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Artigo

Spasmolytic activity of lapachol and its derivatives, α and β-lapachone, on the guinea-pig ileum involves blockade of voltage-gated calcium channels

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RESUMO: "Atividade espasmolítica do lapachol e seus derivados, α e β-lapachona, em íleo de cobaia envolve bloqueio dos canais de cálcio dependentes de voltagem". O lapachol, α e β-lapachona são naftoquinonas obtidas de espécies de *Tabebuia*, apresentam propriedades antiinflamatória, antibacteriana, anticâncer e tripanossomicida. O objetivo deste trabalho foi investigar um possível efeito espasmolítico destas naftoquinonas em íleo de cobaia, uma vez que, outras naftoquinonas inibem a atividade contrátil de músculos lisos. O lapachol, α e β-lapachona inibiram as contrações fásicas induzidas tanto por carbacol (CI_{50} = 1,5 ± 0,2 x 10^{-4}; 7,3 ± 0,9 x 10^{-5} e 3,2 ± 0,5 x 10^{-5} M, respectivamente) quanto por histamina (CI_{50} = 3,6± 0,5; 3,6 ± 0,7 e 3,3 ± 0,6 x 10^{-5} M, respectivamente). Estes compostos também relaxaram o íleo pré-contraído com KCl (CE_{50} = 1,2 ± 0,4; 4,3 ± 0,8 e 2,7 ± 0,2 x 10^{-5} M, respectivamente); carbacol (CE_{50} = 2,6 ± 0,7; 3,5 ± 0,5 e 2,2 ± 0,7 x 10^{-5} M, respectivamente) ou histamina (CE_{50} = 3,0 ± 0,8; 1,1 ± 0,3 e 3,3 ± 0,6 x 10^{-5} M, respectivamente) de maneira dependente de concentração. Este efeito é provavelmente devido à inibição do influxo de Ca^{2+} através dos canais de Ca^{2+} dependentes de voltagem (Ca_V). β-lapachona antagonizou (pD'$_2$ = 5,73 ± 0,12; "slope" = 1,51 ± 0,05) as contrações induzidas por $CaCl_2$ em meio despolarizante nominalmente sem Ca^{2+}. O achado de que a β-lapachona inibiu as contrações tônicas induzidas por S-(-)-Bay K8644 (CE_{50} = 1,4 ± 0,1 x 10^{-5} M) é sugestivo que o Ca_V envolvido é o do tipo L. Em conclusão, lapachol, α e β-lapachona apresentam atividade espasmolítica não seletiva em íleo de cobaia, e β-lapachona exerce este efeito pelo bloqueio dos canais Ca_V tipo L.

Unitermos: Lapachol, α-lapachona, β-lapachona, espasmolítico, íleo de cobaia, Ca_V tipo L.

ABSTRACT: Lapachol, α and β-lapachone are naphthoquinones extracted from species of *Tabebuia* that have shown antiinflammatory, antibacterial, anticancer and trypanosomicidal properties. The aim of this work was to investigate the spasmolytic effect of these naphthoquinones on the guinea-pig ileum, since other naphthoquinones are known to depress the contractile activity of smooth muscles. Lapachol, α and β-lapachone inhibited the phasic contractions induced by both carbachol (IC_{50} = 1.5 ± 0.2 x 10^{-4}; 7.3 ± 0.9 x 10^{-5} and 3.2 ± 0.5 x 10^{-5} M, respectively) and histamine (IC_{50} = 3.6± 0.5; 3.6 ± 0.7 and 3.3 ± 0.6 x 10^{-5} M, respectively). These compounds also relaxed the ileum pre-contracted with KCl (EC_{50} = 1.2 ± 0.4; 4.3 ± 0.8 and 2.7 ± 0.2 x 10^{-5} M, respectively); carbachol (EC_{50} = 2.6 ± 0.7; 3.5 ± 0.5 and 2.2 ± 0.7 x 10^{-5} M, respectively) or histamine (EC_{50} = 3.0 ± 0.8; 1.1 ± 0.3 and 3.3 ± 0.6 x 10^{-5} M, respectively) in a concentration-dependent manner. This effect is probably due to inhibition of calcium influx through voltage-gated calcium channels (Ca_V). β-lapachone antagonized (pD'$_2$ = 5.73 ± 0.12; slope = 1.51 ± 0.05) $CaCl_2$-induced contractions in depolarizing medium nominally without Ca^{2+}. The finding that β-lapachone inhibited the tonic contractions induced by S-(-)-Bay K8644 (EC_{50} = 1.4 ± 0.1 x

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10^{-5} M) is suggestive that the L-type Ca_V is involved. In conclusion, lapachol, α and β-lapachone showed non-selective spasmolytic activity in guinea-pig ileum, and β-lapachone exerts this effect by to blockade of L-type Ca_V channels.

Keywords: Lapachol, α-lapachone, β-lapachone, spasmolytic, guinea-pig ileum, L-type Ca_V.

INTRODUCTION

Lapachol (2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone) is a naphthoquinone extracted from the bark and wood of *Tabebuia* spp. (Bignoniaceae) popularly known as Pau d'arco, Ipê-roxo, Lapacho, among others. Several species that contain lapachol and other naphthtoquinones are extensively used in folk medicine for the treatment of cancer, lupus, infections, wound healing and many other illnesses (Duke, 1985; Morais et al., 2005; Agra et al., 2007; Oliveira et al., 2007). Among the naturally occurring naphthoquinones in *Tabebuia* spp, lapachol, α-lapachone (2,2-dimethyl-2H-benzo[g]chromene-5,10-dione) and β-lapachone (2,2-dimethyl-3,4-dihydro-2H-benzo [h]chromene-5,6-dione) are the most abundant.

A large spectrum of therapeutic activities has been attributed to lapachol and many of its heterocyclic derivatives, such as prevention of cercarial skin penetration of *Schistossoma mansoni* (Pinto et al., 1977; Lima et al., 2002), trypanosomicidal (Austin, 1974; Goijman & Stoppani, 1985; Saúde-Guimarães & Faria, 2007), antiinflammatory (Almeida, 1990; Moon et al., 2007), antimicrobial (Antunes et al., 2006), antineoplastic; antimalarial activity (Carvalho et al., 1988) and against enteroviruses (Pinto et al., 1987; Subramanian & Ferreira, 1998; Teixeira et al., 2001).

The pharmacological activities of *Tabebuia* species are often related to the presence of saponins, flavonoids, coumarins and natural antibiotics (Miranda et al., 2001; Machado et al., 2003; Falcão et al., 2005), while the chemical profile presented by most to these studies have shown the quinones as the main active substances (Santana et al., 1968; Ueda et al., 1994; Pinto et al., 2000; Miranda et al., 2001; Machado et al., 2003).

In a previous study Auyong et al. (1963) described that the juglone (5-hydroxy, 1-4 naphthoquinone), isolated from *Juglans nigra,* dilates coronary arteries of the rabbit heart and depresses the activity of smooth muscle of rat intestine and uterus. Moreover, vitamins K1 (2-methyl-3 methyl-fitil-1,4-naphthoquinone) and K3 (2-methyl-1,4-naphthoquinone) exerted spasmolytic action on coronary vessels of rats (Lider et al., 1987). Additionally, 7-methyl-juglone and plumbagin (2-methyl-juglone) showed strong spasmolytic activity (Krahl & Gordonoff, 1955; Neuhaus-Carlisle et al., 1997; Krenn et al., 1998). Therefore the aim of the present study was to evaluate the spasmolytic activity of the lapachol, α and β-lapachone on the guinea-pig ileum, since no other information on the agent's spasmolytic activity has been reported for lapachol and its derivatives.

MATERIAL AND METHODS

General

The tissues were suspended in 6 mL organ baths under a resting load of 1.0 g at 37 °C. Force generation was monitored using an isometric transducer (7003-Ugo Basile, Italy) coupled to a polygraph (7070-Ugo Basile, Italy). The modified Krebs solution (mM): NaCl (117.0), KCl (4.7), $MgSO_4.7H_2O$ (1.3), $NaH_2PO_4.H_2O$ (1.2), $CaCl_2.2H_2O$ (2.5), glucose (11.0), $NaHCO_3$ (25.0); and high-K^+ isosmotic solution for KCl 70 mM: NaCl (51.7), KCl (70.0), $MgSO_4.7H_2O$ (1.3), $NaH_2PO_4.H_2O$ (1.2), glucose (11.0), $NaHCO_3$ (25.0) were bubbled with a 95 % O_2 and 5 % CO_2 gas mixture continuously. All experimental procedures were performed in accordance with the guidelines approved by the Animal Research Ethic Committee of LTF/UFPB (Protocol CEPA/LTF Nº 0706/06).

Drugs

All buffer salts were purchased from Vetec (Rio de Janeiro, RJ, Brazil). S-(-)-Bay K8644; cremophor and histamine dihydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lapachol was isolated from the bark of *Tabebuia* spp. and α and β-lapachone were synthesized as previously described (Camara et al., 2001; Hooker, 1936). Stock-solutions of all the chemicals were prepared in distilled water. All stock-solutions were stored at 0 °C and the working solutions were freshly prepared daily.

Effect of the naphthoquinones on carbachol- and histamine-induced phasic contractions

Adult guinea-pigs (*Cavia porcellus,* 300-500 g) of both sexes were fasted for about 18 h (only water was given to them during this period). Animal were then killed by cervical dislocation and exsanguinated. The distal ileum was excised rapidly and carefully washed with modified Krebs solution (pH 7.4) at room temperature. Segments of the ileum oriented along their longitudinal axis (2-3 cm in length) were suspended in a 6 mL organ bath, which contained modified Krebs solution maintained at 37 °C, and allowed to stabilize

for 30 min. Two phasic contractions were obtained for 1 μM of carbachol or histamine with intervals of 15 min among them. Lapachol, α or β-lapachone 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 procedure was repeated in the absence and in the presence of various concentrations of the substances. The molar concentration of a substance that inhibits the response to an agonist by 50 % (IC_{50}) was obtained by non-linear regression from the individual values of inhibition for each substance.

Effect of the naphthoquinones 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. Contractile agents remained in contact with the preparation until a plateau of contraction was reached (approximately 8 min), and then the tissue was washed. After a further 30 min the process was repeated lapachol, α or β-lapachone were added cumulatively (10^{-7} up to 3 x 10^{-4} M) at the plateau phase, in different preparations. Relaxation was expressed as the reverse percentage of initial contraction elicited by contractile agents. The molar concentration of a substance that produces 50 % of its maximal possible effect (EC_{50}) was obtained graphically from concentration-response curves.

Effect of β-lapachone on Ca^{2+}-induced contractions in depolarizing medium nominally without Ca^{2+}

After a 30 min stabilization period, modified Krebs solution 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 were then induced at 60 min interval. After this procedure the organ baths were washed and several concentrations of β-lapachone were incubated for 15 min in different preparations and then a third $CaCl_2$ cumulative curve was obtained. The maximal contraction obtained with the first concentration-response curve to $CaCl_2$ was considered as 100 %, and all contractions were calculated proportionally to this value.

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

The guinea-pig ileum was prepared as described before. After stabilizing 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 S-(-)-Bay K8644, a selective L-type Ca^{2+}-channel agonist (Conte-Camerino et al., 1987) was induced. During the stabilization of the tonic phase of this contraction, β-lapachone was added cumulatively in order to obtain a concentration-response curve. Relaxation was expressed as described before.

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 IC_{50} and EC_{50} values were determined by non-linear regression (Jenkinson et al., 1995). Differences between means were statistically compared using Student's t-test 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 non-competitive when the slope of the Schild's plot was significantly different from unity (Arunlakshana & Schild, 1959) and depression of the maximum response was observed.

RESULTS

Effect of the naphthoquinones on carbachol- or histamine-induced phasic contractions

All the compounds tested antagonized in a significant ($p < 0.05$) and concentration-dependent manner (10^{-7} - 3 x 10^{-4} M) the phasic contractions induced by 1 μM carbachol or histamine in guinea-pig ileum. The IC_{50} values for lapachol, α and β-lapachone were respectively of 1.5 ± 0.2 x 10^{-4}; 7.3 ± 0.9 x 10^{-5} and 3.2 ± 0.5 x 10^{-5} M to carbachol and 3.6 ± 0.5 x 10^{-5}; 3.6 ± 0.7 x 10^{-5} and 3.3 ± 0.6 x 10^{-5} M to histamine (Table 1). The responsiveness of the ileum was recovered 45 min after withdrawal of the naphthoquinones from the bath.

Effect of the naphthoquinones on KCl-, carbachol- or histamine-induced tonic contractions

Both lapachol and its derivatives α and β-lapachone (Figure 1) relaxed in a significant ($p < 0.05$) and dependent-concentration manner the ileum pre-contracted with 40 mM KCl (EC_{50} = 1.2 ± 0.4; 4.3 ± 0.8 and 2.7 ± 0.2 x 10^{-5} M, respectively) 1 μM carbachol (EC_{50} = 2.6 ± 0.7; 3.5 ± 0.5 and 2.2 ± 0.7 x 10^{-5} M, respectively) or 1 μM histamine (EC_{50} = 3.0 ± 0.8; 1.1 ± 0.3 and 3.3 ± 0.6 x 10^{-5} M, respectively). An analysis of the EC_{50} values indicates that α-lapachone was more potent in inhibiting the induced contractions for histamine than KCl and carbachol. Differently, lapachol and β-lapachone showed similar potency in inhibiting the contractions induced for these contractile agents.

Effect of β-lapachone on Ca^{2+}-induced contractions in depolarizing medium nominally without Ca^{2+}

Figure 2 shows the mean cumulative concentration-response curves for $CaCl_2$ alone and in the presence of different concentrations of β-lapachone (10^{-5}, 3 x 10^{-5}, 10^{-4} and 3 x 10^{-4} M). β-lapachone produced a non-parallel and concentration-dependent shift to the right of the concentration-response curve to $CaCl_2$ significantly reducing the maximal effect [E_{max} = 73.9 ± 6.2; 50.7 ± 6.5; 29.2 ± 4.4 and 19.6 ± 1.8]. Analysis of the data by linear regression yielded a correlation coefficient (r^2) of 0.81 ± 0.06. The pD'$_2$ and Schild slope values were 5.73 ± 0.12 and 1.51 ± 0.05, respectively, indicating a non-competitive blockade. The antagonism of β-lapachone was reversed after washing the preparation with depolarization medium for about 60 min (data not shown).

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

Cumulative addition of β-lapachone (3 x 10^{-8} - 3 x 10^{-4} M) on the tonic component of the contractions elicited by 3 x 10^{-7} S-(-)-Bay K8644 M resulted in a concentration-dependent relaxation. This relaxant effect was more potent ($p < 0.05$) than when the contraction was evoked by KCl 40 M (Figure 3). The EC_{50} values of β-lapachone were 1.4 ± 0.1 and 2.7 ± 0.2 x 10^{-5} M against S-(-)-Bay K8644 and KCl, respectively.

DISCUSSION

In the present work, the spasmolytic effect of lapachol, α and β-lapachone, was investigated on intestinal smooth muscle. The most important finding is the demonstration for the first time that these naphthoquinones show non-selective spasmolytic action on the guinea-pig ileum, and that the mechanism of action of β-lapachone is due to the inhibition of Ca^{2+} influx probably through L-type Ca_v channels.

A comparison of the IC_{50} values on carbachol-induced phasic contractions shows that α and β-lapachone were more potent than lapachol, being β-lapachone the most potent one. However, when the contractions were evoked by histamine, no significant difference was observed between them. So, it would be reasonable to affirm that naphthoquinones have a non-selective spasmolytic effect to the agonists tested.

On the other hand, lapachol and α-lapachone were more potent (3 and 2 fold, respectively) in inhibiting the phasic contractions induced by histamine than those induced by carbachol. The absence of significant difference between the IC_{50} values of β-lapachone on carbachol- or histamine-induced phasic contractions in guinea-pig ileum suggests that β-lapachone may be acting on a common pathway related to the cascade of events that leads to smooth muscle contraction by these agonists.

The major trigger for smooth muscle contraction is a rise in intracellular calcium concentration ($[Ca^{2+}]_i$. Increased $[Ca^{2+}]_i$ enhances binding of Ca^{2+} to calmodulin (CaM) and this complex activates MLC kinase (MLCK) to phosphorylate MLC and promote interaction of myosin II with actin, leading to cross-bridge cycling and thereby causing contraction. In smooth muscle, contraction can be achieved either via K^+-depolarization of the membrane, leading to an increase in $[Ca^{2+}]$i and muscle contraction, or by agonist-induced contraction which can be membrane potential-independent. Agonists such as serotonin, carbachol, and histamine bind to G protein coupled receptors (GPCRs) and activate the phosphoinositide cascade, usually by Gq mediated production of inositol (1,4,5)-trisphosphate (IP_3), which stimulates Ca^{2+} release from the sarcoplasmic reticulum (SR). Contractile agonists can also elevate Ca^{2+} release through ryanodine receptors and stimulate Ca^{2+} entry through multiple channel types, including voltage-, receptor-, and store-operated Ca^{2+} channels (Watterson et al., 2005).

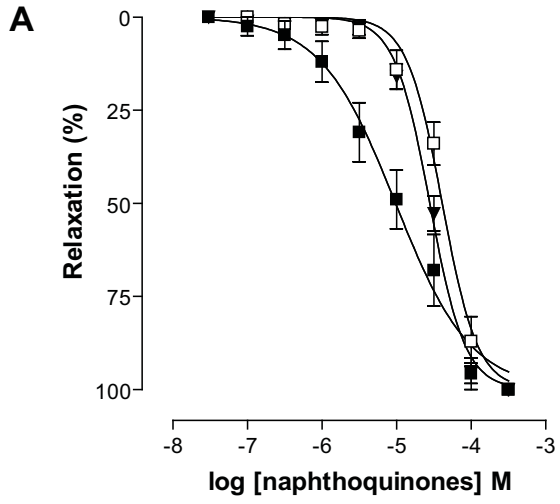
As the maintenance of tonic contraction induced by these contractile agents involves Ca^{2+} influx through voltage-gated Ca^{2+} channels (Bolton, 1979; Bolton et al., 2006), it is suggestive that naphthoquinones-induced relaxation in smooth muscle may be due to the blockade of Ca^{2+} influx through these channels. In order to verify this hypothesis, we evaluated their effect on the tonic component of the contractile response induced by KCl, carbachol or histamine on the guinea-pig ileum. As shown in Figure 1, all the naphthoquinones tested inhibited in a concentration-dependent manner the tonic contractions induced by all contractile agents. Independent of whether the contraction is evoked by either pharmacomechanical or electromechanical coupling, the maintenance of the tonic component involves activation of the Ca_V channel (Rembold, 1996). Therefore, we can suggest that these naphthoquinones may inhibit the Ca^{2+} influx through these channels to produce non-selective spasmolytic effects.

Since β-lapachone showed similar potency at inhibiting both phasic and tonic contractions, we decided to further investigate its action mechanism. The ileum is

Table 1. Concentration values of lapachol and derivatives that reduce to 50 % a maximal response of carbachol- or histamine-induced phasic contractions (IC_{50}) (n = 5).

	IC_{50} (M)	
Naphthoquinones	Carbachol	Histamine
Lapachol	1.5 ± 0.2 x 10^{-4} [a]	3.6 ± 0.5 x 10^{-5}**
α-Lapachone	7.3 ± 0.9 x 10^{-5} [c]	3.6 ± 0.7 x 10^{-5}*
β-Lapachone	3.2 ± 0.5 x 10^{-5} [b]	3.3 ± 0.6 x 10^{-5}

Student's *t*-test, *$p < 0.05$; **$p < 0.001$ (carbachol *vs.* histamine); carbachol: [a]lapachol *vs.* α-lapachone, [b]lapachol *vs.* β-lapachone, [c]α-lapachone *vs.* β-lapachone.



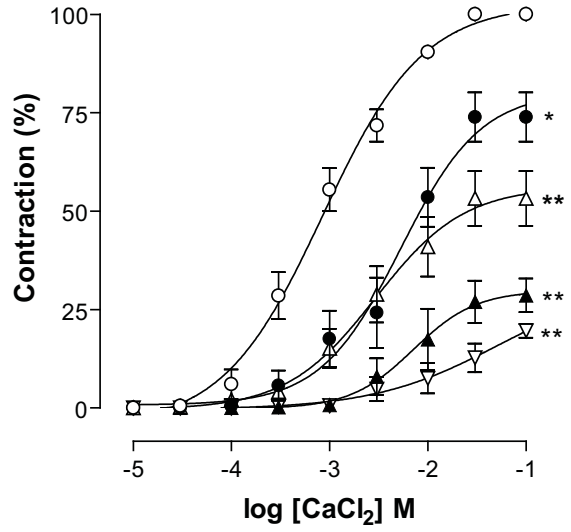
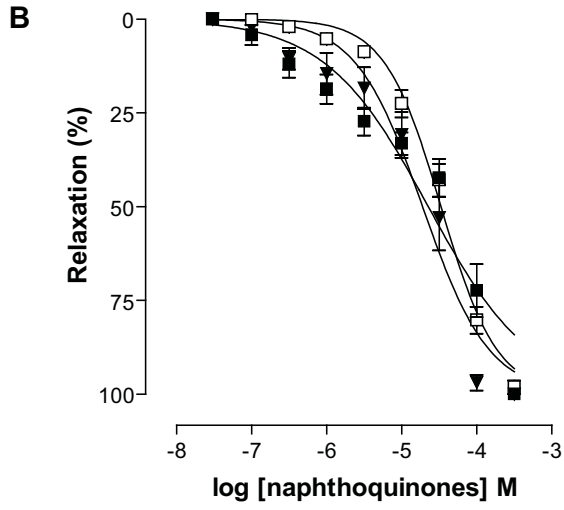


Figure 2. Cumulative concentration-response curves to $CaCl_2$ with tissue strips bathed in depolarizing medium nominally without Ca^{2+} in the absence (○) and presence of the β-lapachone 10^{-5} (●), 3 x 10^{-5} (△), 10^{-4} M (▲) and 3 x 10^{-4} M (▽) (n = 5). Symbols and vertical bars represent mean and S.E.M., respectively. One-way ANOVA followed by Bonferroni's test, significant differences are indicated by $^{*}p < 0.05$ and $^{**}p < 0.001$ (control *vs.* β-lapachone).



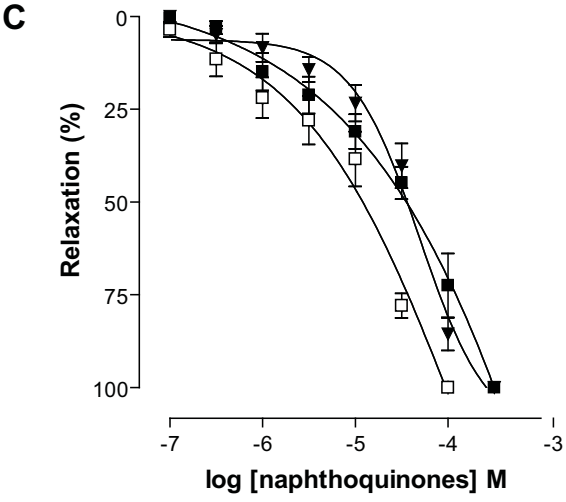


Figure 1. Effect of the lapachol (■), α-lapachone (□) and β-lapachone (▼) on the tonic contractions induced by 40 mM KCl (**A**), 1 μM carbachol (**B**) and 1 μM histamine (**C**) (n = 5). Symbols and vertical bars represent the mean and S.E.M., respectively.

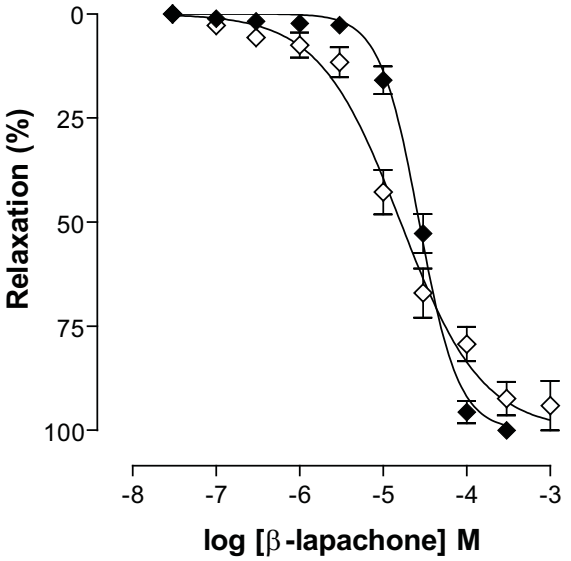


Figure 3. Effect of β-lapachone on the tonic contraction elicited by 40 mM KCl (◆) or 3 x 10^{-7} M S-(-)-Bay K8644 (◇) (n = 5). Symbols and vertical bars represent mean and S.E.M., respectively.

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 Ca^{2+} influx through Ca_V, we tested the hypothesis that β-lapachone could be acting by blocking Ca^{2+} influx through Ca_V. This hypothesis was confirmed by the observation that β-lapachone ($pD'_2 = 5.73 \pm 0.12$) inhibited Ca^{2+}-induced contractions in a depolarizing medium nominally without Ca^{2+}, showing a non-competitive antagonism (slope = 1.51 ± 0.05) with a shift of the concentration-response curve to the right in a non-parallel and concentration-dependent manner, reducing significantly E_{max} (Figure 2). The most abundantly expressed voltage-gated Ca^{2+} channels in the ileum are of the L-subtype (Ca_V-L) (Bolton, 1979; Tomita, 1981), recently reported as Ca_V 1.2 (Caterral et al., 2005). To evaluate if the Ca_V channel involved in the response of β-lapachone was of the L-subtype, the effect of the naphthoquinone on S-(-)-Bay K8644-precontracted ileum was investigated. S-(-)-Bay K8644 is an L-type Ca_v agonist that acts by directly binding to the channel´s α_1 subunit and not by depolarization (Spedding & Paoletti, 1992). In these conditions β-lapachone induced a concentration-dependent relaxation ($CE_{50} = 1.4 \pm 0.1 \times 10^{-5}$ M, Figure 3), suggesting that the L-subtype channel is involved. The observation that β-lapachone was more potent in relaxing the ileum pre-contracted with S-(-)-Bay K8644 than with KCl can be explained by the fact that KCl, in addition to induce Ca_V-L activation by depolarization, utilizes other mechanisms to sustain the tonic phase of smooth muscle contraction, such as Ca^{2+} sensitization involving translocation and activation of RhoA Kinase (Ratz et al., 2005). On the other hand, S-(-)-Bay K8644 keeps contraction mainly by direct activation of Ca_V-L (Spedding & Paoletti, 1992).

In conclusion, we demonstrate for the first time that lapachol, α and β-lapachone have non-selective spasmolytic activity on the guinea-pig ileum. In the functional level, β-lapachone exerts this effect due to inhibition of Ca^{2+} influx through Ca_V-L.

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