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Nantenine blocks muscle contraction and Ca^{2+} transient induced by noradrenaline and K^+ in rat vas deferens

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Abstract

The effect of nantenine, an aporphine alkaloid isolated from *Ocotea macrophylla* H.B.K., was studied on contractions and Ca^{2+} translocation induced by noradrenaline, Ca^{2+}, or K^+ in the isolated rat vas deferens from reserpinized animals. Concentration–response curves of calcium chloride ($CaCl_2$) were performed in the vas deferens, in a Ca^{2+}-free nutrient solution, using potassium chloride (KCl, 80 mM) as a depolarizing agent. In these conditions, nantenine (2.35×10^{-4} and 4.7×10^{-4} M) significantly reduced the maximum contractions (E_{max}) of Ca^{2+} ($IC_{50} = 2.6 \times 10^{-4}$ M) and noradrenaline ($IC_{50} = 2.9 \times 10^{-4}$ M). The contractile responses were totally recovered after the withdrawal of nantenine. In addition, experiments performed to measure simultaneously the contraction and the increase of intracellular Ca^{2+} induced by noradrenaline (10^{-5} M) or KCl (80 mM) showed that nantenine (2.35×10^{-4} and 4.7×10^{-4} M) significantly decreased both effects. The results suggest that a reversible block of Ca^{2+} entry could be involved on the non-competitive-like antagonism of nantenine in rat vas deferens.

Keywords: Nantenine; Aporphine; Ca^{2+} entry blocker; Fura-2; Smooth muscle; Vas deferens, rat

1. Introduction

Nantenine is an aporphine alkaloid found in several vegetal species. It was first isolated in Japan by Takase and Ohashi (1926) from the fruit of *Nandina domestica* Thunberg. Derived from benzyltetrahydroisoquinoline, nantenine presents structural likeness with papaverine and a series of benzyltetraisoquinoline alkaloids (isocrasifoline, laudanosine, antioquine and tetrandrine) described as Ca^{2+} antagonists (Anselmi et al., 1992; D'Ocon et al., 1989, 1991; King et al., 1988).

In spite of being structurally similar, the mechanism of action exhibited by aporphine molecules is different from the non-specific relaxant agent papaverine (Anselmi et al., 1992). Aporphine alkaloids, such as glaucine and boldine, have been described to have a selective smooth muscle relaxant activity through the block of Ca^{2+} influx via voltage-operated calcium channels. They have no effect on the contractile machinery or on Ca^{2+} release or Ca^{2+} redistribution from intracellular storage sites. In addition, radioligand binding assays have demonstrated that these alkaloids act at the benzothiazepine site in the calcium channel as well as at the α_1-adrenoceptor (Ivorra et al., 1992, 1993a,b).

The nantenine alkaloid shares some activities with other aporphines, as well as with Ca^{2+} entry blockers. In vitro assays have shown that nantenine, in small concentrations, antagonizes the contractions induced by both α_1-adrenoceptor (Bricola et al., 1981; Alzueta et al., 1992; Indra et al., 2002a) and 5-HT_2 receptor agonists in isolated aorta (Shoji et al., 1984; Alzueta et al., 1992; Indra et al., 2002b); furthermore, similarly to Ca^{2+} entry blockers (Murad, 1993), it causes hypotension, bradycardia and negative inotropism (Takase and Ohashi, 1926).

In preliminary studies with isolated rat vas deferens, nantenine showed an α_1-adrenoceptor antagonist activity at low concentrations that was followed by a non-compet-

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itive component at higher concentrations (Bricola et al., 1981). Taking into account the critical role of Ca^{2+} in regulating the smooth muscle activity (Godfraind et al., 1986; Smaili et al., 1998), including the rat vas deferens (Jurkiewicz et al., 1975; Castillo et al., 1992; Belevych et al., 1999), and the structural and pharmacological likeness between nantenine alkaloid and Ca^{2+} entry blockers, we have examined if the non-competitive antagonism observed in isolated rat vas deferens could be due to a Ca^{2+} antagonist activity.

2. Materials and methods

2.1. Drugs and chemical

Nantenine was isolated from *Ocotea macrophylla* H.B.K., trivial name "louro fofo", collected at the Ducke Forest Reserve, near Manaus, and identified by the botanist W. Rodrigues upon comparison of voucher specimen (42226) with specimen 14721, both deposited at the Instituto Nacional de Pesquisas da Amazônia, Manaus, Amazon, Brazil. It was kindly provided by Profs Otto R. Gottlieb and Massayoshi Yoshida from the Institute of Chemistry of the University of São Paulo, Brazil.

The alkaloid was dissolved in 0.01 N HCl solution and the pH was adjusted to 5.0–5.5 with 0.1 N NaOH. Noradrenaline (L-arterenol chloride), reserpine and tyramine HCl were from Sigma (USA), and Fura-2/AM from Molecular Probes (USA). All other chemicals used were from Merck analytical grade.

2.2. Rat vas deferens preparation

Adult male rats (5–6 months old) from our own colony, BAW-2 (Festing, 1980) were killed with an overdose of ether. Both vasa deferentia were removed, cleaned from surrounding tissues, and the lumens carefully washed with a nutrient solution (pH = 7.4) of the following composition (mM): NaCl 138, KCl 5.7, $CaCl_2$ 1.8, NaH_2PO_4 0.36, $NaHCO_3$ 15 and glucose 5.5, prepared in glass distilled water. Each organ was suspended in a 10 ml, chamber containing continuously aerated nutrient solution at 31 °C. Isotonic contractions were recorded on smoked drums with tangential levers, with a load of 1.0 g, and sixfold amplification. Contractions were also measured with transducers coupled to a microcomputer, when calcium measurements were made simultaneously, with Fura-2, as described below.

After an initial equilibration period of 30 min, cumulative concentration–response curves of noradrenaline (van Rossum, 1963) were obtained at 30–35 min intervals.

In Ca^{2+} experiments, animals were pretreated with intraperitoneal injections of 10 mg/kg reserpine 24 h before sacrifice, in order to deplete catecholamine stores. In this case, in isolated preparations, after an initial equilibration period of 30 min, tyramine 10^{-4} M was added to confirm catecholamine depletion. Twenty minutes after, the organ responsiveness was tested with barium chloride 10^{-2} M. After barium washout, the vas deferens preparations were maintained in regular nutrient solution for 20 min before changing to a Ca^{2+}-free + EDTA 0.03 mM nutrient solution. After about 1 h, $CaCl_2$ cumulative concentration–response curves (1 μM to 30 mM) were performed in the presence of KCl 80 mM, added 2 min before Ca^{2+}.

In some preparations, after achieving stable responses (after two to three curves) nantenine was incubated 10 min (or 20 min in Ca^{2+} experiments) prior and during the performance of the next agonist cumulative curve. Usually up to four alkaloid concentrations in rising sequence were tested in the same organ (5.9×10^{-5}, 1.17×10^{-4}, $2{,}35 \times 10^{-4}$ and 4.7×10^{-4} M or 2, 4, 8 and 16 μg/ml, respectively). Only one agonist was used in each experiment.

Calcium chloride (10^{-2} M) time–effect curves, in the presence and absence of a single concentration of nantenine (IC_{50}), were performed to verify the reversibility of its effect. In this experiment, the contractile response was followed during 10 min before washout. Interval between curves was about 30–35 min.

The effect of nantenine on the agonists contractile response was calculated as a percentage of the E_{max} of the last curve obtained after achieving stable responses (e.g. third curve) in each tissue preparation.

Apparent affinities for agonists were estimated graphically from individual experiments, as the concentrations inducing 50% of maximal effect (EC_{50}). The values of pD_2 were determined as the negative logarithm of the EC_{50} (Ariëns and van Rossum, 1957). A modified Schild plot adapted for non-competitive antagonism (Martini et al., 1997) was used to determine pD_2' values. By definition pD_2' is the negative logarithm of antagonist concentration that decreases agonist effect by 50% (IC_{50}). Contralateral rat vas deferens preparations were used as controls, in which the antagonist was not used.

The logarithms of dose ratios (log[DR]), determined as the difference of the log of the doses of agonist inducing a 50% effect in absence and presence of nantenine, were used to evaluate the displacement of the curves, induced by the alkaloid.

2.3. Ca^{2+} measurements with Fura-2

A strip (about 1.0×0.1 cm) of prostatic segment of rat vas deferens was mounted in a quartz cuvette filed with 2.5 ml Krebs solution (pH = 7.4), at 37 °C, bubbled with a mixture of 95% O_2 and 5% CO_2. One end of the strip was connected to a strain gauge transducer to monitor the isometric tension under a resting tension of 1.0 g. Simultaneous registration of isometric tension and fluorescence

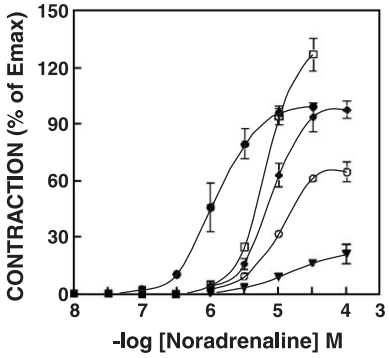

Fig. 1. Mean cumulative concentration–response curves for noradrenaline in the presence and absence of nantenine (incubation time = 10 min). The noradrenaline concentrations are expressed as $-\log$[concentration] M. Values represent the mean of five to six experiments. Vertical bars indicate S.E.M. Control (●), Nantenine: 5.9×10^{-5} M (□), 1.17×10^{-4} M (♦), 2.35×10^{-4} M (○), 4.7×10^{-4} M (▼).

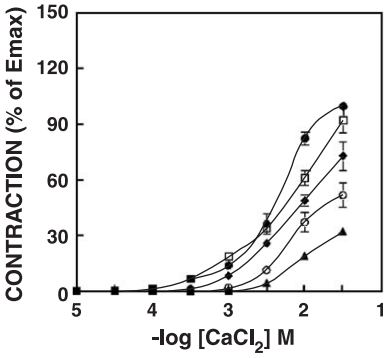

Fig. 2. Mean cumulative concentration–response curves for $CaCl_2$ in the presence and absence of nantenine (incubation time = 20 min). The $CaCl_2$ concentrations are expressed as $-\log$ [concentration] M. Values represent the mean of five to six experiments. Vertical bars indicate S.E.M. Depolarisation was elicited by 80 mM KCl (incubation time = 2 min). Control (●), Nantenine: 5.9×10^{-5} M (□), 1.17×10^{-4} M (♦), 2.35×10^{-4} M (○), 4.7×10^{-4} M (▼).

were recorded. Fluorescence measurements were performed in a PTI System (Photon Technology International, USA) with excitation wavelengths at 340 and 380 nm and emission at 505 nm. At the beginning of experiments, the ratio F_{340}/F_{380} was calculated before and after drugs applications. After 15 min washing the buffer was replaced with a Krebs solution containing fura-2/AM (8 μM) and non-cytotoxic detergent Pluronic F-127 (0.08%) for 5–6 h at room temperature. After the loading, the muscle strip was washed with fresh Krebs solution at 37 °C. Noradrenaline- or KCl-induced contractions were recorded for 300 s in the absence and presence of nantenine 2.35×10^{-4} or 4.7×10^{-4} M incubated for 20 min before noradrenaline or KCl addition. At the end of experiments, the fura-2-Ca^{2+} signal was calibrated. The maximal ratio (R_{max}) was measured in Ca^{2+} saturating medium by addition of digitonin (0.1 mM). To be sure that the registered signals came really from the fluorescence of the complex fura-2-Ca^{2+}, 2 mM $MnCl_2$ was added and fura-2 fluorescent signal was quenched. Finally, 4 mM EGTA was added and the minimum ratio (R_{min}) was obtained.

Table 1
Shifts to the right (as log [DR]) and E_{max} (mean ± S.E.M.) of curves for noradrenaline and calcium chloride

Concentration of nantenine	Noradrenaline		Calcium chloride	
(M)	log [DR]	E_{max}	log [DR]	E_{max}
0	–	100	–	100
5.90×10^{-5}	0.67 ± 0.10^{a}	126.9 ± 8.0	0.10 ± 0.08	92.3 ± 6.6
1.17×10^{-4}	0.83 ± 0.15^{a}	97.7 ± 4.7	0.14 ± 0.10	73.1 ± 7.6^{a}
2.35×10^{-4}	0.95 ± 0.18^{a}	64.8 ± 5.3^{a}	0.16 ± 0.07^{a}	52.1 ± 6.6^{a}
4.70×10^{-4}	1.00 ± 0.28^{a}	21.4 ± 5.3^{a}	0.25 ± 0.05^{a}	32.5 ± 2.3^{a}

[a] $P < 0.05$ with respect of values without nantenine.

2.4. Statistics

Student's paired *t*-test was performed to determine significance of analysed parameters differences. The significance level was considered as $P < 0.05$.

3. Results

3.1. Effect on noradrenaline-induced contractions

Nantenine induced a significant displacement of noradrenaline curve to the right (Fig. 1), leading to a shift of more than half log unit, in the presence of the lowest concentration used (5.9×10^{-5} M). This was accompanied by an increase of the maximum response (E_{max}) of about 20%. However,

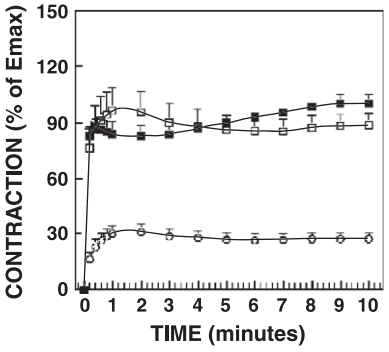

Fig. 3. Effect of nantenine on mean time–response curves for $CaCl_2$. Values represent the mean of five to six experiments. Vertical bars indicate S.E.M. The nantenine and $CaCl_2$ concentrations were 2.6×10^{-4} and 10^{-2} M, respectively. Nantenine incubation time was 20 min. $CaCl_2$ was added in a Ca-free medium after depolarisation elicited by 80 mM KCl. The later was added 2 min before calcium. Control (□), Nantenine 2.6×10^{-4} M (○), Calcium-Recovery (■).

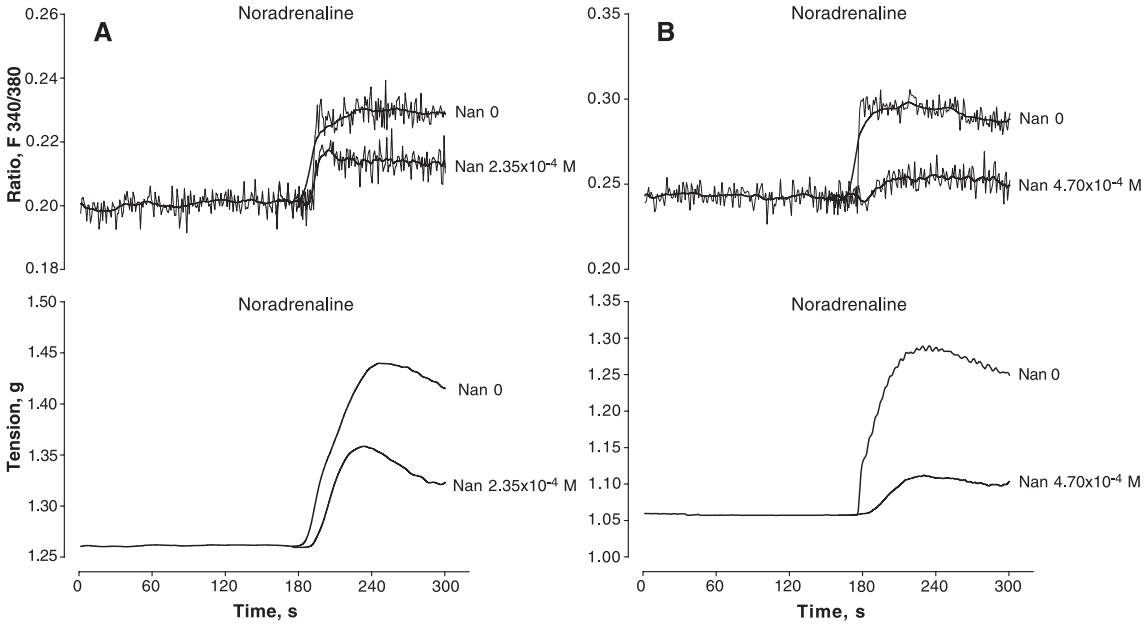

Fig. 4. (A) Typical recording of 10^{-5} M noradrenaline-induced fluorescence ratio ($F_{340/380}$) in fura-2 loaded strips and respective contraction of rat vas deferens, in absence (Nan 0) and presence of nantenine (Nan 2.35×10^{-4} M). Incubation time for nantenine was 20 min. (B) Similar experiment after a higher dose of nantenine (Nan 4.70×10^{-4} M). Essentially identical results were obtained in three similar independent experiments. The thick lines in (A) were automatically computer-calculated as means of the respective thinner lines.

higher concentrations of nantenine (2.35×10^{-4} and 4.7×10^{-4} M) while inducing a shift to the right, caused instead a decrease of E_{max}. Based on the decline of E_{max}, the corresponding pD_2' value, determined according to Martini et al. (1997), was 3.52. In addition, the IC_{50} value, calculated as antilog of pD_2' was 2.95×10^{-4} M. Furthermore, the values of log [DR] and percentage of maximum response (E_{max}) are shown in Table 1.

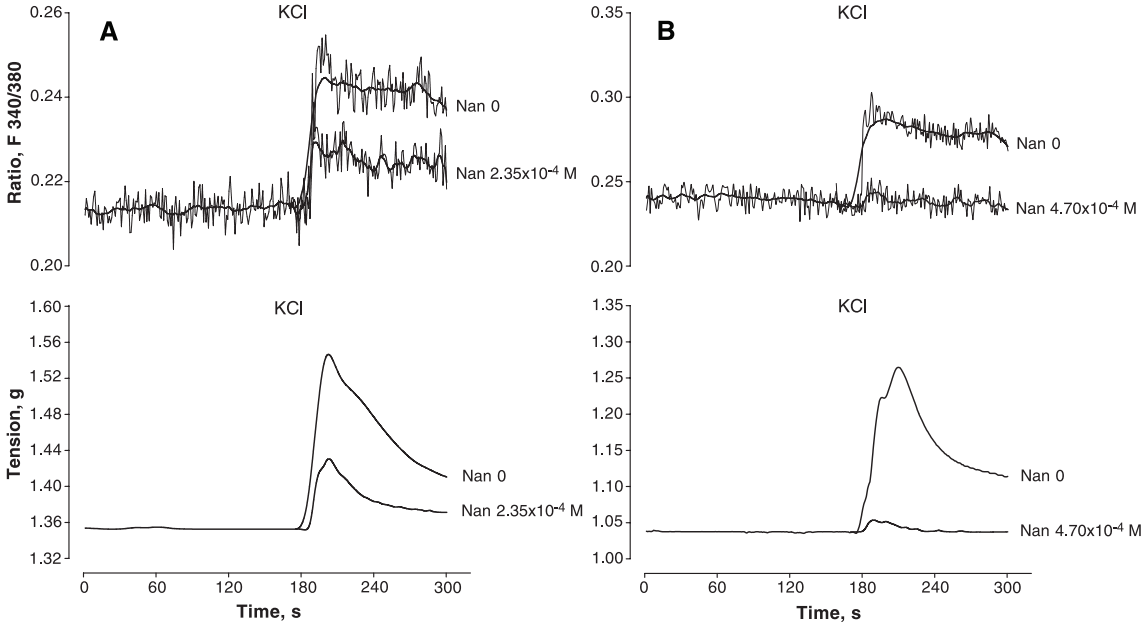

Fig. 5. (A) Typical recording of 80 mM KCl-induced fluorescence ratio ($F_{340/380}$) in fura-2 loaded strips and respective contraction of rat vas deferens, in absence (Nan 0) and presence of nantenine (Nan 2.35×10^{-4} M). Incubation time for nantenine was 20 min. (B) Similar experiment after a higher dose of nantenine (Nan 4.70×10^{-4} M). Essentially identical results were obtained in three similar independent experiments. The thick lines in (A) were automatically computer-calculated as means of the respective thinner lines.

3.2. Effect on $CaCl_2$-induced contractions

At concentrations of 1.17×10^{-4} to 4.7×10^{-4} M, nantenine significantly reduced the E_{max} of cumulative Ca^{2+} curves in tissues depolarised with 80 mM KCl, with a 50% inhibitory concentration (IC_{50}) equal to 2.61×10^{-4} M (Fig. 2). A significant displacement to the right was observed only at higher concentrations (2.35×10^{-4} and 4.7×10^{-4} M). The pD_2' value determined according to Martini et al. (1997) was 3.58. The values of log [DR] and E_{max} are shown in Table 1.

3.3. Effect on $CaCl_2$ contractile response recovery

Fig. 3 shows that a single dose of $CaCl_2$ (10^{-2} M) caused a sustained contraction, when added in a calcium-free medium, in the presence of a depolarising dose of 80 mM KCl. This contraction was strikingly reduced by nantenine and was totally recovered after nantenine withdrawal.

In parallel experiments, used as time-matched controls, no significant variations were observed in the contractile response of contralateral rat vas deferens in the absence of nantenine. In additional experiments, where rising concentrations of nantenine were used in the same preparation, the effect of each concentration was totally reverted after nantenine washout (data not shown).

3.4. Effect on Ca^{2+} measurements with Fura-2

Fig. 4A shows a typical recording, in which the contraction and increase in calcium internal concentration ($[Ca^{2+}]_i$) induced by 10^{-5} M noradrenaline were measured simultaneously. The preincubation with nantenine (2.35×10^{-4} M) significantly decreased both contraction and $[Ca^{2+}]_i$ by $51 \pm 13\%$ and $56 \pm 2\%$, respectively.

The effects of nantenine on KCl-induced contraction (80 mM) and fluorescence ratio are represented in Fig. 5A. In this case, the reduction of contractile response and $[Ca^{2+}]_i$ was $40 \pm 12\%$ and $62 \pm 4\%$, respectively.

A larger dose of nantenine (4.7×10^{-4} M) reduced by about 80% the effect of noradrenaline (Fig. 4B) and almost completely extinguished the effects of KCl-induced contraction and fluorescence ratio (Fig. 5B).

All effects were reverted after washout (data not shown).

4. Discussion

The effects already described for nantenine in isolated smooth muscle include α_1-adrenoceptor (Bricola et al., 1981; Alzueta et al., 1992; Indra et al., 2002a) and 5-HT_2 receptor antagonism (Shoji et al., 1984; Alzueta et al., 1992; Indra et al., 2002b). The data obtained in the present work, using isolated preparations of rat vas deferens, confirmed some of these activities. Nantenine presented an α_1-adrenoceptor antagonistic activity accompanied of a non-competitive component at higher concentrations. This is indicated by the fact that the alkaloid produced a significant shift to the right of concentration–response curve of noradrenaline with a fall in the maximum effect (E_{max}) after the largest doses of nantenine. The fact that the lowest dose of nantenine induced an increase instead of a reduction of E_{max} is compatible with results previously shown, in which a number of α-adrenoceptor antagonists, besides causing a shift to the right, increased the height of noradrenaline and adrenaline curves in this preparation (Jurkiewicz and Jurkiewicz, 1976). In the rat vas deferens, stimulation of α_1-adrenoceptors is associated with an increase in cytosolic Ca^{2+} concentrations. However, the source of Ca^{2+} mobilized upon α_1-adrenoceptors activation in smooth muscles remains uncertain (Khoyi et al., 1993). It is believed that both extra- and intracellular pools are mobilized during noradrenaline-induced contraction (Jurkiewicz et al., 1975, 1977). The phasic component of noradrenaline response would be related mainly to the release of intracellular Ca^{2+} whereas the tonic phase of contraction would involve Ca^{2+} influx via membrane voltage-sensitive channels (Vesperinas et al., 1989). The later model of Ca^{2+} mobilization is more consistent with the observation that Ca^{2+} deprivation causes a reduction (Amobi et al., 1999) or even a complete loss of contractile response of adrenoceptor agonists (Jurkiewicz et al., 1975) and that the vas deferens contractions induced by noradrenaline are inhibited by Ca^{2+} channel blockers (Feddersen et al., 1988).

In contrast to noradrenaline, the contraction elicited by KCl-induced depolarisation is reported to be absolutely dependent of extracellular Ca^{2+} (Hay and Wadsworth, 1982). It is well known that the L-type voltage-dependent calcium channels (VDCC) are the main portal of entry of Ca^{2+} inward current that is gated by potential in smooth muscles. Briefly, membrane depolarisation causes the opening of Ca^{2+} channel which allows the rapid entry of Ca^{2+} down its electrochemical gradient. Ca^{2+} antagonists (e.g. nifedipine, verapamil and diltiazem) bind reversibly to voltage-sensitive calcium channels preventing Ca^{2+} influx upon membrane depolarisation (Vaghy et al., 1987).

In order to investigate the involvement of a Ca^{2+} antagonist activity on the non-competitive antagonism described above, cumulative $CaCl_2$ curves were performed. In these conditions, nantenine partially inhibited the contractile response induced by $CaCl_2$ in a concentration-dependent and reversible manner, clearly showing its involvement in Ca^{2+} translocation. Therefore, it could be suggested that the antagonism promoted by nantenine on Ca^{2+} curves is consistent with an inhibitory action on the inward movement of the Ca^{2+}, an effect which, in part, could be also involved in the non-competitive antagonism that was here shown in relation to noradrenaline. This suggestion is reinforced by the recent data described by Orallo and Alzueta (2001) and Orallo (2003), in which the authors concluded that the vasorelaxing effect of nantenine

observed in rat aorta preparations involves a block of Ca^{+2} influx through transmembrane calcium channels.

The nantenine inhibitory action on intracellular Ca^{2+} was also analysed using Fura-2 measurements. We have shown (Figs. 4 and 5) that the increase of cytosolic Ca^{2+} and of the contraction induced either by 10^{-5} M noradrenaline or 80 mM KCl was inhibited in a dose-dependent manner. Considering that, in rat vas deferens, increase of intracellular Ca^{2+} induced by high concentrations of KCl is mainly caused by membrane depolarisation and Ca^{2+} influx through L-type voltage-dependent Ca^{2+} channels (see above), it could be concluded that the decrease in $[Ca^{2+}]_i$ induced by nantenine may reflect a decline of Ca^{2+} influx through calcium channels.

In summary, our results indicate that nantenine presents a reversible inhibitory action on the inward movement of Ca^{2+} through Ca^{2+} channels, a mechanism that seems to be involved in the non-competitive component of its antagonism in rat vas deferens.

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