Effects of Different Agents on the Contractile Response Elicited by Extracellular Calcium after Depletion of Internal Calcium Stores in Rat Isolated Aorta

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Abstract—Noradrenaline, 1 μM, induced a sustained contractile response in rat isolated aorta in the presence and in the absence of extracellular Ca^{2+}. After depleting the noradrenaline-sensitive intracellular Ca^{2+} stores, an increase in the basal tone of the aorta was observed during the incubation period in the presence of Ca^{2+} and in the absence of the agonist. We have tested the possible pathways through which Ca^{2+} enters the cell to refill the previously depleted Ca^{2+} pools, a process that is accompanied by an increase in tension. The magnitude of this increase does not depend on the presence of Mg^{2+} in the extracellular medium nor on the temperature, suggesting that it is mediated by an event that does not depend on intracellular energy or Ca^{2+}, Mg^{2+}-ATPase. It is inhibited in a concentration-dependent manner by an unspecific relaxing compound, caffeine, and an organic Ca^{2+} entry blocker, verapamil, but not by an inorganic Ca^{2+} entry blocker, lanthanum. Caffeine (10 mM) and verapamil (10^{-5} M) completely inhibited the increase in the resting tone, but only verapamil abolished the refilling of the noradrenaline-sensitive Ca^{2+} pools, indicating that the extracellular Ca^{2+} enters the cell through voltage-operated Ca^{2+} channels. Caffeine inhibited the increase in the resting tone without blocking the refilling process of the stores at 37°C, but at 25°C a partial inhibition of the repletion of internal Ca^{2+} pools was observed. These results confirm previous work that showed a temperature-dependent activity of caffeine.

Noradrenaline induces contractions in vascular smooth muscle by mobilizing Ca^{2+} from intracellular stores and by opening Ca^{2+} channels in the plasma membrane, thus permitting a sustained influx of extracellular Ca^{2+} (Chiu et al 1986; Minnemann 1988; Koch et al 1990; Nishimura et al 1991). Recent evidence suggests the existence of two independent intracellular Ca^{2+} stores sensitive to noradrenaline in rat aorta (Noguera & D'Ocon 1992) which are responsible for noradrenaline-induced contraction in Ca^{2+}-free medium (Koch et al 1990). The noradrenaline-induced Ca^{2+}-release by these intracellular pools could be a regulatory process which induces the opening of membrane Ca^{2+} channels and permits the entry of the extracellular Ca^{2+} (Von Tscharner et al 1986; Putney 1990). Depletion of intracellular Ca^{2+} stores by an agonist results in an increased permeability of the plasma membrane that does not depend on the continued presence of the agonist (Putney 1990) and remains activated as long as the intracellular Ca^{2+} pools are not permitted to refill (Takemura & Putney 1989; Shattleworth 1990).

Our study indicates that after depleting noradrenaline-releasable pools in Ca^{2+}-free medium, a loading time in Ca^{2+}-containing solution, but in the absence of the agonist, induces a considerable increase in the resting tone of aorta. Similar findings were reported by Deth & Lynch (1981) in rabbit aorta, but no explanation was given by those authors.

The present study analyses the mechanisms involved in the genesis of this increase in resting tone by studying different temperatures, the presence or the absence of Mg^{2+}, and the addition of lanthanum (La^{3+}), caffeine and verapamil during the repletion of internal Ca^{2+}-stores.

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Materials and 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 Krebs bicarbonate solution, maintained at 37°C and gassed with 95% O_2–5% 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 previously contracted with noradrenaline (1 μM) indicated the absence of a functional endothelium (Furchgott & Zawadzki 1980).

Drugs and solutions

The following drugs were obtained from Sigma (St Louis, MO): anhydrous caffeine, verapamil hydrochloride and acetylcholine; L-noradrenaline L-tartrate from Merck (Darmstadt, Germany) and lanthanum chloride from Aldrich-Chemie, Steinheim, Germany. 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 Krebs solution (prepared by omission of $CaCl_2$ and the addition of 0·1 mM EDTA). Krebs solution was of the

following composition (mM): NaCl 118, KCl 4·75, CaCl$_2$ 1·8, MgCl$_2$ 1·2, KH$_2$PO$_4$ 1·2, NaHCO$_3$ 25, and glucose 11.

Ca^{2+}, Mg^{2+}-free solution had the same composition except that CaCl$_2$ and MgCl$_2$ were omitted and EDTA (0·1 mM) was added.

Experimental procedures

Relaxation dose-response curves to caffeine, verapamil and La^{3+} were obtained by addition of cumulative concentrations of the agents to tissues in which sustained contractions were induced by 1 μM noradrenaline or by exposure to 80 mM KCl-containing solution (prepared by equimolar substitution of KCl for NaCl in the Krebs solution). Relaxations were expressed as a percentage of the maximum increment in tension obtained by agonist addition. E$_{max}$ represents the maximal relaxation (100%) obtained after addition of the highest concentration of each compound tested. A regression of response against −log C of tested compounds was performed by the least squares method for each preparation. The concentration needed to produce 50% inhibition (IC50) was obtained from the linear regression plot of all points between 20 and 80% of the maximal response.

A separate series of experiments assessed the effects of caffeine, verapamil and La^{3+} on the increase in tension (Fig. 2a). The aorta was treated with different concentrations of these compounds 15 min before the increase in resting tone of aorta was induced. The magnitude of this increase in the presence of different concentrations of the agents is expressed as a percentage of the reference increase in resting tone, and to determine the IC50 values for each compound, a linear regression plot of all points obtained was performed. Each point was the mean of 4–7 experiments.

Analysis of results

Contractions in Krebs 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 Krebs solution.

Results are presented as a mean ± s.e.m. for n determinations obtained from different animals. Statistical significance was evaluated by Student's *t*-test for unpaired data. Differences were considered significant when $P < 0.05$.

Results

Influence of Mg^{2+} and temperature on the increase in resting tone of aorta after depletion of intracellular Ca^{2+}

Addition of noradrenaline 1 μM to rat aortic strips in Ca^{2+}-containing solution generated a sustained maximal contractile response with a magnitude of 406·6 ± 51·4 mg (n = 10). After 15 min in Ca^{2+}-free solution (Fig. 1a), addition of noradrenaline induced a contraction with two components: a phasic one followed by a smaller tonic one, both of them due to the release of intracellular Ca^{2+}. The magnitude of these
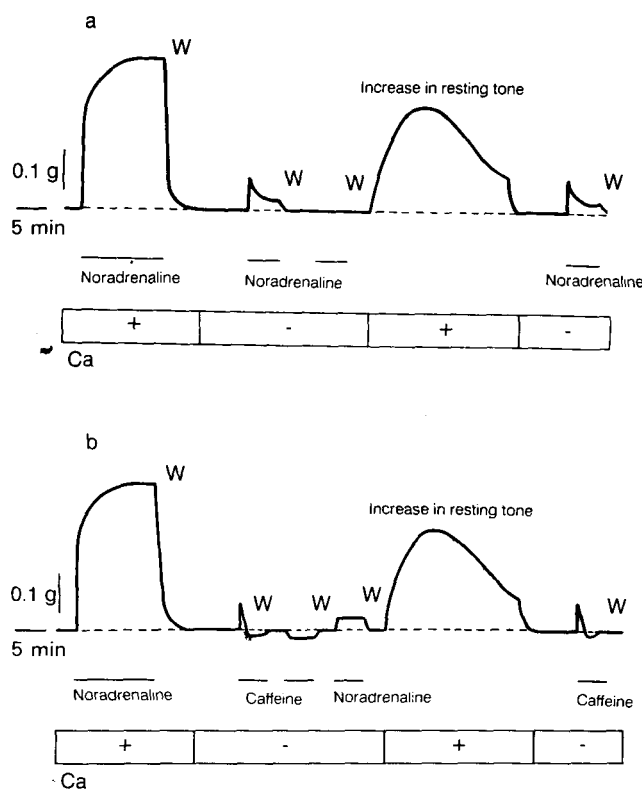

Fig. 1. a. Representative trace recordings of the increase in the resting tone of aorta obtained after depletion of noradrenaline-sensitive intracellular Ca^{2+} stores. Preincubation time in Ca^{2+}-free medium = 15 min. b. Experimental procedure used to analyse the increase in the resting tone observed after noradrenaline- and caffeine-induced depletion of intracellular Ca^{2+} stores. Preincubation time in Ca^{2+}-free medium = 15 min. W = washing. This experimental procedure was also performed but adding caffeine and noradrenaline in reversed order in Ca^{2+}-free medium.

Table 1. Increase in the resting tone of rat aortic segments elicited by noradrenaline 1 μM in the presence or the absence of Mg^{2+}, and at 25°C.

n	Temperature (°C)	Mg^{2+} (mM)	Krebs (Contraction (mg))	Ca^{2+}-free (NA_1 (%)) Phasic	Ca^{2+}-free (NA_1 (%)) Tonic	Krebs (Increase in resting tone (%))	Ca^{2+}-free (NA_2 (%)) Phasic	Ca^{2+}-free (NA_2 (%)) Tonic
10	37	1·2	406·6±51·4	23·2±1·9	15·3±1·7	71·7±5·8	22·5±2·0	12·9±1·6
6	37	0	360·9±37·1	26·0±0·6	12·3±0·9	76·0±5·7	11·8±1·2*	5·7±0·7*
6	25	1·2	410·3±34·8	26·6±2·7	13·3±1·7	76·2±11·0	30·3±2·9	10·8±2·0

Increases in tone and contractions in Ca^{2+}-free medium are expressed as a percentage of the noradrenaline-induced contraction in Krebs solution. All values represent mean±s.e.m., n=number of experiments. * $P<0·001$, significantly different from NA_1. NA_1, first addition of noradrenaline, NA_2 second addition of noradrenaline.

contractile components in Ca^{2+}-free medium is shown in Table 1, relative to the contraction induced by noradrenaline in the presence of 1·8 mM Ca^{2+}. After washing, no contraction was evoked by the second application of noradrenaline in Ca^{2+}-free solution (Fig. 1a), which indicates a complete depletion of the noradrenaline-sensitive intracellular Ca^{2+} pools. Furthermore, when the tissue was loaded for 20 min in Krebs solution, an increase in the resting tone of aorta was observed during this incubation period. The magnitude of this increase was 71·7±5·8% (n=10) relative to the noradrenaline-induced contraction in Krebs solution.

When noradrenaline was added again after an incubation period of 15 min in Ca^{2+}-free solution, the magnitude of the contraction was similar to that of the first one in Ca^{2+}-free medium (Table 1). This indicates that loading with 1·8 mM Ca^{2+} for 20 min refilled the Ca^{2+} storage sites.

Experiments performed in the absence of Mg^{2+} to test the effects of this cation on the increase in tone indicate that the muscle tension generated by adding noradrenaline in Ca^{2+}, Mg^{2+}-free solution is slightly higher (Table 1) than that obtained in the presence of Mg^{2+}. Moreover, an increase in the resting tone of similar magnitude to that induced in the presence of Mg^{2+} was obtained during the incubation in Mg^{2+}-free Krebs. Our previous results (Noguera & D'Ocon 1992) have also shown that the Ca^{2+}-storage sites sensitive to noradrenaline cannot be completely refilled in the absence of Mg^{2+} (Table 1).

Experiments performed at 25°C to test the effects of temperature on the generation of increases in tension indicate that it is similar at 37 and 25°C (Table 1).

The same experimental procedure was applied at 25°C using caffeine (10 mM) instead of noradrenaline, but no increase in the resting tone was observed during the refilling of the Ca^{2+} stores in the presence of Ca^{2+} after depletion of caffeine-sensitive intracellular Ca^{2+}-pools (unpublished results).

Increase in the resting tone of aorta after depletion of intracellular Ca^{2+} induced by noradrenaline and caffeine independently

Experiments were performed to investigate increases in the resting tone of aorta obtained when the two intracellular Ca^{2+} stores sensitive to noradrenaline, one of them common to caffeine (Noguera & D'Ocon 1992), were depleted independently. For this purpose, the two agonists were added in reversed order in Ca^{2+}-free medium (Fig. 1b). Caffeine did not induce any contractile response after noradrenaline

pretreatment (n=6). In contrast, a small contraction evoked by noradrenaline was observed after depletion of caffeine-sensitive Ca^{2+}-stores (10·0±1·1%, n=6; relative to the noradrenaline-induced contraction in Krebs solution; Fig. 1b). This response is due to the release of Ca^{2+} from the store sensitive only to noradrenaline (Noguera & D'Ocon 1992). During the repletion of the internal noradrenaline- and caffeine-sensitive Ca^{2+} stores in the presence of Ca^{2+}, an increase in the resting tone of aorta was observed. The magnitude of this increase was similar to that obtained when only noradrenaline was added (58·4±4·9%, n=12; relative to the noradrenaline-induced contraction in Krebs solution). After 15 min incubation in Ca^{2+}-free medium the noradrenaline- or caffeine-induced contraction was similar in magnitude to that obtained by the first addition of the agonist in Ca^{2+}-free medium (Fig. 1b). This indicated complete repletion of the internal Ca^{2+}-storage sites.

Modification of the increase in the resting tone of aorta by preincubation with different concentrations of caffeine, verapamil and La^{3+}

In Krebs solution, contractile responses to noradrenaline (405·2±44·4 mg) and KCl (225·0±15·0 mg) were relaxed concentration-dependently by caffeine (5 × 10^{-5}–10^{-2} M) and verapamil (10^{-10}–10^{-4} M). The IC50 values for each compound are summarized in Table 2. $LaCl_3$ (2 mM) failed to relax contractions induced by noradrenaline and promoted only a partial inhibition (19·5±2·1%, n=4) of the contractile response generated by KCl. Higher concentrations of $LaCl_3$ could not be tested because it precipitates in Krebs solution.

An experimental procedure (Fig. 2a) was performed to determine the modification of the increase in resting tone produced by preincubation (15 min) with different concentrations of caffeine (10^{-5}–10^{-2} M), verapamil (5 × 10^{-7}–10^{-5} M) and La^{3+} (2 mM). Caffeine and verapamil elicited a concentration-dependent inhibition of the contractile process. The number of experiments for each concentration of the test agents was 4–7. Conversely, $LaCl_3$ (2 mM) did not modify the magnitude of the increase in tension (n=6). The calculated IC50 values for caffeine and verapamil relative to the inhibition of the increase in the resting tone are summarized in Table 2.

Influence of caffeine and verapamil on the refilling of the intracellular Ca^{2+} stores

A series of exeriments was carried out, in which the refilling

Table 2. —Log IC50 values of the test compounds on the increase in the resting tone of aorta and on KCl- and noradrenaline-induced contractions.

	−Log IC50 (M)		
	Noradrenaline	KCl	Increase in resting tone
Verapamil	6·45 ± 13* (n = 6)	7·31 ± 0·10 (n = 4)	5·62[a]
Caffeine	3·43 ± 0·11* (n = 7)	2·76 ± 0·15 (n = 4)	3·22[a]

Values represent mean ± s.e.m., n = number of experiments, except the values of −log IC50 for the increase in resting tone, which are presented as mean values. *$P < 0.01$; significantly different from IC50$_{KCl}$, [a] mean of duplicates. *$P < 0.01$ compared with the corresponding value for KCl.

of the internal Ca^{2+}-stores was performed in the presence of Ca^{2+} and in the presence of the highest concentration of caffeine (10 mM) and verapamil (10^{-5} M) that completely inhibited the increase in resting tone. Afterwards, the testing agents were washed out, and after 15 min of incubation in Ca^{2+}-free medium, noradrenaline, 1 μM (Fig. 2a) or caffeine, 10 mM (Fig. 2b) was added to the organ bath.

When caffeine (10 mM) was added during the repletion of the internal Ca^{2+}-stores, a recovery of noradrenaline-induced contractile response in Ca^{2+}-free medium (Fig. 2a) was observed (Table 3). Thus, caffeine does not modify the refilling of intracellular Ca^{2+}-stores, although it completely inhibits the increase in the resting tone of aorta.

Conversely, in tissues treated with verapamil (10^{-5} M) during the repletion of the internal Ca^{2+}-stores, noradrena-

line (Fig. 2a; Table 3) promoted no contractile response when added subsequently in Ca^{2+}-free solution.

In order to clarify the possible action of caffeine on the refilling of intracellular Ca^{2+} storage sites and in view of the fact that caffeine modifies its activity as the temperature changes (Sato et al 1988; Noguera & D'Ocon 1992), we carried out similar experiments at 25°C. During the refilling period in the presence of caffeine (10 mM) and Ca^{2+} (1·8 mM), no increase in the resting tone was observed (n = 5); the results obtained, therefore, were the same as at 37°C. However, later addition of noradrenaline after 15 min of loading in Ca-free medium gave different results from those obtained at 37°C. Noradrenaline showed a significantly lower response (Table 3) compared with that generated by the first addition of the agonist in Ca^{2+}-free medium and
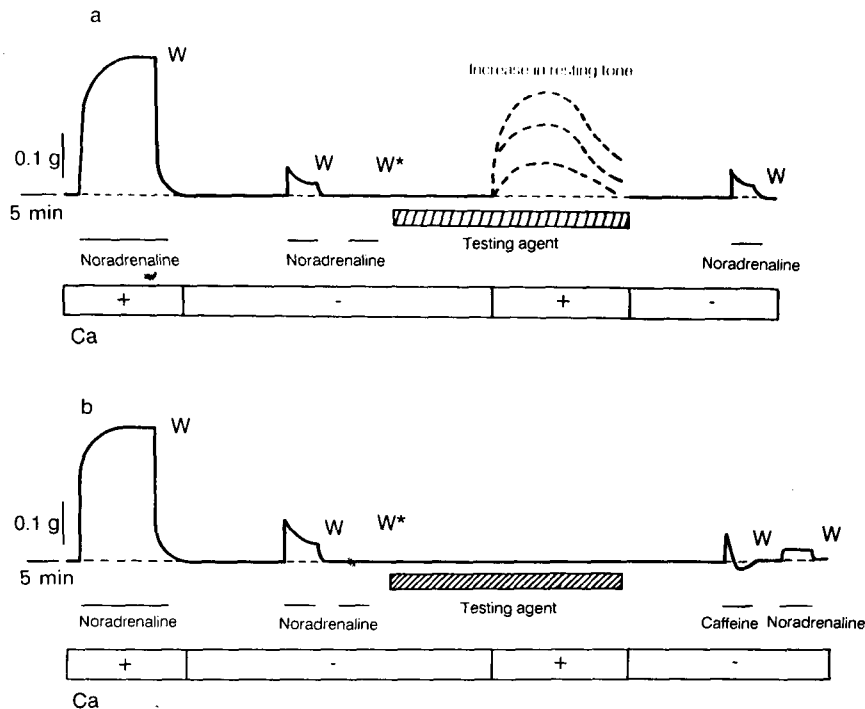

FIG. 2. a. Experimental procedure used to analyse the modification of the increase in the resting tone of aorta obtained in the presence of different concentrations of the testing agents. Preincubation time in Ca^{2+}-free medium = 15 min. W = washing, W* = washing with Ca^{2+}-EDTA-free solution. b. Experimental procedure used to analyse the refilling of internal Ca^{2+}-storage sites in the presence of the testing agents 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+} EDTA-free solution.

Table 3. Contracile responses to noradrenaline in Ca^{2+}-free medium in tissues treated with the test agents during the repletion of Ca^{2+} stores (see Fig. 2a).

Agent	Temperature (°C)	Concn (M)	n	First addition[a]	Second addition[b]
Caffeine	37	10^{-2}	4	24.4 ± 2.8	22.1 ± 3.9
Caffeine	25	10^{-2}	5	29.5 ± 3.1	$12.3 \pm 1.2*$
Verapamil	37	10^{-5}	3	21.4 ± 2.4	—

Contractions are expressed as a percentage of the noradrenaline-induced contraction in Krebs solution. All values represent mean ± s.e.m., n = number of experiments. $*P < 0.001$, significantly different from the first addition of noradrenaline. [a] Addition of noradrenaline after 15 min in Ca^{2+}-free medium. [b] Further addition of noradrenaline in Ca^{2+}-free medium after an incubation period (20 min) in the presence of Ca^{2+}.

Table 4. Contractile responses to caffeine and noradrenaline in Ca^{2+}-free medium in tissues treated with the test agents during the repletion of Ca^{2+}-stores (see Fig. 2b).

Agent	M	n	Noradrenaline[a]	Caffeine[b]	Noradrenaline[c]
Control		4	31.5 ± 1.6	18.3 ± 3.5	8.3 ± 0.8
Caffeine	10^{-2}	8	29.5 ± 3.1	$6.9 \pm 1.0**$	$4.6 \pm 0.8*$ (n = 3) — (n = 5)
Verapamil	10^{-5}	3	29.6 ± 3.2	—	—

Contractions are expressed as a percentage of the noradrenaline-induced contraction in Krebs solution. All values represent mean ± s.e.m. n = number of experiments. $*P < 0.05$, $**P < 0.01$ compared with control. [a] Addition of noradrenaline after 15 min in Ca^{2+}-free medium. [b] Addition of caffeine after 20 min in the presence of Ca^{2+} and 15 min in Ca^{2+}-free medium. [c] Further addition of noradrenaline in Ca^{2+}-free medium.

subsequent application of caffeine did not evoke a contractile response (n = 5).

In another series of experiments caffeine (10 mM), instead of noradrenaline, was added after the refilling period in Ca^{2+}-containing solution and after 15 min of incubation in the absence of Ca^{2+} (Fig. 2b), and a transient contraction was obtained (Table 4). Later application of noradrenaline promoted a small contractile response (control experiments). Conversely, in tissues in which the repletion of the Ca^{2+} stores was performed in the presence of caffeine, later addition of this agonist in Ca^{2+}-free medium yielded a significantly smaller contraction (Table 4) compared with the control value. Subsequent addition of noradrenaline induced a significantly smaller contraction in some of the experiments (n = 3; Table 4), while in others no contraction was observed (n = 5).

In tissues treated with verapamil during the repletion of internal Ca^{2+} stores at 25°C, caffeine and noradrenaline did not elicit any contractile response when they were added in Ca^{2+}-free medium after washing out the testing agent (n = 3; Table 4).

Discussion

Noradrenaline-induced contraction in Ca^{2+}-free medium is mediated by Ca^{2+} release from two different intracellular pools, one of which is common to caffeine, and the other one sensitive only to noradrenaline. This contraction involves the emptying of the intracellular stores sensitive to the agonist. Later loading in Ca^{2+}-containing solution refills these stores when Mg^{2+} is present in the medium, which suggests the

existence of a process mediated by a Ca^{2+}-Mg^{2+} ATPase (Noguera & D'Ocon 1992; present results).

Intracellular stores emptied by noradrenaline are replenished by Ca^{2+} that enters the cell through specific channels or by passive diffusion or exchange with other ions and can be refilled during the tonic component of the contractile response (Karaki et al 1979). This process is closely related to the stimulation of α-adrenoceptors and has been observed to occur when the agonist is not present in the incubating medium (Putney 1986, 1990). This increased permeability of the cell membrane for Ca^{2+} entry is common to different agonists and different preparations (Putney 1990) but the subsequent stimulation of the contractile machinery in the absence of the agonist during the refilling of noradrenaline-sensitive Ca^{2+} stores is linked only to noradrenaline-binding to α-adrenoceptors and does not occur after depletion of intracellular Ca^{2+} stores induced by other agonists such as caffeine or 5-hydroxytryptamine (unpublished observations).

The finding that the magnitude of the contractile process does not depend on variations in the temperature of incubation suggests that it is mediated by an event that does not depend on intracellular energy. The magnitude decreases proportionally with the time of exposure in Ca^{2+}-containing medium, which means that the accelerated entry of Ca^{2+} to replenish the intracellular pool inactivates the entry mechanism.

The results obtained show that the magnitude of the increase in resting tone after depletion of intracellular Ca^{2+} stores is similar in the presence and in the absence of Mg^{2+} in the incubating medium, but the process of Ca^{2+} refilling of intracellular stores sensitive to noradrenaline, depends on the presence of Mg^{2+}. Many biochemical systems subserving contractility are known to be sensitive to the free Mg^{2+} concentration (Flatman & Lew 1981; Moreland & Ford 1981, 1982; Masini et al 1983; Ikebe et al 1984). Present results suggest that the biochemical process involved in this increased tone is not dependent on the presence of Mg^{2+} in the extracellular medium, although we cannot determine whether it depends on variations in intracellular Mg^{2+}. Present results also indicate that this Ca^{2+} entry pathway is not related to Ca^{2+}-Mg^{2+} exchange or activation of the Mg^{2+}-dependent ATPase located in the cell membrane of many smooth muscles (Karaki et al 1983; Altura et al 1987; Singh 1987).

$LaCl_3$ is well known as an inorganic calcium blocker that nonspecifically modifies transport across the cell membrane

(Högestätt & Andersson 1984). However, it failed to inhibit the increase in the resting tone or to relax the noradrenaline- and KCl-induced contractions. This suggests that the Ca^{2+} entry pathway is not sensitive to La^{3+} in our experimental conditions.

In order to determine if Ca^{2+} enters through voltage-operated channels to refill intracellular pools, a series of experiments was designed in which the increase in tension was induced in the presence of different concentrations of verapamil. In our experimental conditions, verapamil generates a concentration-dependent inhibition of the increase in the resting tone. Its inhibitory action would seem to relate the increase in the resting tone to Ca^{2+} entry via voltage-operated Ca^{2+} channels. Moreover, recovery of the contractile response to noradrenaline or caffeine in Ca^{2+}-free medium was totally abolished when the increase in tone was blocked by verapamil. This indicates that entry of extracellular Ca^{2+} and, in consequence, refilling of intracellular Ca^{2+} pools have been suppressed by verapamil.

Finally, we tested the effect of a well known non-specific relaxant of smooth muscle, caffeine (Iino et al 1988; Sato et al 1988). Caffeine did not block the refilling pathway, but due to the increased levels of cAMP or a direct action on the contractile machinery (Iino et al 1988; Sato et al 1988), the entry of Ca^{2+} was not able to activate the contractile proteins. These results suggest a separation of the Ca^{2+} entry pathway and the subsequent stimulation of contractile proteins.

Experiments based on the assumption that caffeine contracts smooth muscle at a lower temperature in Ca^{2+}-free medium (Karaki et al 1987; Sato et al 1988; Noguera & D'Ocon 1992) were performed at 25°C and different results were obtained. The presence of caffeine during the refilling period blocked the generation of the increase in the resting tone, as it did in experiments carried out at 37°C. When recovery of the contractile response to noradrenaline or caffeine was analysed, the results were different, and this response was clearly diminished with respect to the first one in Ca^{2+}-free medium. This suggests that the refilling process was inhibited by caffeine treatment at 25°C, but not at 37°C.

In summary, the present work shows the existence of an increase in the resting tone of rat aorta produced by incubation in Ca^{2+}-containing solution after depletion of noradrenaline-sensitive intracellular calcium stores. This process was inhibited in a concentration-dependent way by a calcium-channel blocker verapamil, which also inhibited the refilling of the Ca^{2+} pools sensitive to noradrenaline and caffeine. Caffeine inhibits the increase in the resting tone without blocking the refilling process of the stores at 37°C, but at 25°C a partial inhibition of the repletion of internal Ca^{2+} pools by caffeine treatment was observed.

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