ca^{2+}-free medium: 15 min. W = washing. NA1, Caf1, 5-HT_1: addition of the agonist after 15 min incubation in Ca^{2+}-free medium. NA1', Caf1', 5-HT_1': addition of the agonist after washing (W) in Ca^{2+}-free medium. NA2, Caf2, 5-HT_2: addition of the agonist after 20 min resting period in physiological solution and 15 min in Ca^{2+}-free medium.

Table 1 Increase in the resting tone of rat aortic segments elicited by 1 μM noradrenaline (NA), 10 mM caffeine (Caf) and 1 μM 5-hydroxytryptamine (5-HT)

	n	T (°C)	Ca^{2+}-containing Agonist (mg)	Ca^{2+}-free Agonist 1 (%) phasic	tonic	Ca^{2+}-containing 20 min Increasing tone (%) (IRT)	Ca^{2+}-free Agonist 2 (%) phasic	tonic
NA	6	37	358.0 ± 55.3	22.8 ± 1.4	15.2 ± 1.5	73.1 ± 4.0	21.0 ± 0.8	12.8 ± 1.5
Caf	4	25	123.1 ± 9.3	42.2 ± 9.7		NR	46.1 ± 10.1	
5-HT	6	37	215.3 ± 11.4	19.7 ± 1.5		NR	14.0 ± 1.2	

Increases in tone and contractions in Ca^{2+}-free medium are expressed as a percentage of the noradrenaline-, caffeine- or 5-hydroxytryptamine-induced contractions in physiological solution respectively.
All values represent mean ± s.e.mean, n = number of experiments.
Agonist1: first addition of the agonist after 15 min of incubation in Ca^{2+}-free medium.
Agonist2: second addition of the agonist after 20 min in Ca^{2+}-containing and 15 min in Ca^{2+}-free medium.
NR: no response.

but in Ca^{2+}-free medium no contractile response was observed. At 25°C, exposure to caffeine induced a transient contraction both in Ca^{2+}-containing solution and after 15 min in Ca^{2+}-free medium (CAF1). The size of these contractile responses are shown in Table 1. During the 20 min exposure to physiological solution, refilling of the intracellular Ca^{2+} stores was observed (since a contraction was obtained at CAF2) but there was no increase in the resting tone of the tissues (Figure 4b).

Similar results were obtained when 1 μM 5-hydroxytryptamine was added to the organ bath at 37°C, eliciting a sustained contraction in physiological solution of smaller magnitude than that elicited by noradrenaline (215.3 ± 11.4 mg; n = 6). In Ca^{2+}-free medium, 5-hydroxytryptamine induced a transient phasic contraction (5-HT_1) (Figure 4, Table 1). Subsequent exposure to Ca^{2+}-containing physiological solution (20 min) produced no increase in the resting tone of the aorta (Figure 4c), but after returning to a Ca^{2+}-free

medium 5-hydroxytryptamine again induced a contraction (5-HT_2), similar in size to that first obtained in Ca^{2+}-free medium.

As shown in Figure 1, when tissues had not been exposed to noradrenaline in the absence of extracellular Ca^{2+}, exposure to Ca^{2+}-containing solution (after an exposure to Ca^{2+}-free solution) did not result in the development of a contraction and subsequent contractile responses to noradrenaline in Ca^{2+}-containing solution were unaffected ($n = 5$; Figure 1b). Conversely, if noradrenaline was included during exposure to Ca^{2+} 1.8 mM, the result was a sustained and significantly higher contraction than that obtained in the presence of Ca^{2+} at the beginning of the experiment ($n = 6$; $P < 0.001$; Figure 1c).

Modification of the increase in the resting tone of aorta by preincubation with different concentrations of the testing agents

To investigate the possible relation between the increase in the resting tone of aorta and α-adrenoceptors or voltage-dependent Ca^{2+} channels, we studied the actions of prazosin, phentolamine, yohimbine, nifedipine and papaverine on this increase in tension. In physiological solution, contractile responses to noradrenaline or KCl (408.8 ± 57.4 mg and 340.0 ± 26.8 mg respectively) were relaxed in a concentration-dependent manner by exposure to prazosin (10^{-13}–10^{-4} M), phentolamine (10^{-12}–10^{-4} M), yohimbine (10^{-11}–10^{-3} M), nifedipine (10^{-12}–10^{-6} M) or papaverine (10^{-8}–5×10^{-5} M).

Table 2 pIC_{50} values of compounds on the increase in the resting tone of aorta (IRT) and on KCl- and noradrenaline (NA)-induced contractions

		KCl	*NA*	*IRT*
Prazosin	pIC_{50}	4.42 ± 0.19	9.70 ± 0.09*	10.20
		$n = 4$	$n = 5$	
	E_{max} (%)	67.1 ± 7.7	106.4 ± 5.2	
Phentolamine	pIC_{50}	5.19 ± 0.05	7.01 ± 0.20*	8.53
		$n = 4$	$n = 7$	
	E_{max} (%)	83.3 ± 16.5	107.4 ± 4.8	
Yohimbine	pIC_{50}	4.26 ± 0.06	6.80 ± 0.09*	9.09
		$n = 4$	$n = 6$	
	E_{max} (%)	101.9 ± 2.1	108.0 ± 4.3	
Nifedipine	pIC_{50}	9.16 ± 0.10	8.07 ± 0.11*	10.23
		$n = 5$	$n = 5$	
	E_{max} (%)	98.1 ± 12.6	93.2 ± 8.7	
Papaverine	pIC_{50}	5.51 ± 0.10	5.29 ± 0.07	5.24
		$n = 8$	$n = 6$	
	E_{max} (%)	104.8 ± 8.5	111.5 ± 8.3	

All values represent mean ± s.e.mean, n = number of experiments, except the values of pIC_{50} (IRT), which are presented as mean.
*$P < 0.001$, significantly different from pIC_{50} (KCl).

IC_{50} values for each compound are summarized in Table 2.

The experimental procedure shown in Figure 2 was used in order to determine how the increase in tone was modified by preincubation (15 min) in the presence of different concentrations of prazosin (10^{-13}–10^{-8} M), phentolamine (10^{-11}–10^{-7} M), yohimbine (10^{-12}–10^{-7} M), nifedipine (10^{-13}–10^{-6} M) or papaverine (10^{-7}–10^{-4} M). All of these compounds concentration-dependently inhibited the contractile process. The calculated IC_{50} values for each compound to inhibit the increase in the resting tone are summarized in Table 2. A complete inhibition (100%) of the contractile response was obtained in the presence of 10^{-8} M prazosin, 10^{-7} M phentolamine or yohimbine, 10^{-6} M nifedipine or 10^{-4} M papaverine.

Influence of agents on the refilling of intracellular Ca stores sensitive to noradrenaline

After a 20 min incubation period in physiological solution in the presence of concentrations of phentolamine and yohimbine that completely inhibited the increase in the resting tone (10^{-7} M), the α-adrenoceptor antagonists were washed out and a recovery of the noradrenaline-induced contraction in Ca^{2+}-free medium (NA2; Figure 2) was observed: 21.8 ± 4.0% ($n = 6$) after phentolamine treatment and 19.9 ± 4.9% ($n = 5$) after yohimbine treatment, relative to noradrenaline-induced contractions in physiological solution. Therefore, this concentration of the α-adrenoceptor antagonists does not seem to modify the repletion of intracellular Ca^{2+} stores sensitive to noradrenaline. On the other hand, when the refilling of Ca^{2+} stores was performed in the presence of 10^{-8} M prazosin, 10^{-6} M nifedipine or 10^{-4} M papaverine, later addition of noradrenaline in Ca^{2+}-free medium (NA2) did not promote any contractile response.

Influence of inhibitors on the refilling of intracellular Ca-stores sensitive to caffeine or noradrenaline

In order to clarify the possible action of these compounds on the repletion of intracellular Ca^{2+} stores, another series of experiments were carried out at 25°C in which caffeine (10 mM), instead of noradrenaline, was added to Ca^{2+}-free medium after the refilling period in the presence of Ca^{2+} (Figure 3). The magnitude of the caffeine-induced contraction (CAF1) in Ca^{2+}-free medium was 21.0 ± 3.1%, $n = 3$; relative to the noradrenaline-induced contraction in Ca^{2+}-containing medium. After washing in Ca^{2+}-free solution, addition of noradrenaline (NA2) elicited a small contractile response (6.5 ± 0.6%, $n = 3$; relative to the contraction induced by noradrenaline in physiological solution; Table 3). This response is due to the release of Ca^{2+} from a store sensitive only to noradrenaline (Noguera & D'Ocon, 1992). After 20 min exposure to Ca^{2+}-containing solution, complete recovery of the noradrenaline-induced contraction (NA3) was obtained.

Table 3 Contractile responses to caffeine (Caf1) and noradrenaline (NA2) in Ca^{2+}-free medium after an incubation period in the presence of testing agents in Ca^{2+}-containing solution (see Figure 2) and recovery of noradrenaline-elicited response in Ca^{2+}-containing solution (NA3)

Agent	(M)	n	*Caf1*	*NA2*	*NA3*
–	–	3	21.0 ± 3.1	6.5 ± 0.6	114.0 ± 5.0
Prazosin	10^{-8}	4	17.2 ± 4.2	–	92.6 ± 12.7
Phentolamine	10^{-7}	5	22.7 ± 3.7	10.1 ± 1.9	96.3 ± 15.4
Yohimbine	10^{-7}	5	19.6 ± 2.8	8.0 ± 0.8	81.5 ± 9.3
Nifedipine	10^{-6}	3	19.3 ± 4.1	–	38.4 ± 7.2 *
Papaverine	10^{-4}	3	–	–	7.3 ± 0.2*

Contractions are expressed as a percentage of the noradrenaline-induced contraction in physiological solution. All values represent mean ± s.e.mean, n = number of experiments.
*$P < 0.001$, significantly different from control.
Caf1: Addition of caffeine after 20 min in the presence of Ca^{2+} and 15 min in Ca^{2+}-free medium.
NA2: Addition of noradrenaline in Ca^{2+}-free medium after removing caffeine from the organ bath.
NA3: Addition of noradrenaline in physiological solution after 20 min of exposure to Ca^{2+}-containing solution.

Using the same experimental procedure, except that either phentolamine or yohimbine (10^{-7} M) were added during the refilling of the stores in the presence of Ca^{2+}, washout of the α-adrenoceptor antagonists in Ca^{2+}-free medium and addition of caffeine elicited a phasic contraction (CAF1). Subsequent addition of noradrenaline in Ca^{2+}-free solution produced a small tonic contraction (NA2) that was not modified by previous exposure of tissues to phentolamine or yohimbine (Table 3). After a further incubation period of 20 min in physiological solution the size of the contractile response to noradrenaline was restored (NA3).

When the stores were refilled in the presence of either 10^{-8} M prazosin or 10^{-6} M nifedipine, addition of caffeine in Ca^{2+}-free medium (Caf1) yielded a contractile response similar in magnitude to that obtained in the absence of the antagonists (Table 3). Later addition of noradrenaline (NA2) in Ca^{2+}-free medium did not induce any response. After 20 min in the presence of Ca^{2+}, the response to noradrenaline (NA3) after tissue exposure to nifedipine was significantly smaller than that obtained in the absence of nifedipine, but recovery of the response to noradrenaline was complete after prazosin treatment (Table 3).

When 10^{-4} M papaverine was present during the repletion of the internal Ca^{2+} stores neither caffeine nor noradrenaline elicited any contractile response when they were added in Ca^{2+}-free medium in the absence of papaverine (CAF1, NA2; Table 3). Furthermore, noradrenaline induced a significantly smaller contraction ($P<0.001$) when applied after 20 min exposure to Ca^{2+}-containing solution (NA3; Table 3).

Discussion

The results show that in rat aorta, noradrenaline-, 5-hydroxytryptamine- or caffeine-induced contractions in Ca^{2+}-free medium are associated with the emptying of intracellular Ca^{2+} stores of limited capacity and that further addition of the agonists evokes no mechanical response. These intracellular Ca^{2+} pools, when emptied, can be rapidly replenished from the extracellular space by incubation in Ca^{2+}-containing solution. The rapid refilling of the pools with extracellular Ca^{2+} occurs in the absence of the contractile agonist. It might be assumed that any means of depleting the intracellular Ca^{2+} pool, even in the absence of receptor activation or increases in inositol polyphosphates, activates the same Ca^{2+} entry mechanism normally activated by agonists (Putney, 1986; 1990; Rowena *et al.*, 1992).

A question arises from these results about the pathway for Ca^{2+} entry because there are at least two possible routes. Ca^{2+} may enter the cytoplasm directly on its way to the internal compartments sensitive to the agonists (Putney, 1990; Rowena *et al.*, 1992) or Ca^{2+} entry may involve direct replenishment of the intracellular pool in the absence of the agonist and its continuous discharge to the cytoplasm when the agonist is present (Itoh *et al.*, 1981; 1985; Hisayama & Takayanagi, 1988; Low *et al.*, 1991).

The observation that exposure of the tissues to Ca^{2+} after emptying of intracellular Ca^{2+} stores by exposure to caffeine or 5-hydroxytryptamine did not result in a contractile response suggests that the pathway for refilling could be a relatively direct one that does not involve transfer of Ca^{2+} through the cytosol to the intracellular storage sites. However, after depletion of noradrenaline-sensitive Ca^{2+} stores, there was an increase in the resting tone of aorta on re-exposure to Ca^{2+}. This Ca^{2+}-dependent contraction has been described in previous studies carried out in rabbit aorta (Deth & Lynch, 1981), although no explanation for the observation was advanced.

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. This increase in tone takes place in the absence of the agonist, indicating that it is a consequence of the previous activation of adrenoceptors. Removal of the endothelium rules out possible involvement of endothelium-derived factors in this mechanical response.

When a contractile response to noradrenaline was elicited in the presence of Ca^{2+} and the tissue was washed in Ca^{2+}-free medium and then in a Ca^{2+}-containing solution, without attempting to deplete intracellular Ca^{2+} stores in the absence of Ca^{2+}, there was no increase in the resting tone of the tissue in Ca-containing solution (Figure 1b), suggesting that depletion of intracellular Ca^{2+} pools was required for an increased permeability of the plasma membrane permitting Ca^{2+} entry. This could explain the fact that noradrenaline-induced contractions in Ca^{2+}-containing solution after depletion of intracellular stores were of a significantly greater magnitude than the standard response (Figure 1c). On this basis, it can be assumed that the depletion of noradrenaline-sensitive Ca^{2+} stores are the signal for the entry of extracellular Ca^{2+} not only to refill organelles, but also to activate contractile proteins. 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.

According to Sato *et al.* (1988), noradrenaline has the ability to augment the efficacy of contraction (i.e. the response to a given concentration of intracellular Ca^{2+}) in vascular smooth muscle, and this may be responsible for the increase in the resting tone if it continues when the agonist is removed from the incubating medium.

We assessed the influence of three α-adrenoceptor antagonists (prazosin, phentolamine and yohimbine) on the magnitude of this increase in the resting tone. Treatment with α-adrenoceptor antagonists induced a concentration-dependent inhibition of the response and it was completely abolished in the presence of the highest concentrations tested. This seems to indicate that α-adrenoceptors are involved in the increase in resting tone. An explanation for this effect in the absence of an α-adrenoceptor agonist may be that mobilization of an intracellular Ca^{2+} pool sensitive to noradrenaline could determine the fixing of the α-adrenoceptor in an activated state until the intracellular Ca^{2+} pool is refilled and that these antagonists bind to the α-adrenoceptor in the absence of Ca^{2+} and fix it in an inactivated state, in a manner similar to that of Ca^{2+}-channel blockers on the voltage-dependent Ca^{2+} channels (Trautwein & Pelzer, 1985; Godfraind *et al.*, 1986). 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 conformation change unfavourable to the coupling of the receptor to its G protein.

The inhibition of this increase in tension in a concentration-dependent manner by nifedipine would seem to relate this mechanical response to Ca^{2+}-entry via voltage-operated Ca^{2+} channels. The great sensitivity of the increase in the resting tone to nifedipine suggests that a change in membrane potential might be involved.

According to previous work (Noguera & D'Ocon, 1992; Low *et al.*, 1991; present results) noradrenaline induces a biphasic contractile response in Ca^{2+}-free medium. The present study provides the novel observation that this biphasic response is mediated by two different intracellular Ca^{2+} pools with different refilling processes: compartment 1 is sensitive to noradrenaline and caffeine, nifedipine and prazosin do not modify its refilling process; compartment 2 is sensitive only to noradrenaline; nifedipine and prazosin completely inhibit its repletion. Papaverine blocks the repletion of both compartments and the presence of phentolamine or yohimbine affects neither.

In view of the above findings, we propose a model for Ca^{2+} entry into intracellular stores sensitive to noradrenaline (one of them common to caffeine). The first component of the noradrenaline-induced contraction in Ca^{2+}-free medium indicates Ca^{2+} release from the endoplasmic reticulum (com-

partment 1) (Low *et al.*, 1991). The second component of the biphasic response to noradrenaline in the absence of extracellular Ca^{2+} might be represented by compartment 2. This Ca^{2+} storage compartment lies close to, and may be directly connected to, the plasma membrane (Devine *et al.*, 1972; Van Breemen & Saida, 1989; Noguera & D'Ocon, 1992). This second component is susceptible to modulation by nifedipine and prazosin and includes the passage of Ca^{2+} through L-type Ca^{2+}-channels for the refilling of the internal Ca^{2+} pool. The gate at this passage may be voltage-operated, although the α_{1a}-adrenoceptor subtype is directly related to it (Minneman, 1988). There is some evidence (Saida & Van Breemen, 1983; Leijten *et al.*, 1985; Wakui *et al.*, 1990) that the intracellular Ca^{2+} pool specific to noradrenaline can be released by receptor activation and the release of this small pool might trigger Ca^{2+} release from the internal store common to noradrenaline and caffeine. Thus, the fact that prazosin and nifedipine block the uptake of Ca^{2+} into the small pool specific to noradrenaline (compartment 2) explains the lack of response to noradrenaline in Ca^{2+}-free medium: the first component of the biphasic response is abolished because the release of the internal Ca^{2+} pool common to noradrenaline and caffeine (compartment 1) depends on the previous release of Ca^{2+} from the pool specific to noradrenaline (compartment 2) when this agonist is employed. However, complete recovery of the contractile response induced by caffeine in Ca^{2+}-free medium is observed, indicating that the refilling and release of compartment 1 may, in fact, be independent of the presence of the antagonists.

Phentolamine and yohimbine did not block these Ca^{2+} refill pathways but the fact that the increase in the resting tone related to α-adrenoceptors activation was abolished may suggest that the entry of Ca^{2+} in the presence of these antagonists is not able to activate the contractile proteins because of the blockade of the process of sensitization mediated by α-adrenoceptors.

In summary, the present study shows that incubation in Ca^{2+}-containing solution after depletion of noradrenaline-sensitive intracellular Ca^{2+} stores increases the resting tone of rat aorta. This increase in tension was inhibited by α-adrenoceptor antagonists such as prazosin, phentolamine and yohimbine, and by Ca^{2+} channel blockers such as nifedipine. Papaverine also inhibited the increase. During this contractile process, the intracellular Ca^{2+} stores sensitive to noradrenaline refill, but the repletion of these internal pools is differentially modulated by the agents tested.

References

BENHAM, C.D. & TSIEN, R.W. (1988). Noradrenaline modulations of calcium channels in single smooth muscle cells from rabbit ear artery. *J. Physiol.*, **404**, 767–784.

BOLTON, T.B. (1979). Mechanisms of action of transmitters and other substances on smooth muscle. *Physiol. Rev.*, **59**, 606–718.

BRAY, K.M., WESTON, A.H., DUTY, S., NEWGREEN, D.T., LONGMORE, J., EDWARDS, G. & BROWN, T.J. (1991). Differences between the effects of cromakalim and nifedipine on agonist-induced responses in rabbit aorta. *Br. J. Pharmacol.*, **102**, 337–344.

DETH, R.C. & LYNCH, C.J. (1981). Relative contributions of Ca influx and cellular Ca release during drug-induced activation of the rabbit aorta. *Am. J. Physiol.*, **240**, C239–C247.

DEVINE, C.E., SOMLYO, A.V., SOMLYO, A.P. (1972). Sarcoplasmic reticulum and excitation-contraction coupling in mammalian smooth muscle. *J. Cell. Biol.*, **52**, 690–718.

FUKUMITSU, T., HAYASHI, H., TOKUNO, H. & TOMITA, T. (1990). Increase in calcium channel current by β-adrenoceptor agonists in single smooth muscle cells isolated from porcine coronary artery. *Br. J. Pharmacol.*, **100**, 593–599.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GODFRAIND, T., MILLER, R. & WIBO, M. (1986). Ca antagonism and Ca entry blockade. *Am. Soc. Pharmacol. Exp. Ther.*, **38**, 312–415.

HISAYAMA, T. & TAKAYANAGI, I. (1988). Ryanodine: its possible mechanism of action in the caffeine-sensitive Ca-store of smooth muscle. *Pflügers Arch.*, **412**, 376–381.

IINO, M., KOBAYASHI, T. & ENDO, M. (1988). Use of ryanodine for functional removal of the calcium store in smooth muscle cells of the guinea-pig. *Biochem. Biophys. Res. Commun.*, **152**, 417–422.

IMAIZUMI, Y., TAKEDA, M., MURAKI, K. & WATANABE, M. (1991). Mechanisms of NE-induced reduction of Ca current in single smooth muscle cells from guinea-pig vas deferens. *Am. J. Physiol.*, **260**, C17–C25.

ITOH, T., KURIYAMA, H. & SUZUKI, H. (1981). Excitation-contraction coupling in smooth muscle cells of the guinea-pig mesenteric artery. *J. Physiol.*, **321**, 513.

ITOH, T., UENO, H. & KURIYAMA, H. (1985). Calcium-induced calcium release mechanism in vascular smooth muscles-assessments based on contractions evoked in intact and saponin-treated skinned muscles. *Experientia*, **41**, 989.

LEIJTEN, P., CAUVIN, C., LODGE, N., SAIDA, K. & VAN BREEMEN, C. (1985). Ca^{2+} sources mobilized by α1-receptor activation in vascular smooth muscle. *Clin. Sci.*, **68**, 47–50.

LOW, A.M., GASPAR, V., KWAN, C.Y., DARBY, P.J., BOURREAU, J.P. & DANIEL, E.E. (1991). Thapsigargin inhibits repletion of phenylephrine-sensitive intracellular Ca pool in vascular smooth muscles. *J. Pharmacol. Exp. Ther.*, **258**, 1105–1113.

LUGNIER, C., SHOEFFTER, P., LE BEC, A., STROUTHON, E. & STOCLET, J.C. (1986). Selective inhibition of cyclic nucleotide phosphodiesterase on human bovine and rat aorta. *Biochem. Pharmacol.*, **35**, 1743–1751.

MINNEMAN, K.P. (1988). α1-adrenergic receptor subtypes, inositol phosphates, and sources of cell calcium. *Pharmacol. Rev.*, **40**, 87–119.

MOREL, N. & GODFRAIND, T. (1991). Characterization in rat aorta of the binding sites responsible for blockade of noradrenaline-evoked calcium entry by nisoldipine. *Br. J. Pharmacol.*, **102**, 467–477.

NISHIMURA, K., OTA, M. & ITO, K. (1991). Existence of two components in the tonic contraction of rat aorta mediated by α_1-adrenoceptor activation. *Br. J. Pharmacol.*, **102**, 215–221.

NOGUERA, M.A. & D'OCON, M.P. (1992). Different and common intracellular calcium-stores mobilized by noradrenaline and caffeine in vascular smooth muscle. *Naunyn Schmied Arch. Pharmacol.*, **345**, 333–341.

PUTNEY, J.W. (1986). A model for receptor-regulated calcium entry. *Cell Calcium*, **7**, 1–12.

PUTNEY, J.W. (1990). Capacitative calcium revisited. *Cell Calcium*, **11**, 611–634.

ROWENA, J.D., HURWITZ, L.M., MIRZA, Z., STRAUSS, H.C. & WHORTON, A.R. (1992). Regulation of extracellular calcium entry in endothelial cells: role of intracellular Ca pool. *Am. J. Physiol.*, **262**, C171–C181.

SAIDA, K. & VAN BREEMEN, C. (1983). A possible Ca^{2+}-induced Ca^{2+}-release mechanism mediated by norepinephrine in vascular smooth muscle. *Pflügers Arch.*, **397**, 166–167.

SATO, K., OZAKI, H. & KARAKI, H. (1988). Changes in cytosolic calcium levels in vascular smooth muscle strip measured simultaneously with contraction using fluorescent calcium indicator fura 2^1. *J. Pharmacol. Exp. Ther.*, **246**, 294–300.

SCHUTZ, W. & FREISSMUTH, M. (1992). Reverse intrinsic activity of antagonists on G protein-coupled receptors. *Trends Pharmacol. Sci.*, **13**, 376–380.

TRAUTWEIN, W. & PELZER, D. (1985). Voltage-dependent gating of single calcium channels in the cardiac cell membrane and its modulation by drugs. In *Calcium and Cell Physiology*. ed. Marmé, D. pp. 53–93. Berlin Heildelberg: Springer-Verlag.

VAN BREEMEN, C. & SAIDA, K. (1989). Cellular mechanisms regulating Cai smooth muscle. *Annu. Rev. Physiol.*, **51**, 315–329.

WAKUI, M., OSIPCHUK, Y.V. & PETERSEN, O.H. (1990). Receptor-activated cytoplasmic Ca spiking mediated by inositol trisphosphate is due to Ca-induced Ca release. *Cell*, **63**, 1025–1032.

YATANI, A., SEIDEL, C.L., ALLEN, J. & BROWN, A.M. (1987). Whole cell and single-channel calcium currents of isolated smooth muscle cells from saphenous vein. *Circ. Res.*, **60**, 523–533.

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