Synthetic Lapachol Derivatives Relax Guinea-Pig Ileum by Blockade of the Voltage-Gated Calcium Channels

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The present study was designed to further evaluate a possible spasmolytic activity of synthetic lapachol derivatives, norlapachol, α-norlapachone, β-norlapachone and hydro-hydroxy-norlapachol (HH-norlapachol), on guinea-pig ileum. In guinea-pig ileum, except for norlapachol, all naphthoquinones inhibited the phasic contractions induced by carbachol or histamine. Even when the ileum was pre-contracted with KCl, carbachol or histamine, all naphthoquinones induced relaxation, suggesting that these naphthoquinones could be acting on the voltage-gated calcium channels (Ca_V). As the tonic component this contraction is maintained mainly by the opening of the Ca_V, we hypothesized that these naphthoquinones might be acting on these channels. This hypothesis was confirmed by the observation that norlapachol ($pD'_2 = 4.99$), α-norlapachone ($pD'_2 = 4.49$), β-norlapachone ($pD'_2 = 6.33$), and HH-norlapachol ($pD'_2 = 4.53$) antagonized the contractions induced by $CaCl_2$ in depolarizing medium nominally without Ca^{2+}. As β-norlapachone was the most potent we decided to continue the study of its action mechanism. The fact that this naphthoquinone has inhibited the tonic contractions induced by S-(-)-Bay K8644 [$EC_{50} = (1.6 \pm 0.30) \cdot 10^{-5}$ M] suggests that the Ca^{2+} channel involved belongs to the type L ($Ca_V1.2$). In addition, in the functional level, the spasmolytic effect of β-norlapachone does not involve participation of free radicals, since its curve of relaxation was unchanged in the presence of glutathione, an antioxidant agent.

Key words: Synthetic Lapachol Derivatives, Guinea-Pig Ileum, Ca_V.

Introduction

Naphthoquinone derivatives are widespread in nature and their biological and pharmacological activities are of great interest (Salmon-Chemin *et al.*, 2001). In particular, lapachol and the related quinones, α- and β-lapachones, are natural naphthoquinones extracted from a wide range of species from the genus *Tabebuia* (Bignoniaceae). Norlapachol is a semi-synthetic derivative of natural lapachol (Hooker, 1936), a naphthoquinone readily extracted from a number of species of *Tabebuia* abundant in Brazil and South America (Souza and Lorenzi, 2005).

It has been reported that several naphthoquinones including juglone (5-hydroxy-1,4-naphthoquinone), isolated from *Juglans nigra,* present a spasmolytic effect, dilate coronary arteries of the rabbit heart, and depresse the activity of smooth muscles of rat intestine and uterus (Auyong *et al.*, 1963). Moreover, vitamins K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) and K_3 (2-methyl-1,4-naphthoquinone) exerted a spasmolytic action on coronary vessels of rats (Lider *et al.*, 1987). In addition, 7-methyl-juglone and 2-methyl-juglone (plumbagin) showed strong spasmolytic activity (Krahl, 1956; Neuhaus-Carlisle *et al.*, 1997; Krenn *et al.*, 1998). In previous studies we showed that lapachol, α- and β-lapachones, which are natural naphthoquinones, present spasmolytic activity on the guinea-pig ileum (Cavalcante *et al.*, 2008).

Therefore the aim of the present study was to evaluate the spasmolytic activity of the synthetic lapachol derivatives norlapachol, α- and β-norlapachones and hydrohydroxy (HH)-norlapachol on the guinea-pig ileum, since no other information on the spasmolytic activity of these synthetic lapachol derivatives has been reported.

Material and Methods

Synthesis of the synthetic lapachol derivatives

Norlapachol was synthesized from lapachol, extracted from its natural source (*Tabebuia* wood) encountered in our Campus, and the other naphthoquinones were synthesized in our Laboratory from norlapachol, following the procedure described by Fieser and Fieser (1948) and Hooker (1936).

Norlapachol

2.42 g of lapachol were added to a stirred mixture of 25 mL of dioxane and 25 mL of water containing 1.2 g of sodium carbonate. The mixture was heated with 2 mL of 30% hydrogen peroxide at 70 ºC until the solution became colourless, then cooled in ice, treated with 2 mL of 36% hydrochloric acid and with 10 mL of water saturated with sulfur dioxide. The mixture was treated with 20 mL of 25% sodium hydroxide and 50 mL of a solution containing 10 g of copper sulfate and then heated for 10–30 min on the steam-bath. The solution was filtered by suction, and the filter pad was washes with water until the washing water by colourless. The resultant solution was acidified, cooled on ice, and the organic matter was extracted with 4 × 25 mL of chloroform. The organic layer was dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. The crude material, red oil, was purified by column chromatography with choloform/hexane (99:1) yielding 63% of norlapachol.

β-Norlapachone

456 mg of norlapachol were dissolved in 5 mL of cold 80% sulfuric acid, and after standing for 20 min the brownish-red solution was poured into 100 mL of cold water. After extraction and purification by column chromatography with chloroform, the product was obtained as red crystals in 92% yield.

α-Norlapachone

456 mg of norlapachol were dissolved in 25 mL of 15% sulfuric acid and refluxed for 90 min. The yellow solution was poured into 100 mL of cold water. After extraction and purification by column chromatography with chloroform the product was obtained as yellow crystals in 82% yield.

HH-norlapachol

456 mg of norlapachol were dissolved in 5 mL of cold 80% sulfuric acid, and after standing for 15 min the brownish-red solution was neutralized and boiled with an excess of 5% sodium hydroxide solution for 15 min. The solution was acidified with acetic acid, and, after purification by column chromatography with chloroform, a yellow product was obtained in 85% yield.

Animals

The experiments were performed with guinea-pig ileum *(Cavia porcellus*, 300–500 g). All experimental procedures were performed in accordance with the guidelines approved by the Animal Research Ethic Committee of the Universidade Federal da Paraíba (Protocol CEPA/LTF No. 0706/2006).

Measurements of tension on guinea-pig ileum

Adult guinea-pigs of both sexes were fasted for about 18 h (only water was given to them during this period). The animals were then killed by cervical dislocation and exsanguinated. The ileum segments were suspended under 1 g load in an organ bath containing Krebs solution (mM): NaCl (117.0), KCl (4.7), $MgSO_4 \cdot 7H_2O$ (1.3), $NaH_2PO_4 \cdot H_2O$ (1.2), $CaCl_2 \cdot 2H_2O$ (2.5), glucose (11.0), $NaHCO_3$ (25.0). The solution maintained at 37 °C was continuously oxygenated with a mixture of 95% O_2 and 5% CO_2.

Effect of synthetic lapachol derivatives on carbachol- and histamine-induced phasic contractions in guinea-pig ileum

After stabilization for 30 min, two phasic contractions evoked by 1 μM of carbachol or histamine were recorded using isotonic levers coupled to kymographs and smoked drums (DTF, São

Paulo, SP, Brazil). The synthetic lapachol derivatives were then added, and after an incubation period of 15 min (the drug incubation period was established in preliminary experiments) a third concentration-response curve was induced in the presence of various concentrations of naphthoquinones in different preparations. The molar concentration of a substance that reduces the response to an agonist by 50% (IC_{50}) was obtained by nonlinear regression from the individual values of inhibition for each substance.

Effect of synthetic lapachol derivatives on KCl-, carbachol- or histamine-induced tonic contractions

After stabilization of the preparations, an isometric contraction was elicited with 40 mM KCl, 1 μM carbachol or 1 μM histamine and was measured with a force displacement transducer (World Precision Instruments, Sarasota, Florida, USA). After further 30 min the process was repeated and the synthetic lapachol derivatives were added cumulatively (10^{-7} up to $3 \cdot 10^{-4}$ M) at the plateau phase, in different preparations. The molar concentration of a substance that produces 50% of its maximal possible effect (EC_{50}) was obtained graphically from concentration-inhibition curves. Relaxation was expressed as reversal percentage of the initial contraction elicited by contractile agents.

Effect of synthetic lapachol derivatives on Ca^{2+}-induced contractions in depolarizing medium nominally without Ca^{2+}

After 30 min of stabilization, modified Krebs solution (mM) [NaCl (51.7), KCl (70.0), $MgSO_4 \cdot 7H_2O$ (1.3), $NaH_2PO_4 \cdot H_2O$ (1.2), glucose (11.0), $NaHCO_3$ (25.0)] was replaced by a depolarizing solution nominally without Ca^{2+}, and the tissue was allowed to equilibrate in this medium for further 45 min. Two similar $CaCl_2$ cumulative response-concentration curves (control) were then induced at an interval of 60 min. After this procedure several concentrations of naphthoquinones were incubated for 15 min in different preparations, and then a third $CaCl_2$ cumulative curve was obtained. The maximal contraction obtained with the control curves ($CaCl_2$) was considered as 100%, and all contractions were calculated proportionally to this value.

Effect of β-norlapachone on S-(-)-Bay K8644-induced tonic contractions

Previous studies indicated that the contractile responses to Ca^{2+} channel activators, such as S-(-)-Bay K8644 [*S*-(-)-1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl) phenyl]-3-pyridine carboxylic acid methyl ester] (Sigma-Aldrich, St. Louis, MO, USA), a L-type Ca^{2+}-channel selective agonist (Ferrante *et al.*, 1989), were enhanced by raising the level of KCl above that of the Krebs solution (8–20 mM) (Wei *et al.*, 1986; Conte-Camerino *et al.*, 1987; Zheng *et al.*, 1991; Usowicz *et al.*, 1995). Therefore, after stabilization for 30 min in modified Krebs solution, the ileum was partly depolarized by addition of 15 mM KCl for 10 min. In the presence of KCl a contraction with 0.3 μM S-(-)-Bay K8644 was induced. During stabilization of the tonic phase of this contraction, β-norlapachone was added cumulatively in order to obtain a concentration-inhibition curve. Relaxation was expressed as described before.

Effect of β-norlapachone on contractions induced by carbachol in the absence and presence of glutathione

The protocol used was a modification of the method described previously by Moummi *et al.* (1991). To test the effect of glutathione (GSH), an antioxidant agent (Gaté *et al.*, 1999), on β-norlapachone-induced spasmolytic response, the ileum was treated with GSH (60 μM) for 15 min, followed by the addition of the naphthoquinone; even in its presence, a further contraction with carbachol was obtained. Then β-norlapachone was applied cumulatively on the tonic contraction. In another set of experiments the naphthoquinone was mixed prior with GSH (1:1) before addition to the tissue baths. The percentage of inhibition was calculated by comparing the responses before and after the addition of antioxidant agent.

Statistical analysis

Values were expressed as mean ± S. E. M. Statistical analysis was performed using Graph-Pad Prism 3.03 software (GraphPad Software Inc., San Diego, CA, USA). The EC_{50} and IC_{50} values were determined by nonlinear regression (Jenkinson *et al.*, 1995). Differences between means were statistically compared using Student's t-test and/or one-way ANOVA followed by Bonferroni's test, as appropriate, and were considered to differ significantly when $p < 0.05$. Schild plots were analyzed by linear regression. Antagonism was judged to be noncompetitive when the slope of the Schild plot was significantly different from unity and depression of the maximum response was observed (Arunlakshana and Schild, 1959).

Results

Effect of synthetic lapachol derivatives on carbachol- and histamine-induced phasic contractions in guinea-pig ileum

Until 10^{-4} M, both norlapachol and HH-norlapachol did not effective antagonize the phasic contractions induced by 1 μM carbachol or histamine in the guinea-pig ileum ($n = 3$). Their maximum inhibitory effects did not exceed 35%. However, α- and β-norlapachones antagonized in a significant ($p < 0.05$) and concentration-dependent ($10^{-7} - 3 \cdot 10^{-4}$ M) manner the phasic contractions induced by carbachol or histamine in guinea-pig ileum ($n = 5$). The IC_{50} values for α- and β-norlapachones were, respectively, $(1.1 \pm 0.2) \cdot 10^{-4}$ M and $(2.2 \pm 0.4) \cdot 10^{-5}$ M to carbachol and $(1.5 \pm 0.4) \cdot 10^{-4}$ M and $(3.7 \pm 1.0) \cdot 10^{-5}$ M to histamine (Table I). The responsive of the ileum was recovered 45 min after withdrawal of the naphthoquinones from the bath. The values of IC_{50} showed that β-norlapachone presented a potency about 5 times higher than that of α-norlapachone.

Effect of synthetic lapachol derivatives on KCl-, carbachol- or histamine-induced tonic contractions

Norlapachol and its derivatives, α-, β-norlapachones and HH-norlapachol (Fig. 1), relaxed in a significant ($p < 0.05$) and concentration-dependent manner the ileum pre-contracted with 40 mM KCl [$EC_{50} = (3.3 \pm 0.3) \cdot 10^{-5}$ M, $(1.9 \pm 0.3) \cdot 10^{-5}$ M, $(1.2 \pm 0.2) \cdot 10^{-5}$ M, and $(1.2 \pm 0.4) \cdot 10^{-5}$ M], 1 μM carbachol [$EC_{50} = (3.5 \pm 1.4) \cdot 10^{-5}$ M, $(0.8 \pm 0.1) \cdot 10^{-5}$ M, $(1.4 \pm 0.6) \cdot 10^{-5}$ M, and $(2.6 \pm 0.7) \cdot 10^{-5}$ M] or 1 μM histamine [$EC_{50} = (3.3 \pm 0.6) \cdot 10^{-5}$ M, $(1.5 \pm 0.3) \cdot 10^{-5}$ M, $(1.7 \pm 0.3) \cdot 10^{-5}$ M, and $(1.6 \pm 0.7) \cdot 10^{-5}$ M]. An analysis of the EC_{50} values indicated that only α-norlapachone did not present equipotency to the agents tested, since it was more potent than the other naphthoquinones when the ileum was pre-contracted by carbachol.

Effect of synthetic lapachol derivatives on Ca^{2+}-induced contractions in depolarizing medium nominally without Ca^{2+}

Fig. 2 shows the mean cumulative concentration-response curves to $CaCl_2$ in the absence and presence of different concentrations of the synthetic lapachol derivatives. All naphthoquinones produced a nonparallel and concentration-dependent shift to higher concentrations of $CaCl_2$. Analysis of the data by linear regression yielded a correlation coefficient (r^2) of 0.85, 0.96, 0.80, and 0.91, respectively. The pD'_2 values were 4.99 ± 0.61, 4.49 ± 0.02, 6.33 ± 0.16, and 4.53 ± 0.05, respectively. Therefore, β-norlapachone was the most potent naphthoquinone. The Schild plot slope values were 1.52 ± 0.19, 3.92 ± 0.46, 1.26 ± 0.13, and 2.60 ± 0.09, respectively, indicating a noncompetitive blockade.

Effect of β-norlapachone on S-(-)-Bay K8644-induced tonic contractions

Cumulative addition of β-norlapachone (10^{-7}–$3 \cdot 10^{-4}$ M) to the tonic component of the contractions elicited by 0.3 μM S-(-)-Bay K8644 resulted in a concentration-dependent relaxation (Fig. 3). This effect was similar to the relaxant

Table I. IC_{50} and E_{max} values of norlapachol and its derivatives on the guinea-pig ileum ($n = 3–5$).

Naphthoquinone	Carbachol		Histamine	
	E_{max} (%)	IC_{50} [M]	E_{max} (%)	IC_{50} [M]
Norlapachol	24.2 ± 3.9**	ND	18.3 ± 2.7**	ND
α-Norlapachone	71.3 ± 4.5**	$(1.1 \pm 0.2) \cdot 10^{-4a}$	76.5 ± 5.8*	$(1.5 \pm 0.4) \cdot 10^{-4a}$
β-Norlapachone	98.4 ± 1.0	$(2.2 \pm 0.4) \cdot 10^{-5a}$	97.1 ± 1.2	$(3.7 \pm 1.0) \cdot 10^{-5a}$
HH-Norlapachol	38.1 ± 0.3**	ND	8.2 ± 3.2**	ND

Student's t-test; * $p < 0.05$ and ** $p < 0.001$ [control (100%) *vs.* naphthoquinones].
[a] $p < 0.05$ (α-norlapachone *vs.* β-norlapachone).
ND, non determined.

effect of β-norlapachone when the ileum was pre-contracted with KCl (Fig. 4). The EC_{50} values of β-lapachone were $(1.6 \pm 0.3) \cdot 10^{-5}$ M and $(1.2 \pm 0.2) \cdot 10^{-5}$ M against S-(-)-Bay K8644 and KCl, respectively.

Effect of β-norlapachone on contractions induced by carbachol in the absence and presence of glutathione

Pre-incubation for 15 min of the guinea-pig ileum with glutathione, an antioxidant agent, did not affect the relaxing effect induced by β-norlapachone [$EC_{50} = (1.4 \pm 0.2) \cdot 10^{-5}$ M]. Similarly, the mixture β-norlapachone/glutathione (1:1) *per se* [$EC_{50} = (1.4 \pm 0.1) \cdot 10^{-5}$ M] also did not alter the β-norlapachone-induced relaxation [$EC_{50} = (1.4 \pm 0.6) \cdot 10^{-5}$ M] (data not shown).

Discussion

In the present work, the spasmolytic effect of synthetic lapachol derivatives (norlapachol, α-norlapachone, β-norlapachone, and HH-norlapachol) on intestinal smooth muscle was investigated. The most important finding was that these naphthoquinones show nonselective spasmolytic action on the guinea-pig ileum, and that the mechanism of action by which β-norlapachone induces relaxation in the guinea-pig ileum involves the participation of voltage-gated calcium channels (Ca_V) belonging to the type L ($Ca_V1.2$), but not of free radicals.

Since lapachol and its natural derivatives showed a spasmolytic effect in the guinea-pig ileum (Cavalcante *et al.*, 2008), we investigated whether its synthetic derivatives norlapachol, α-norlapachone, β-norlapachone, and HH-norlapachol also present this effect. Interestingly, we found that only α- and β-norlapachones inhibited the phasic contractions in guinea-pig ileum induced by carbachol and histamine in an equipotent and concentration-dependent manner (Table I), suggesting that the furane naphthoquinones, α-norlapachone and β-norlapachone, may be acting on a common pathway related to the cascade of events that leads to smooth muscle contraction by these agonists.

It has to be noted that β-norlapachone was about 5 times more potent than α-norlapachone (Table I). These results are in agreement with other studies, which reported that *ortho*-naphthoquinones

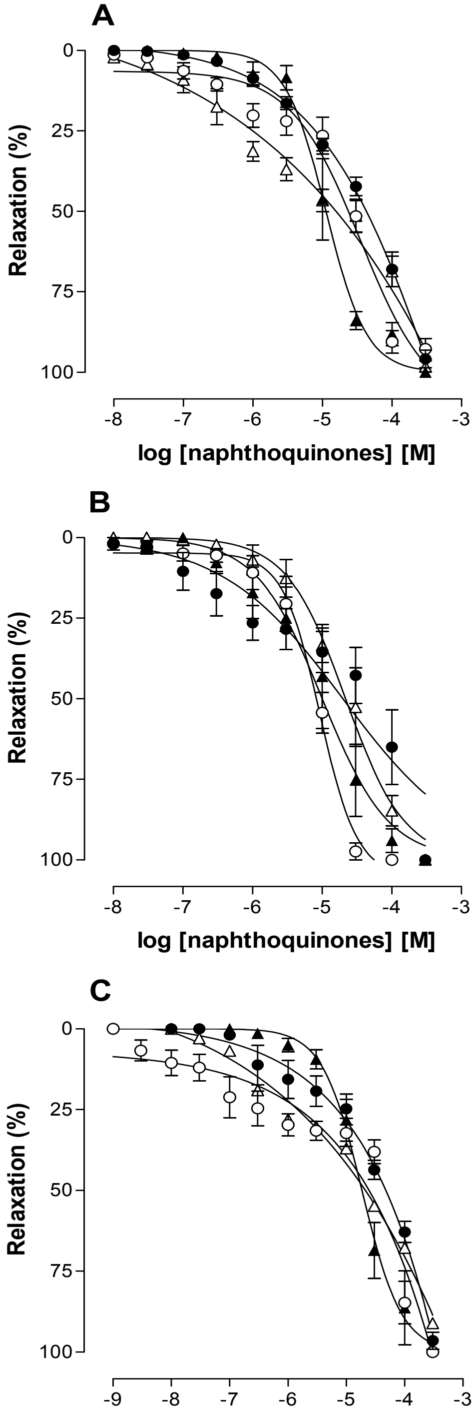

Fig. 1. Effect of norlapachol (●), α-norlapachone (○), β-norlapachone (▲), and HH-norlapachol (△) on the tonic contractions induced by (A) 40 mM KCl, (B) 1 μM carbachol, and (C) 1 μM histamine ($n = 5$). Symbols and vertical bars represent the mean ± S. E. M.

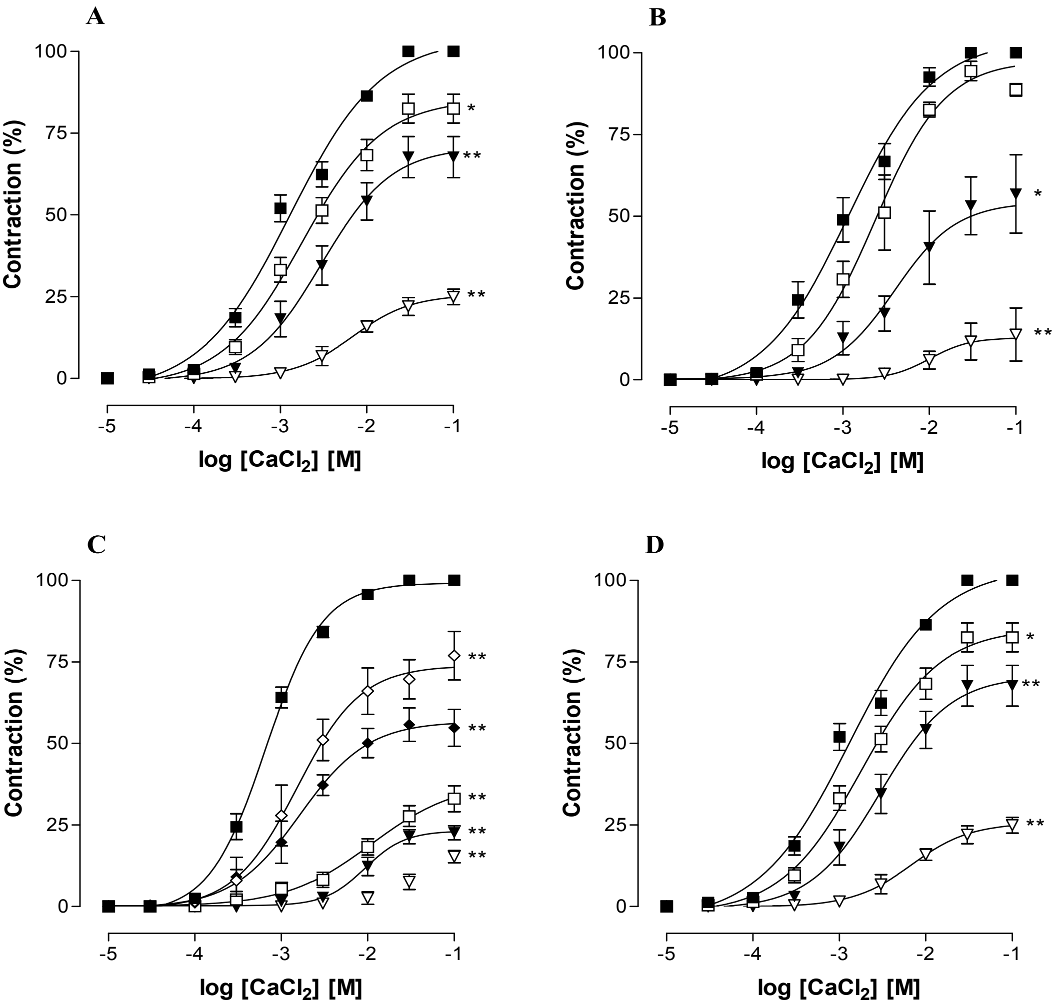

Fig. 2. Cumulative concentration-response curves to $CaCl_2$ with tissue strips bathed in depolarizing medium nominally without Ca^{2+} in the absence (■) and presence of (A) norlapachol, (B) *α*-norlapachone, (C) *β*-norlapachone, and (D) HH-norlapachol: $3 \cdot 10^{-6}$ (◇), 10^{-5} (◆), $3 \cdot 10^{-5}$ (□), 10^{-4} (▼), and $3 \cdot 10^{-4}$ M (▽) ($n = 5$). Symbols and vertical bars represent the mean ± S. E. M. One-way ANOVA followed by Bonferroni's test, significant differences are indicated by $^{*}p < 0.05$ and $^{**}p < 0.001$ (control *vs.* *β*-lapachone).

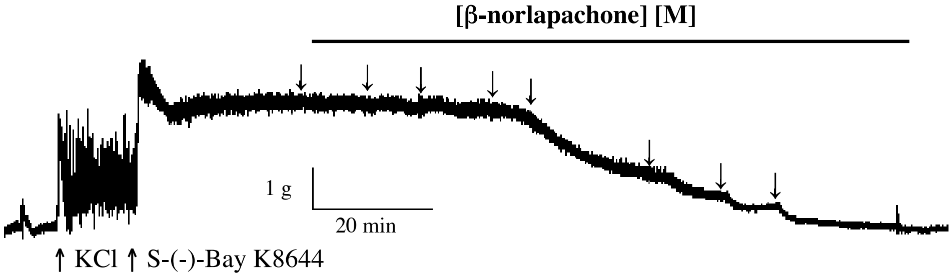

Fig. 3. Representative tracing showing the effect of the cumulative addition of *β*-norlapachone on ileum partially depolarized by the addition of 15 mM KCl (10 min) and pre-contracted with 0.3 *µ*M S-(-)-Bay K8644. Arrows down show the time course of *β*-norlapachone (10^{-7} to $3 \cdot 10^{-4}$ M).

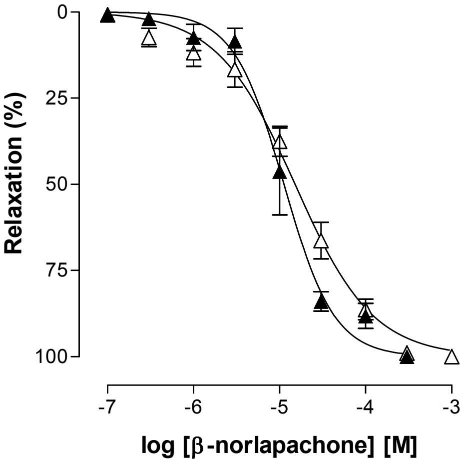

Fig. 4. Effect of β-norlapachone on the tonic contraction elicited by 40 mM KCl (▲) or 0.3 μM S-(-)-Bay K8644 (△) (n = 5). Symbols and vertical bars represent the mean ± S. E. M.

(β-lapachone and β-norlapachone) are more active than *para*-naphthoquinones (α-lapachone and α-norlapachone) regarding trypanocidal, topoisomerase II inhibition, antibacterial, and cytotoxic activity (Goijman and Stoppani, 1985; Krishnan and Bastow, 2000; Antunes *et al.*, 2006; Pereira *et al.*, 2006).

The major trigger for smooth muscle contraction is a rise in the intracellular calcium concentration, $[Ca^{2+}]_i$. Two general forms of excitation initiate the contraction of smooth muscles: innervation and consequent depolarization of the membrane's resting potential, termed electromechanical coupling, and activation by ligands of cell surface receptors, termed pharmacomechanical coupling (Somlyo and Somlyo, 2000).

Many agonists that induce guinea-pig ileum contraction cause a biphasic contraction: in the first phase the muscle exhibits a fast and transient contraction followed by a long-lasting second phase, which is characterized by the maintained tonic contraction (Horie *et al.*, 2005). On the other hand, pharmacomechanical coupling involves activation of cell surface receptors to augment the increase in Ca^{2+}, either by the release of Ca^{2+} from intracellular stores or through cell signaling-mediated mechanisms that increase the Ca^{2+} sensitivity of the contractile apparatus (Stevens *et al.*, 2000). Based on the fact that the mechanisms involved in the maintenance tonic component of the contraction are different in relation to the phasic on the guinea-pig ileum (Abdel-Latif, 1989; Honda *et al.*, 1996), we decided to check if the naphthoquinones promote the relaxation of the ileum pre-contracted with KCl (electromechanical coupling) and carbachol or histamine (pharmacomechanical and electromechanical couplings).

As shown in Fig. 1, all the naphthoquinones tested relaxed in a concentration-dependent manner the ileum pre-contracted with KCl, carbachol or histamine. Independently whether the contraction is evoked by pharmacomechanical or electromechanical coupling, the maintenance of the tonic component involves activation of the Ca_V (Rembold, 1996).

As the maintenance of the tonic contraction induced by these contractile agents involves Ca^{2+} influx through voltage-gated Ca^{2+} channels (Bolton, 1979, 2006), it is suggestive that naphthoquinones-induced relaxation in guinea-pig ileum may be due to the blockade of Ca^{2+} influx through these channels.

The ileum is an organ completely dependent upon membrane potential variation (Nouailhetas *et al.*, 1985). Thus, since the tonic component of mixed-coupling agonists or depolarizing agents is almost exclusively sustained by calcium influx through the Ca_V, we tested the hypothesis that theses naphthoquinones could act by blocking the Ca^{2+} influx through the Ca_V. This hypothesis was confirmed by the observation that norlapachol, α-norlapachone, β-norlapachone, and HH-norlapachol inhibited Ca^{2+}-induced contractions in a depolarizing medium nominally without Ca^{2+}, showing a noncompetitive antagonism (slope $\neq$ unity) with a shift of the concentration-response curve to higher $CaCl_2$ concentrations in a non-parallel and concentration-dependent manner, reducing significantly E_{max} (Fig. 2).

Ca_V are present in the membrane of most excitable cells. The 10 cloned a-subunits can be grouped into three families: (1) the high-voltage activated dihydropyridine-sensitive (L-type, Ca_V1) channels; (2) the high-voltage activated dihydropyridine-insensitive (Ca_V2) channels; and (3) the low-voltage activated (T-type, Ca_V3) channels (Catterall *et al.*, 2005; Alexander *et al.*, 2007). The most abundantly expressed Ca_V in the ileum are of the L-subtype (Ca_V-L) (Bolton, 2006; Tomita, 1981), recently reported as $Ca_V1.2$ (Catterall *et al.*, 2005).

As β-norlapachone was the most potent derivative, we decided to investigate which subtype of Ca_V is involved in the spasmolytic action mechanism. To evaluate if the Ca_V involved in the response of β-norlapachone was of the L-subtype, the effect of the naphthoquinone on S-(-)-Bay K8644 pre-contracted ileum was investigated. S-(-)-Bay K8644 is an L-type Ca_V agonist that acts by direct binding to the channel's α_1 subunit and not by depolarization (Spedding and Paoletti, 1992). Under these conditions β-norlapachone induced a concentration-dependent relaxation [$EC_{50} = (1.4 \pm 0.1) \cdot 10^{-5}$ M, Fig. 3), suggesting that at the least functional level the L-type Ca_V are involved.

Recent experiments have shown that reactive oxygen species (ROS) stimulate signal transduction processes for transcription factor activation, gene expression, muscle contraction, cell growth, apoptosis, and chemotaxis. Oxidants elicit different biological responses depending on their concentrations. For example, low doses of oxidants may elicit cell growth, higher levels induce apoptosis, and even higher concentrations cause necrosis. Similarly, low doses of oxidants induce or enhance muscle contraction whereas higher levels suppress the ability of muscles to contract (Suzuki and Ford, 1999).

As quinones induce oxidative stress (Thompson, 1971, 1997; Goijman and Stoppani, 1985; Kappus, 1986; Marr and Docampo, 1986; Monks *et al.*, 1992; Abreu *et al.*, 2002) we hypothesized that β-norlapachone could induce the relaxation of guinea-pig ileum and did not to block the Ca_V directly, but by increasing the oxidative stress through the production of free radicals.

If this possibility was correct, glutathione, an antioxidant agent (Gaté *et al.*, 1999), should interfere in the response of relaxing β-norlapachone. Interestingly, glutathione was unable to change the effect of this naphthoquinone. That is, β-norlapachone-induced relaxation has not changed in the presence of this antioxidant agent, suggesting that β-norlapachone causes relaxation of guinea-pig ileum by a mechanism independent of free radicals. This fact is very interesting since the induction of oxidative stress could cause various pathological processes (Freeman and Crapo, 1982).

Moummi *et al.* (1991) reported that depending on the way glutathione was incubated its action on the oxidants was different. To produce its inhibitory action, glutathione had to be mixed with the oxidant before addition to the tissue baths. Preincubation of the tissue with glutathione, followed by addition of the oxidant to the bath did not prevent the response to the reactive oxygen metabolites. It is possible that glutathione may adhere to the tissue and/or permeate the cell and react with other sites, thus preventing it from reacting with the oxidant. Therefore, to establish whether glutathione was preventing β-norlapachone to produce free radicals, glutathione was mixed with β-norlapachone before addition to the tissue baths.

Interestingly, even though glutathione was mixed with β-norlapachone previously, its was unable to prevent the relaxant effect produced by this furane naphthoquinone, confirming that β-norlapachone exerts its spasmolytic effect on the guinea-pig ileum by a mechanism that does not involve free radicals.

In summary, we demonstrated for the first time that, on the guinea-pig ileum, norlapachol, α-norlapachone, β-norlapachone, and HH-norlapachol have nonselective spasmolytic activity. At the functional level, β-norlapachone exerts this effect due to inhibition of calcium influx through Ca_V-L, but without the involvement of free radicals.

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