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INHIBITORY EFFECT OF LINALOOL IN PREPARATIONS OF ISOLATED SMOOTH MUSCLE OF RAT TRACHEA WITH EPITHELIUM STIMULATED BY ELECTROMECHANICAL COUPLING

(Efeito inibitório do linalol em preparações isoladas de músculo liso traqueal de rato com epitélio estimulado por acoplamento eletromecânico)

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ABSTRACT

Plants have been used as source of therapeutic elements employed to treat several disorders, such as hypertension and asthma. The discovery of pharmacological agents that act on the contractility of airways smooth muscle can be considered to help in treating diseases of the respiratory tract. In this study, we evaluated the effect of linalool, a terpenic constituent of various aromatic and medicinal plants as an antispasmodic agent in preparations of isolated rat trachea. Linalool fully reversed the electromechanical induced contraction in isolated preparations with (188.46±13.05 µM) and without (135.21±12.76 µM) epithelium. A lower inhibitory potency was observed in phamaco mechanical contractions induced in tracheal rings with preserved epithelium (261.34±38.22 µM) or not (593.45±11.32 µM). Linalool (100 µM and 1000 µM) was able to inhibit the curves of controlled influx for calcium and barium. In the present study, the inhibitory effect of linalool on pre-contracted rat tracheal rings was more effective in electromechanical conditions, suggesting a possible effect of this monoterpene on calcium influx through voltage-dependent channels.

Keywords: Monoterpene, smooth muscle, trachea.

RESUMO

As plantas têm sido utilizadas como fonte de elementos terapêuticos empregados para tratar vários distúrbios, como hipertensão e asma. A descoberta de agentes farmacológicos que atuam sobre a contratilidade do músculo liso das vias aéreas pode ser útil no tratamento de doenças do trato respiratório. Neste estudo, avaliamos o efeito do linalol, um constituinte terpênico de várias plantas aromáticas e medicinais como agente antiespasmódico em preparações de traqueia de rato isolada.

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O linalol reverteu completamente a contração eletromecânica induzida em preparações isoladas com (188,46±13,05 μM) e sem epitélio (135,21±12,76 μM). Observou-se uma menor potência inibitória nas contrações fármaco mecânicas induzidas em anéis traqueais com epitélio preservado (261,34±38,22 μM) ou não (593,45±11,32 μM). O linalol (100 μM e 1000 μM) foi capaz de inibir as curvas de influxo controlado para cálcio e bário. No presente estudo, o efeito inibitório do linalol em anéis traqueais de ratos pré-contraídos foi mais eficaz em condições eletromecânicas, sugerindo um possível efeito deste monoterpeno sobre o influxo de cálcio através de canais dependentes da tensão.
Palavras-chave: Monoterpeno, músculo liso, traqueia.

INTRODUCTION

The essential oils of Brazilian plant biodiversity, such as *Aniba rosaeodora* (Rosewood) have the terpenic constituent linalool (ALMEIDA *et al*., 2013; BAKKALI *et al*., 2008). Linalool (LNL), or 3,7-dimethyl-1,6-octadien-3-ol is an acyclic tertiary monoterpene alcohol having two active chemical isomers. Biological effects of linalool-rich rosewood oil include sedative (DE ALMEIDA *et al*., 2009), anticancer (SOEUR *et al*., 2011) and cardiovascular actions (DE SIQUEIRA *et al*., 2014). These cardiovascular effects are related to the induction in vivo of a vago-vagal reflex in addition to vasodilatory properties in isolated smooth muscle preparations of rat aorta (DE SIQUEIRA *et al*., 2014).

The constituent linalool is able to relax the smooth muscle of isolated vascular preparations from rats (ANJOS *et al*., 2013). This vaso relaxant effect was also observed in mouse aortic rings and involves the participation of soluble guanylyl cyclase and K+ channels (KANG and SEOL, 2015). Therefore, this study aimed to investigate the effects of linalool on smooth muscle preparations of rat trachea stimulated by electromechanical and pharmaco mechanical coupling.

MATERIAL AND METHODS

Ethical Aspects

This study was conducted in compliance with the international rules established by the Guide for the Care and Use of Laboratory Animals and was submitted and approved by the Ethics Committee for the Use of Animals of State University of Ceará with the protocol number 10244898-1.

Animals

In this study were used male Wistar rats (250±50g) from the local colonies maintained in the Federal

University of Ceará, Fortaleza, Brazil. These animals were maintained in polypropylene boxes, at a temperature of 24±2 ºC, fed with ration and water *ad libitum*, and at a light-dark cycle of 12 houres.

Isolated Preparations of Trachea Smooth Muscle

Animals were euthanized by pentobarbital anesthesia (50 mg/kg, i.p.) followed by fast aorta exsanguination. The trachea were dissected, isolated in Petri dishes with nutrient solution and divided in sections of 5 mm length allocated in steel triangular devices (0.3 mm diameter) coupled with isometric force transducers (ML870B60/C-V, ADInstruments, Bella Vista, Australia) and a data acquiring system (PowerLab 8/30, ADInstruments, Bella Vista, Australia). The tracheal rings were placed in 5 mL organ baths of Tyrode at 36.5 ºC and continuously bubbled with a 95% O_2 and 5% CO_2 air mixture generated by a perfusion system. The epithelium mechanical denudation was performed with a slight friction in the lumen of the tracheal ring and confirmed after histological analysis.

Experimental Protocols

Series 1: Preparations with or without epithelium were stimulated by the electromechanical (added KCl 80 mM) or pharmaco mechanical (added carbachol 1 µM) coupling. Once identified the maximal contraction, cumulative concentrations of LNL (1 to 3000 µM) were added to the preparation. After the LNL application, Tyrode washings were performed in every 15 min. during the recuperation period of 60 min or longer, if necessary. Only then, a new contraction was stimulated with KCl 80 mM, in order to assess the tissue response after the protocol.

Series 2: In another experimental series designed to reinforce the mechanism of action of LNL related to voltage-operated calcium channels (VOCC), tracheal rings with epithelium were maintained in Ca^{2+} - free medium in the presence of KCl (80 mM) and EGTA (1 mM). After 20 min Ca^{2+} (0.1 to 10 mM) was cumulatively added to the organ bath in the absence or presence of LNL (10 µM, 100 µM and 1000 µM). In another group of preparations, Ca^{2+} was replaced by the cumulative addition of Ba^{2+} (0.1 to 10 mM).

Solutions and Drugs

All the salts and substances necessary for this study, and the LNL in its racemic form as well, were acquired from Sigma Chemical Corporation (St. Louis, USA) and Reagen (Rio de Janeiro,

BRAZIL). Tissues were maintained in modified Tyrode preparation composed of (in mM) NaCl 136.0; KCl 5.0; $MgCl_2$ 0.98; NaH_2PO_4 0.36; $NaHCO_3$ 11.9; $CaCl_2$ 2.0; glucose 11.0, with the due modifications necessary for the concentration-effect ratio experiments of calcium (Ca^{2+}) and barium (Ba^{2+}). The pH of the solution was stabilized in 7.4 (37 ^oC; continuous bubbling with 5% CO_2 in 95% O_2) before the experiments. The LNL was dispersed and homogenized in nutrient solution applied in the protocols added Tween 80 in a 0.5% proportion. The other substances were diluted or dispersed in distilled water.

Statistical Analysis

The results were expressed as mean ± SEM and n indicates the number of experiments. For each protocol, the IC_{50} (i.e., the concentration of LNL at which 50% of a contractile response was inhibited) values were calculated by logarithm interpolation. Paired or unpaired Student's t-tests and One-way ANOVA followed by Bonferroni *post-hoc* test were used for comparing, respectively, two groups and more than two groups. Statistical significance was accepted at $p<0.05$. The data were analyzed using Sigma Plot 10 (Systat Software Inc., Chicago, USA).

RESULTS AND DISCUSSION

The LNL, but not the vehicle, was able to reverse the contraction evoked by KCl-induced electromechanical coupling (Fig. 1). According to the results, both reparations with the preserved epithelium (Fig. 1-A) as well as in the ones where this tissue was removed (Fig. 1-B), the LNL was capable of reversing the contraction. In both preparations, the relaxing occurred significantly from the concentration of 100 µM, achieving complete reversion at 600 µM of LNL. There was no statistically significant difference in IC_{50} values when the reversals of the electromechanical coupling of epithelial (188.46±13.05 µM) and non-epithelial (135.21±12.76 µM) preparations were compared.

The contraction induced by the pharmaco mechanical coupling was reversed by LNL, but not by its vehicle (Fig. 2). For reversal of carbachol precontraction, IC_{50} was significantly lower (261.34±38.22 µM) in preparations with epithelium compared to tracheal preparations epithelium-denuded (593.45±11.32 µM). With the pharmaco mechanical induction, the results also presented contraction reversal in the preparations with (Fig. 2-A) or without the epithelial tissue (Fig. 2-B).

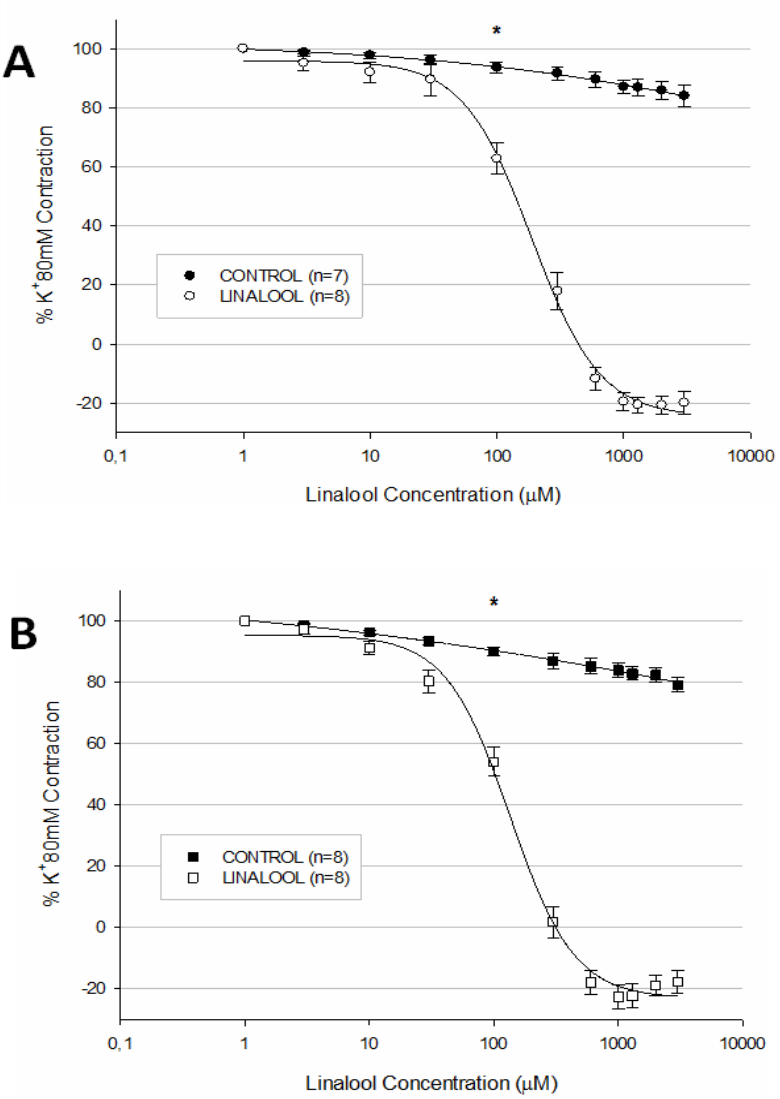

Figure 1: Antispasmodic effect of growing and cumulative concentrations (1 to 3000µM) of LNL on the contraction induced by K^+80mM of tracheal rings isolated from rat.

The data are presented as means±SEM ($p<0.05$). (A) Preparation with the preserved epithelium and; (B) Preparation without epithelium.

Statistical significance was observed between the preparations. In the experiment with the epithelium, the concentration of 30 µM firstly demonstrated significant relaxing, but the reversal, unlike the other experiments, was not complete (2.43±2.73%). In the experiments without the epithelium, the relaxing was not significant until the concentration of 300 µM was achieved, reaching full reversal at the concentration of 2000 µM.

In the concentration-response curves of Ca^{2+} (Fig. 3-A), only the 10 µM LNL concentration did not have a significant difference when compared to control, even though the curve was dislocated to the right. The concentrations

of 100 µM and 1000 µM were significantly different in reducing the effect of the cumulative concentrations of Ca^{2+}, always in comparison to control group.

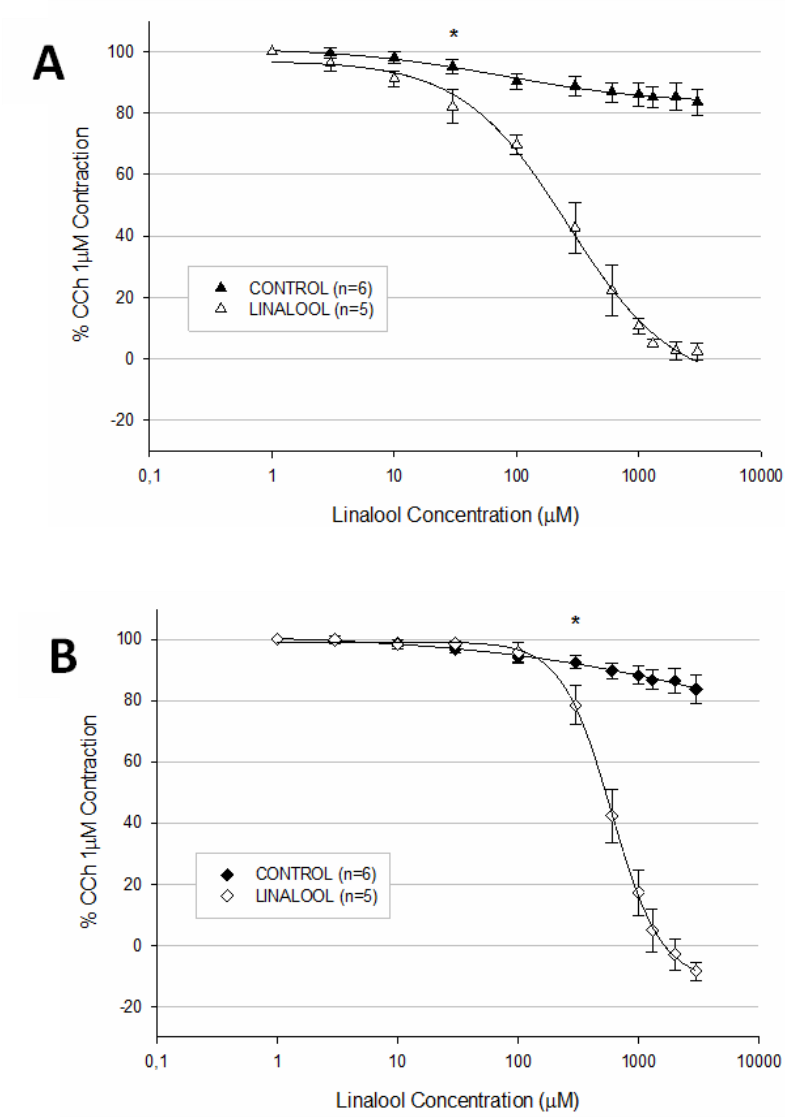

Figure 2: Antispasmodic effect of growing and cumulative concentrations (1 to 3000µM) of LNL on the contraction induced by CCh 1µM of tracheal rings isolated from rat. The data is presented as means±SEM.

(*) Concentration that presented significant difference ($p<0.05$) between control and experimental group. (A) Preparation with the preserved epithelium and (B) preparation without epithelium.

Occurrences on the concentration-response curves for Ca^{2+} were similar to those observed in the protocol for Ba^{2+} (Fig. 3-B). The difference observed was in the greater dislocation to the right in the concentration of 10 µM LNL used, with the statistical difference found until the 3

mM Ba^{2+} concentration, reaching similar results as the control group in the final concentrations. Other LNL concentrations (100 µM and 1000µM) were able to inhibit the contraction after adding more Ba^{2+}. Even without significant difference when the effects of the same concentrations between the Ca^{2+} and Ba^{2+} were compared, it appeared that the blocking in the latter ion curve was greater.

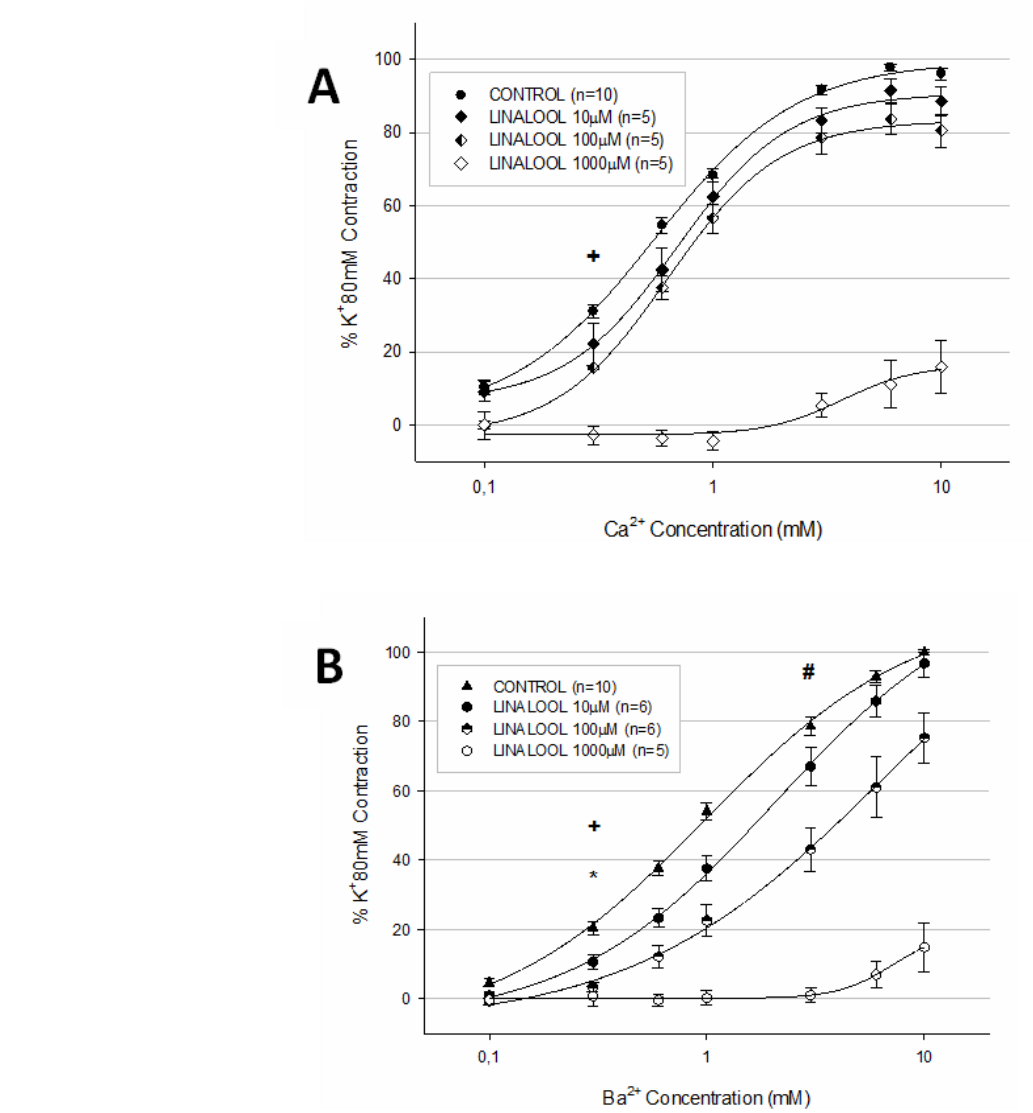

Figure 3: Effect of three concentrations (10, 100 and 1000µM) of LNL on the growing and cumulative concentrations (0.1 to 10mM) of Ca^{2+}(A) and Ba^{2+}(B) on the contraction induced by $K^{+}80$ of the tracheal rings isolated from rat.

The data is presented as means±SEM. (*-#) Interval of concentrations that presented significant difference ($p<0.05$) between the control and experimental group of 10µM LNL. (+) Concentration that presented significant difference ($p<0.05$) between control and experimental group of 100µM and 1000µM LNL.

We could infer that the LNL presented greater relaxant effect in the inductions by electromechanical induction than in those with the pharmaco mechanical contraction since the IC_{50} obtained from the preparations induced by K^+ 80mM were lower than the ones achieved with the CCh stimulation. In addition, statistical differences were observed in comparing the IC_{50} found in both types of induction in the presence of epithelium which was also observed when that tissue was removed.

The differences found between the pharmacological potencies in the results of electromechanical and pharmaco mechanical protocols, may have occurred because ligand stimulation activates intrinsic mechanisms, such as IP_3, cADPR and the RHOK route, besides the opening of calcium membrane channels producing a more sustainable contraction (GOSENS *et al*., 2006; MCFADZEAN and GIBSON, 2002). If this premise endure, and according to the effect on the electromechanical coupling discovered, we might conclude that the LNL did not possess a blocking activity on the muscarinic receptors.

When the most probable mechanisms involving the tracheal smooth muscles were stipulated, the true participation of the epithelium in the muscle relaxing effect of LNL could not be concluded. In the protocols of contraction induced by K^+ 80, the preparations in which the epithelium was removed achieved more potent values, than those in which this tissue was preserved, while in the CCh stimulations, the opposite effect was observed.

Based on the results obtained and that the epithelium releases factors that may act in specific receptors, such as endothelin and gases such as NO (GOLDIE *et al*., 1990; JANSSEN and KILIAN, 2006), this tissue may even potentiate the antispasmodic activity of the LNL in the organism. How the compound acts on the epithelium to achieve the relaxing effects could not be asserted. Actually, since the significant reversal was observed in all four previous situations, we might suggest that the LNL acts directly on the smooth muscle, activating processes that initiate the muscle relaxing or blocking the ionic channels.

This may have occurred due to a blocking in the K^+ channels, often observed in elevated concentrations of Ba^{2+} ion. However, the elevated inhibition in both curves – when stimulated by K^+ 80 – suggested that LNL had an effect on ion flux VOCC type Ca^{2+} channels, activated by electromechanical coupling and the only by which the Ba^{2+} ions permeate

(JANSSEN *et al*., 2004; MURRAY and KOTLIKOFF, 1991).

In conclusion, LNL is capable of reversing the pre-contraction induced by different agents in isolated tracheal preparations. The difference between the two IC_{50} was significant and an elevated dislocation to the right of the preparation without epithelium occurred, indicating that, for this coupling form, the participation of the epithelium-derived factor is important for the relaxing promotion. This inhibitory effect of LNL was more potent in preparations on electromechanical challenge. These findings suggest that linalool interfere with membrane calcium influx through voltage-dependent channels.

CONFLICT OF INTEREST

The authors of this article declare that there is no potential conflicts of interest including employment, consultancies, stock ownership, honoraria, paid expert testimony and patent applications/registrations related to the current manuscript. This manuscript is submitted on behalf of all authors.

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