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Ent-7α-acetoxytrachyloban-18-oic acid and *ent*-7α-hydroxytrachyloban-18-oic acid from *Xylopia langsdorfiana* A. St-Hil. & Tul. modulate K^+ and Ca^{2+} channels to reduce cytosolic calcium concentration on guinea pig ileum

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ABSTRACT

In this study we investigated the mechanism underlying the spasmolytic action of *ent*-7α-acetoxytrachyloban-18-oic acid (trachylobane-360) and *ent*-7α-hydroxytrachyloban-18-oic acid (trachylobane-318), diterpenes obtained from *Xylopia langsdorfiana*, on guinea pig ileum. Both compounds inhibited histamine-induced cumulative contractions (slope = 3.5 ± 0.9 and 4.4 ± 0.7) that suggests a noncompetitive antagonism to histaminergic receptors. $CaCl_2$-induced contractions were nonparallelly and concentration-dependently reduced by both diterpenes, indicating blockade of calcium influx through voltage-dependent calcium channels (Ca_v). The Ca_v participation was confirmed since both trachylobanes equipotently relaxed ileum pre-contracted with S-(−)-Bay K8644 ($EC_{50} = 3.5 \pm 0.7 \times 10^{-5}$ and $1.1 \pm 0.2 \times 10^{-5}$ M) and KCl ($EC_{50} = 5.5 \pm 0.3 \times 10^{-5}$ and $1.4 \pm 0.2 \times 10^{-5}$ M). K^+ channels participation was confirmed since diterpene-induced relaxation curves were significantly shifted to right in the presence of 5 mM tetraethylammonium (TEA^+) ($EC_{50} = 0.5 \pm 0.04 \times 10^{-4}$ and $2.0 \pm 0.5 \times 10^{-5}$ M). ATP-sensitive K^+ channel (K_{ATP}), voltage activated K^+ channels (K_V), small conductance calcium-activated K_+ channels (SK_{Ca}) or big conductance calcium-activated K^+ channels (BK_{Ca}) did not seem to participate of trachylobane-360 spasmolytic action. However trachylobane-318 modulated positively K_{ATP}, K_V and SK_{Ca} ($EC_{50} = 1.1 \pm 0.3 \times 10^{-5}$, $0.7 \pm 0.2 \times 10^{-5}$ and $0.7 \pm 0.2 \times 10^{-5}$ M), but not BK_{Ca}. A fluorescence analysis technique confirmed the decrease of cytosolic calcium concentration ($[Ca^{2+}]_c$) induced by both trachylobanes in ileal myocytes. In conclusion, trachylobane-360 and trachylobane-318 induced spasmolytic activity by K^+ channel positive modulation and Ca^{2+} channel blockade, which results in $[Ca^{2+}]_c$ reduction at cellular level leading to smooth muscle relaxation.

1. Introduction

The genus *Xylopia* is reported in Brazil for its ethnomedicinal and pharmacological uses as diuretic and in skin conditions (Takahashi et al., 2006), antibiotic (Lima et al., 2006) and hypotensive activity (Nascimento et al., 2006). Several natural products were obtained from *Xylopia* species including diterpenes (Campos de Andrade et al., 2004), sesquiterpenes (Moreira et al., 2007), alkaloids (Silva et al., 2009) and flavonoids (Vega et al., 2007).

Xylopia langsdorfiana A. St-Hil. & Tul. (Annonaceae), a tree measuring between 5 and 7 m in height, is popularly known in Northeast Brazil as "pimenteira da terra". Some secondary metabolites as alkaloids, flavonoids and diterpenes (Silva et al., 2009; Tavares et al., 2006) were isolated from it. The diterpenes are compounds biologically poorly studied, showing activity as cytotoxic to tumor cell lines (Castello-Branco et al., 2009), antimicrobial (Li et al., 2005) and osteoclastogenesis blockers (Pan et al., 2006). Moreover, some diterpenes have shown hypotensive activity (Martinsen et al., 2010; Oliveira et al., 2006) and relaxant effect in isolated guinea pig trachea (Ribeiro et al., 2007).

Ent-7α-acetoxytrachyloban-18-oic acid (trachylobane-360) and *ent*-7α-hydroxytrachyloban-18-oic acid (trachylobane-318) are trachylobanes, a rare class of diterpene, isolated from the hexane phase of crude ethanolic extract of *X. langsdorfiana* stem bark. These natural products were studied about their possible spasmolytic

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activity and we observed that both trachylobanes were unable to exert spasmolytic action in male rat aorta or rat uterus, only trachylobane-318 relaxed guinea pig trachea and, interestingly, both compounds significantly and concentration-dependently inhibited contractions and relaxed pre-contracted guinea pig ileum (Santos et al., 2011).

Natural product preparations have historically been the major source of pharmaceutical agents. These products have pointed the way to the future, contributing with many significant advances in science and industry, which inspired the pursuit of recapturing their value (McChesney et al., 2007). Thus, in search for substances with potential therapeutic use for treating intestinal smooth muscle disorders, we aimed to elucidate the mechanisms underlying the spasmolytic action of trachylobane-360 and trachylobane-318 on guinea pig ileum.

2. Materials and methods

2.1. Plant material

Stems of *X. langsdorfiana* A. St-Hil. & Tul. were collected in Cruz do Espírito Santo municipality, Paraíba, Brazil, in July 2002. The plant material was identified by Prof. Maria de Fátima Agra, head of the Botany Section of the Laboratório de Tecnologia Farmacêutica Prof. Delby Fernandes de Medeiros (LTF). A voucher specimen (AGRA 5541) is deposited at the herbarium Prof. Lauro Pires Xavier (JPB) of the Universidade Federal da Paraíba (UFPB).

2.2. Isolation

Dried stems of *X. langsdorfiana* (4 kg) were exhaustively extracted with 95% ethanol. The solvent was evaporated to yield a dark syrup (60 g), which was successively partitioned with hexane, chloroform and ethyl acetate to yield 20, 16, and 12 g of crude residue, respectively. The hexane fraction was subjected to column chromatographic separation using hexane and hexane with increasing amounts of ethyl acetate as eluents and monitored with thin layer chromatography (TLC). Altogether, 95 fractions of 100 ml each were collected and distributed in 12 fractions (F-1–F-12). Fraction F-1 was recrystallized from methanol, yielding **1** (300 mg). Fraction F-4 was purified by preparative TLC with ethyl acetate–hexane (9:1) as developer to obtain compound **2** (56 mg). **1** and **2** were identified according to ^{1}H and ^{13}C nuclear magnetic resonance (NMR) data in spectral and chemical/physical comparison with data reported in literature. Thus, **1** was identified as trachylobane-360 (*ent*-7α-acetoxytrachyloban-18-oic acid) and **2** was identified as trachylobane-318 (*ent*-7α-hydroxytrachyloban-18-oic acid) (Fig. 1). Identification and NMR signal assignment were supported by the analysis of ^{13}C DEPT, ^{1}H–^{1}H COSY, HMQC, HMBC data and are described in literature by Tavares et al. (2006).

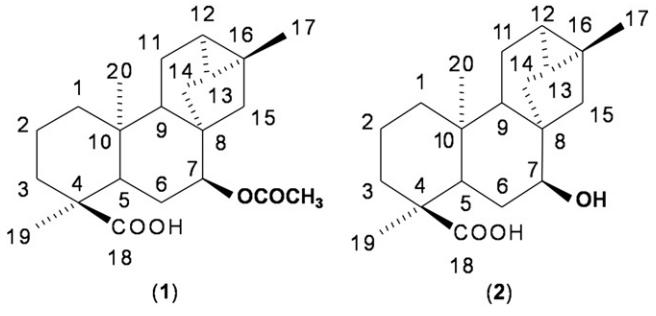

Fig. 1. Chemical structures of *ent*-7α-acetoxytrachyloban-18-oic acid (trachylobane-360) (**1**) and *ent*-7α-hidroxitrachyloban-18-oic acid (trachylobane-318) (**2**).

2.3. Solutions and drugs

Trachylobane-360 and trachylobane-318 were dissolved in Cremophor® and diluted in distilled water. In functional experiments, potassium chloride (KCl), hydrochloric acid (HCl), histamine dihydrochloride, apamin, tetraethylammonium chloride (TEA^{+}), 4-aminopyridine (4-AP), aminophylline, iberiotoxin and Cremophor® were dissolved and diluted in distilled water, while arachidonic acid (AA), S-(−)-Bay K8644 and glibenclamide were dissolved in ethanol and diluted in distilled water. The physiological solution was a freshly modified Krebs solution (pH 7.4) with the following composition (mM): NaCl (117.0), KCl (4.7), $MgSO_4$ (1.3), NaH_2PO_4 (1.2), $CaCl_2$ (2.5), glucose (11.0) and $NaHCO_3$ (25.0). A high K^{+} isosmotic solution (pH 7.4) of the following composition was also used: NaCl (51.7), KCl (70.0), $MgSO_4$ (1.3), NaH_2PO_4 (1.2), glucose (11.0) and $NaHCO_3$ (25.0). Concentrations are presented as final concentration in the bath solution after dissolving the pure substances directly into Krebs solution. In cellular experiments were used bovine fetal serum, Dulbecco's modified eagle medium (DMEM), penicillin, glutamine and trypsin/EDTA solution (1:250). Drugs were purchased from Sigma Aldrich, Reagen (Rio de Janeiro, RJ, Brazil), Cultilab (Campinas, SP, Brazil) and Vetec (Rio de Janeiro, RJ, Brazil).

2.4. Animals

Adult guinea pigs (*Cavia porcellus*) of both sexes from bioterium Prof. Thomas George of LTF/UFPB weighing 300–500 g were used. The animals had free access to food and water, were kept in rooms at 21 ± 1 °C submitted to a 12-h light–dark cycle and fasted for 18 h before the experiments. Actions on reducing pain, stress and any suffering were taken in accordance with the ethical guidelines for animal usage. All experimental procedures were previously approved and performed in accordance with the Animal Research Ethic Committee of LTF/UFPB guidelines (protocol CEPA 0101/08).

2.5. Measurement of ileum contractile activity

Guinea pigs were euthanized by cervical dislocation and exsanguination, the ileum being immediately removed, cleaned of adhering fat and connective tissue, immersed in physiological solution at room temperature and continuously gassed with carbogen mixture (95% O_2 and 5% CO_2). The longitudinal ileum layer was suspended in 5 ml organ baths under resting load of 1.0 g at 37 °C. Isotonic contractions were recorded using isotonic levers coupled to kymographs and smoked drums (DTF, Brazil). An isometric transducer (FORT-10) coupled to an amplifier (TMB4M), both from World Precision Instruments (EUA), connected to an analog/digital converter board (Biodata-Brazil) installed in computer with BioMed© software version RV2 were used to record isometric contractions. Tissues were allowed to stabilize for 30 min. The reversal of trachylobane-360 and trachylobane-318 spasmolytic effect was analyzed by their removal of strip organ bath then physiological solution was added, after 60 min a new contraction was induced and we observed that ileum responsiveness was not altered by trachylobanes (data not shown).

2.6. Cell culture

Guinea pig ileum was collected as described earlier. The longitudinal smooth muscle layer was carefully stripped off and the pieces placed in warmed physiological solution. The organ was successively washed with solution without Ca^{2+} and enriched with penicillin. Afterwards, tissue samples were placed in sterile culture bottles, to which was added 5 ml of DMEM culture medium supplemented with glutamine and 10% bovine fetal serum, and stored in CO_2 incubator. After 24 h, 5 ml of culture medium was added to the bottles (Chamley-Campbell et al., 1979). Each 48 h the bottles were washed with PBS and the culture medium was replaced. When the bottles

were confluent, the medium was removed, added 2 ml of trypsin for 2 min in incubator and the bottles were resuspended in culture medium and transferred to Falcon™ tubes for centrifugation (450×*g*) for 5 min. The supernatant was discarded and the pellets formed were used in the experiments. All procedures were performed in aseptic environment using laminar flow cabinet.

2.7. Experimental protocols

2.7.1. Effect of trachylobane-360 and trachylobane-318 on histamine-induced cumulative contractions

After stabilization, two similar cumulative concentration–response curves for histamine were induced and diterpenes were incubated, in different preparations, in the absence of histamine for 15 min in different concentrations as independent experiments. Afterwards, a new histamine cumulative curve was obtained in presence of each compound (3×10^{-5}, 10^{-4} and 3×10^{-4} M). The average amplitude of concentration–response curves for histamine was considered to be 100% (control) and all contractions were assessed referring to it. Each preparation was exposed to only one diterpene concentration. The antagonism exerted by diterpenes was evaluated based on an analysis of the Schild plot and their potencies with pD'_2 values, which is defined as the negative logarithm to base 10 of molar concentration values of an antagonist that reduces the response to an agonist to 50% of its maximum effect (E_{max}), assessed through concentration–response curves in both absence (control) and presence of diterpenes (Dunne, 1979).

2.7.2. Effect of trachylobane-360 and trachylobane-318 on $CaCl_2$-induced contractions in depolarizing nominally Ca^{2+}-free medium

After the stabilization period, the modified Krebs solution was replaced by a depolarizing (with 40 mM KCl in equimolar exchange for NaCl) and nominally Ca^{2+}-free solution for 45 min. Two similar $CaCl_2$ cumulative concentration–response curves were induced and diterpenes were incubated (3×10^{-5}, 10^{-4} and 3×10^{-4} M), in different preparations, in the absence of $CaCl_2$ for 15 min and a third $CaCl_2$ cumulative curve was obtained in the presence of each compound. The maximal contraction obtained with the first $CaCl_2$ concentration–response curve was considered to be 100% (control), and all contractions were assessed referring to it. Each preparation was exposed to a single diterpene concentration.

2.7.3. Effect of trachylobane-360 and trachylobane-318 on S-(−)-Bay K8644-induced tonic contractions

After the stabilization period, the ileum was partially depolarized by adding 15 mM KCl for 10 min (Usowicz et al., 1995) and a contraction was induced by 3×10^{-7} M S-(−)-Bay K8644, a selective voltage-dependent calcium channel (Ca_v) agonist to L-type or Ca_V1 (Ferrante et al., 1989). In the sustained tonic phase of the contraction, each diterpene was added cumulatively (10^{-7}-3×10^{-4} M), in different preparations, in order to obtain a relaxation curve. The relaxation was expressed as the reversal percentage of initial contraction elicited by contractile agents. The molar concentration of an agonist that produces 50% of its maximal effect (EC_{50}) values was expressed as mean and S.E.M. of EC_{50} individual values assessed through nonlinear regression.

2.7.4. Effect of trachylobane-360 and trachylobane-318 on histamine-induced tonic contractions in both presence and absence of non-selective potassium channel blocker (TEA^+ 5 mM)

After appropriate stabilization of the preparations, tonic contractions were obtained by addition of 10^{-6} M histamine, and trachylobane-360 and trachylobane-318 were cumulatively added (10^{-8}–3×10^{-4} M), in different preparations, to obtain a relaxation curve (control). Afterwards, preparations were washed out and TEA^+ 5 mM, a non-selective potassium channel blocker (Latorre et al., 1989), was added to the preparations for 20 min. Then, other tonic contractions were elicited in the presence of the blocker and trachylobanes were cumulatively added independently. The relaxant potency of the diterpenes was evaluated by comparing EC_{50} values in both the absence and presence of the blocker.

2.7.5. Effect of trachylobane-360 and trachylobane-318 on histamine-induced tonic contractions in both presence and absence of selective potassium channel blockers (glibenclamide, apamin, 4-AP, TEA^+ 1 mM or IbTx)

Histamine-induced tonic contractions were obtained as described earlier.** Afterwards, the selective potassium channel blockers were added to preparations for 20 min. Glibenclamide (10^{-5} M), a blocker of ATP-sensitive K^+ channels (K_{ATP}) (Sun and Benishin, 1994); apamin (10^{-7} M), a blocker of small conductance calcium-activated K^+ channels (SK_{Ca}) (Ishii et al., 1997); 4-AP (3×10^{-7} M), a non-selective blocker of voltage-activated K^+ channels (K_v) (Robertson and Nelson, 1994); TEA^+ 1 mM, a specific blocker of big conductance calcium-activated K^+ channels (BK_{Ca}) (Knot et al., 1996) and iberiotoxin (10^{-7} M) (IbTx), a selective blocker of the BK_{Ca} (Aboulafia et al., 2002), were added in different preparations. Other tonic contractions were elicited in their presence and trachylobanes were cumulatively added independently (10^{-8} - 3×10^{-4} M). Diterpene relaxation potency was evaluated by comparing EC_{50} valuesin both absence and presence of each blocker.

2.7.6. Effect of trachylobane-360 and trachylobane-318 on histamine-induced tonic contractions in both presence and absence of non-selective phosphodiesterase inhibitor (aminophylline)

Histamine-induced tonic contractions were obtained as described earlier. Then, aminophylline (10^{-4} M), a non-selective phosphodiesterase inhibitor (Hirsh et al., 2004), was added to organ baths for 20 min and tonic contractions were induced in the presence of the inhibitor and trachylobanes were cumulatively added (10^{-9}- 3×10^{-4} M), in different preparations. The relaxation potency of each diterpene was evaluated by comparing EC_{50} valuesin both absence and presence of aminophylline.

2.7.7. Effect of trachylobane-360 and trachylobane-318 on cytosolic calcium concentration of myocytes isolated from ileum longitudinal layer

Homogenized pellets with culture medium were cultured in black 96-well microplates, approximately 40.000 cells per well, and stabilized in incubator for 24 h for cell adhesion. After the adherence period, the culture medium of each well was discarded and 50 μL Fluo-4 Direct Calcium Assay Kit, according to the manufacturer's instructions (Molecular Probes/Invitrogen, USA), was added and let rest for 1 h at 37 °C in CO_2 incubator, protected from light. Fluo-4 is a high-affinity calcium indicator that fluoresces when excited at 488 nm and, even in low concentrations, can almost double the fluorescence of other dyes, which is valuable in low density plated cell lines (Paredes et al., 2008). After incorporating fluorophore, the fluorescence was quantified in a FlexStation 3 microplate reader using the Soft Max Pro software (Molecular Devices, USA). The Fluo-4 was excited at 490 nm, and light emission was detected at 525 nm. Records were obtained without interruption for 3 min. The fluorescence intensity was increased by adding 10^{-6} M histamine (control) and later 3×10^{-5} M trachylobane-360 or trachylobane-318 was added, in different experiments, to assess the diterpene action in modify the $[Ca^{2+}]_c$.

2.8. Statistical analysis

Data are expressed as means and S.E.M. EC_{50} and IC_{50} values were determined with nonlinear regression (Neubig et al., 2003). Differences between means were statistically compared using Student's *t*-test and/or one-way ANOVA followed by Bonferroni correction when appropriate. The significance level considered in all tests was 0.05. All values

were obtained using Graph-Pad Prism® 5.01 software (GraphPad Software Inc., USA).

3. Results

3.1. Effect of trachylobane-360 and trachylobane-318 on histamine-induced cumulative contractions

Trachylobane-360 and trachylobane-318 (3×10^{-5}, 10^{-4} and 3×10^{-4} M) concentration-dependently inhibited histamine-induced cumulative contractions shifting the curves nonparallelly to the right, with E_{max} reduction (Fig. 2A and B). Slope values were 3.5 ± 0.9 and 4.4 ± 0.7 to trachylobane-360 and trachylobane-318, respectively, which suggests noncompetitive antagonism. The relaxation potency expressed as pD'_2 was 4.1 ± 0.3 and 4.7 ± 0.06 to trachylobane-360 and trachylobane-318, respectively, which shows equipotency in inhibiting histamine-induced contractions since these values did not differ statistically.

3.2. Effect of trachylobane-360 and trachylobane-318 on $CaCl_2$-induced contractions in depolarizing nominally Ca^{2+}-free medium

Both trachylobanes (3×10^{-5}, 10^{-4} and 3×10^{-4} M) concentration-dependently inhibited Ca^{2+}-induced contractions in Ca^{2+}-free depolarizing medium (Fig. 3A and B). $CaCl_2$ cumulative concentration–response curves were nonparallelly shifted to the right and E_{max} reduced from 100% (control) to 63.7 ± 3.0, 15.0 ± 2.5 and 2.4 ± 1.1% in the presence of trachylobane-360 and to 87.3 ± 4.0, 17.7 ± 3.7 and 3.1 ± 0.5% in the presence of trachylobane-318 which indicates a possible Ca_v blockade and a $[Ca^{2+}]_c$ decrease.

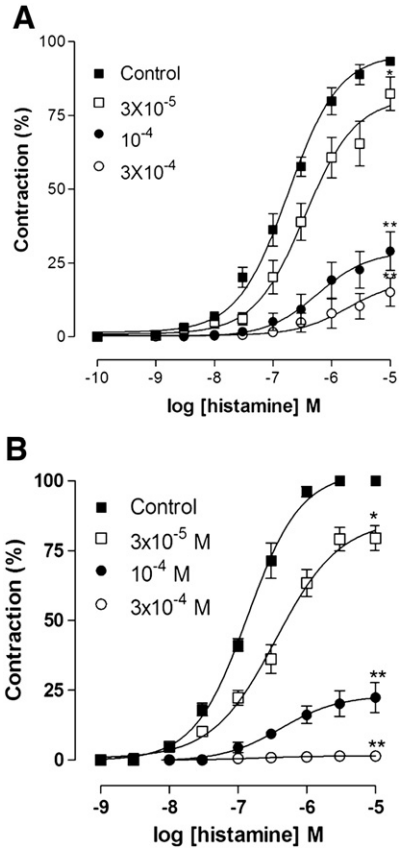

Fig. 2. Cumulative concentration–response curves to histamine in the absence (■) and presence of trachylobane-360 (A) or trachylobane-318 (B): 3×10^{-5} (□), 10^{-4} (●) and 3×10^{-4} M (○) (n = 5). Symbols and vertical bars represent the mean and S.E.M… One-way ANOVA followed by Bonferroni's test, significant differences are indicated by $^{*}P<0.05$ and $^{**}P<0.001$ (control *vs.* trachylobane-360 or trachylobane-318).

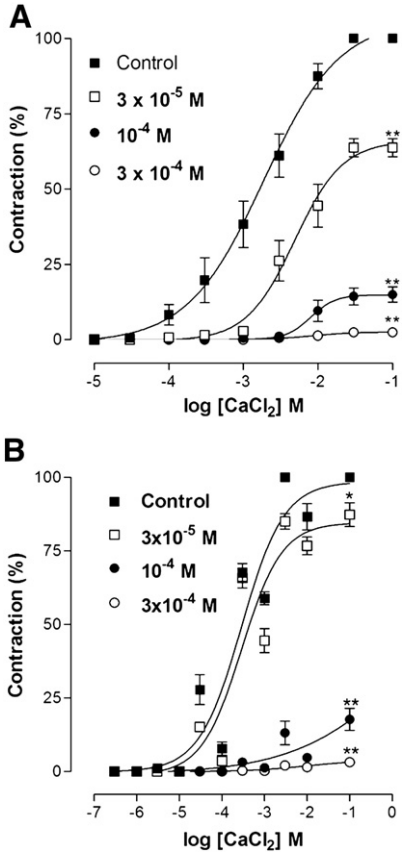

Fig. 3. Cumulative concentration–response curves to $CaCl_2$ in depolarizing medium nominally without Ca^{2+} in the absence (■) and presence of trachylobane-360 (A) or trachylobane-318 (B): 3×10^{-5} (□), 10^{-4} (●) and 3×10^{-4} M (○) (n = 5). Symbols and vertical bars represent the mean and S.E.M. One-way ANOVA followed by Bonferroni's test, significant differences are indicated by $^{*}P<0.05$ and $^{**}P<0.001$ (control *vs.* trachylobane-360 or trachylobane-318).

3.3. Effect of trachylobane-360 and trachylobane-318 on S-(-)-Bay K8644-induced tonic contractions

The cumulative addition of trachylobane-360 (10^{-7} – 3×10^{-4} M) to the tonic component of S-(−)-Bay K8644-induced contraction triggered a concentration-dependent relaxation (Fig. 4A). This effect ($EC_{50} = 3.5\pm0.7\times10^{-5}$ M) was similar to when the contraction was elicited with 40 mM KCl ($EC_{50} = 1.1\pm0.2\times10^{-5}$ M). In the same way, trachylobane-318 significantly and concentration-dependently relaxed the ileum pre-contracted with S-(−)-Bay K8644 (Fig. 4B) ($EC_{50} = 5.5\pm0.3\times10^{-5}$ M) and with 40 mM KCl ($EC_{50} = 1.4\pm0.2\times10^{-5}$ M).

3.4. Effect of trachylobane-360 and trachylobane-318 on histamine-induced tonic contractions in both presence and absence of non-selective potassium channel blocker (TEA^+ 5 mM)

Both diterpenes (10^{-8} – 3×10^{-4} M) relaxed ileum pre-contracted with histamine in presence of TEA^+ 5 mM (Fig. 5A and 5B), a non-selective potassium channel blocker, as can be observed through EC_{50} values, which in the absence of the blocker were $EC_{50} = 1.5\pm0.3$ and $0.1\pm0.01\times10^{-5}$ M, and in the presence of the blocker were $EC_{50} = 0.5\pm0.04\times10^{-4}$ M and $2.0\pm0.5\times10^{-5}$ M to trachylobane-360 and trachylobane-318, respectively. The EC_{50} values of

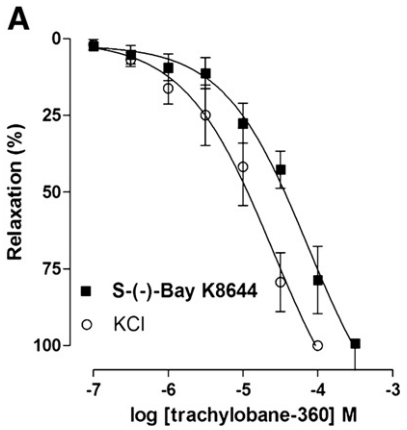

Fig. 4. Effect of trachylobane-360 (A) and trachylobane-318 (B) on the tonic contractions induced by 40 mM KCl (▲) or 3×10^{-7} M S-(−)-Bay K8644 (○) (n = 5), $P<0.05$. Symbols and vertical bars represent the mean and S.E.M., respectively.

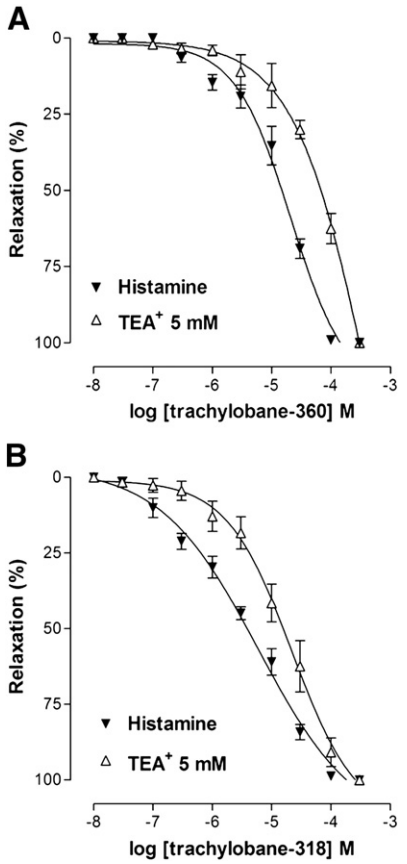

Fig. 5. Effect of trachylobane-360 (A) and trachylobane-318 (B) on the tonic contractions induced by histamine in the absence (▼) and presence (△) of TEA^+ 5 mM (n = 5), $P<0.05$. Symbols and vertical bars represent the mean and S.E.M., respectively.

trachylobane-360 and trachylobane-318 increased approximately 3 and 20 times in the presence of this blocker, respectively.

3.5. Effect of trachylobane-360 and trachylobane-318 on histamine-induced tonic contractions in both presence and absence of selective potassium channel blockers (glibenclamide, apamin, 4-AP, TEA^+ 1 mM or IbTx)

Trachylobane-360 spasmolytic effect ($EC_{50} = 1.5 \pm 0.3 \times 10^{-5}$ M) did not modify statistically in the presence of glibenclamide, a K_{ATP} blocker, ($EC_{50} = 0.8 \pm 0.3 \times 10^{-5}$ M); 4-AP, a K_v blocker, ($EC_{50} = 2.1 \pm 0.7 \times 10^{-5}$ M); apamin, a SK_{ca} blocker, ($EC_{50} = 1.2 \pm 0.3 \times 10^{-5}$ M); TEA^+ 1 mM, a selective BK_{ca} blocker, ($EC_{50} = 1.9 \pm 0.7 \times 10^{-5}$ M) nor IbTx, a BK_{ca} specific blocker ($EC_{50} = 1.1 \pm 0.1 \times 10^{-5}$ M) (Fig. 6A). Differently, trachylobane-318 spasmolytic effect ($EC_{50} = 0.1 \pm 0.01 \times 10^{-5}$ M) was attenuated in the presence of glibenclamide ($EC_{50} = 1.1 \pm 0.3 \times 10^{-5}$ M), 4-AP ($EC_{50} = 0.7 \pm 0.2 \times 10^{-5}$ M) and apamin ($EC_{50} = 0.7 \pm 0.2 \times 10^{-5}$ M), but no significant variation was observed in the presence of TEA^+ 1 mM ($EC_{50} = 0.4 \pm 0.1 \times 10^{-5}$ M) nor IbTx ($EC_{50} = 0.2 \pm 0.07 \times 10^{-5}$ M) (Fig. 6B).

3.6. Effect of trachylobane-360 and trachylobane-318 on histamine-induced tonic contractions in both presence and absence of non-selective phosphodiesterase inhibitor (aminophylline)

Trachylobane-360 and trachylobane-318 relaxation curves ($EC_{50} = 1.5 \pm 0.3 \times 10^{-5}$ M and $0.1 \pm 0.01 \times 10^{-5}$ M, respectively) were not significantly altered in the presence of aminophylline, a non-selective phosphodiesterase inhibitor (Fig. 7A and B).

3.7. Effect of trachylobane-360 and trachylobane-318 on cytosolic calcium concentration of myocytes isolated from ileum longitudinal layer

Histamine (10^{-6} M) induced an increase in the cytosolic calcium in myocytes from the ileum longitudinal layer loaded with calcium fluorophore Fluo-4. A biphasical elevation of cytosolic calcium where the fluorescence peak occurred in 20 seconds after histamine addition, falling slightly and remaining stable throughout the stimulation (180 seconds) (Fig. 8A). After histamine-induced cytosolic calcium stabilization, trachylobane-360 (3×10^{-5} M) was added, reducing the $[Ca^{2+}]_c$ (26.6 ± 14.3%) of cells exposure to diterpene in 10 seconds, sustaining this reduction (36.2 ± 13.8%) throughout the observation period, which indicates a significant $[Ca^{2+}]_c$ decrease (Figs. 8B and 9A). In the same way, trachylobane-318 (3×10^{-5} M) reduced the $[Ca^{2+}]_c$ (56.6 ± 11.7%) in the initial 10 seconds, staying reduced (69.3 ± 7.9%) throughout the observation period, showing a significant $[Ca^{2+}]_c$ decrease (Figs. 8C and 9B).

4. Discussion

In this study we elucidated the spasmolytic action mechanism of trachylolbane-360 and trachylobane-318 at functional and cellular levels. We found differences between the action mechanisms, which probably are due to chemical dissimilarities between them, a substitution of an acetoxy group (trachylobane-360) for a hydroxyl (trachylobane-318) in the basic chemical molecule.

Previously, we have demonstrated that both diterpenes presented spasmolytic activity on tonic and phasic contractions in guinea pig ileum and this effect occurred more potently for trachylobane-318 than trachylobane-360, circa 15 times, when contractions were elicited by histamine (Santos et al., 2011). Thus, a hypothesis that

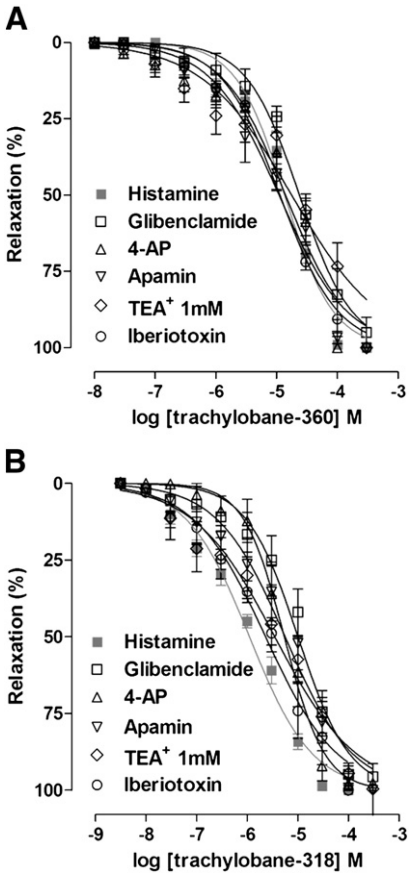

Fig. 6. Effect of trachylobane-360 (A) and trachylobane-318 (B) on the tonic contractions induced by histamine in the absence (■) and presence of glibenclamide (□), 4-AP (Δ), apamin (▽) TEA^+ 1 mM (◊) or iberiotoxin (○) (n = 5), $P<0.05$. Symbols and vertical bars represent the mean and S.E.M., respectively.

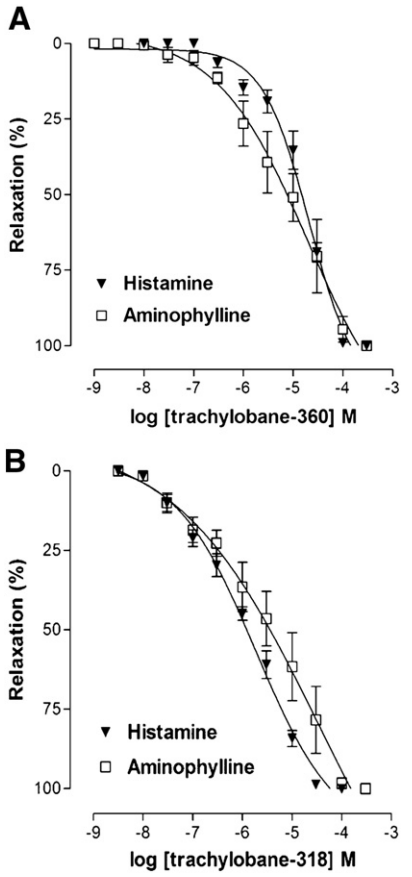

Fig. 7. Effect of trachylobane-360 (A) and trachylobane-318 (B) on the tonic contractions induced by histamine in the absence (▼) and presence of aminophylline (□) (n = 5). Symbols and vertical bars represent the mean and S.E.M., respectively.

trachylobanes might be acting in a direct antagonism on histaminergic receptors to promote its spasmolytic effect was assessed. However, on histamine-induced cumulative contraction protocols, the diterpenes showed the pharmacologic profile of noncompetitive drugs, shifting the histamine cumulative concentration–response curve nonparallelly to the right with E_{max} reduction and showing slope values that differ from the unit, so the competitive antagonism on histaminergic receptors was refuted.

The ileal smooth muscle is known to present biphasic contraction where, in the first phase, the muscle exhibits a fast and transient contraction followed by a long-lasting second phase characterized by the maintained tonic contraction (Horie et al., 2005; Tanahashi et al., 2009). However, both phasic and tonic antagonist-induced contractions depend on extracellular calcium since both are inhibited in its absence (Honda et al., 1996). The removal of extracellular Ca^{2+} prevents contractile responses induced by depolarizing agents (electromechanical coupling), such as KCl, or by agonists of mixed coupling (pharmacomechanical and electromechanical), such as carbachol and histamine, in few seconds, suggesting that the intracellular Ca^{2+} do not contribute significantly to the tension level (Nouailhetas et al., 1985).

Since the antagonism on histaminergic receptors was discarded and trachylobane-360 and trachylobane-318 exerted spasmolytic action in guinea pig ileum contracted by KCl, carbachol or histamine, another relevant hypothesis is that of an action on a common pathway related to the cascade of events elicited by these agents that leads to smooth muscle contraction: the Ca^{2+} influx through Ca_v. Thus, $CaCl_2$-induced contractions in depolarizing nominally Ca^{2+}-free medium were used to assess the possible Ca_v blockade by diterpenes. This protocol is based on the fact that contraction will be obtained almost exclusively by Ca^{2+} from extracellular medium, since depolarization promoted by elevated extracellular potassium concentrations leads to Ca_v opening (Rembold, 1992). Both compounds inhibited significantly $CaCl_2$-induced contractions, shifting them to the right and reducing E_{max}, reinforcing the hypothesis of calcium influx blockade on trachylobane-360 and trachylobane-318 spasmolytic effect.

In smooth muscle, Ca_v1 are the main responsible to calcium influx. These channels are subdivided as $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$ and $Ca_v1.4$ and are sensitive to dihydropyridine and high voltage (Alexander et al., 2008). Ca_v are composed by 4 subunits (2 α, 1 β and 1 γ) where α_1 forms the pore that leads calcium influx (Kuriyama et al., 1995). Thus, the next step was to confirm and identify the Ca_v subtype involved on diterpenes spasmolytic activity. Therefore, tonic contractions were obtained using S-(-)-Bay K8644, a specific dihydropyridine derivative agonist for Ca_v1 that binds directly with α_1-subunit to open these channels, but not by depolarization (Spedding and Paoletti, 1992). Both trachylobanes equipotently and significantly relaxed the ileum pre-contracted with S-(-)-Bay K8644 and in comparison to the relaxant effect on KCl-contracted ileum, an electromechanical agent that activates Ca_v to promote contraction, no significant difference was observed. Furthermore, as the more expressive Ca_v subtype in ileum is $Ca_v1.2$ (Catterall et al., 2005), we confirm that the calcium influx blockade through $Ca_v1.2$ is implicated in the mechanism of spasmolytic action of trachylobane-360 and trachylobane-318 on guinea pig ileum.

Potassium channels play a key role in the regulation of membrane potential and cellular excitability since the contraction of smooth muscle dependents on the balance between increased K^+ ion conductance, leading to hyperpolarization/repolarization, and reduced K^+

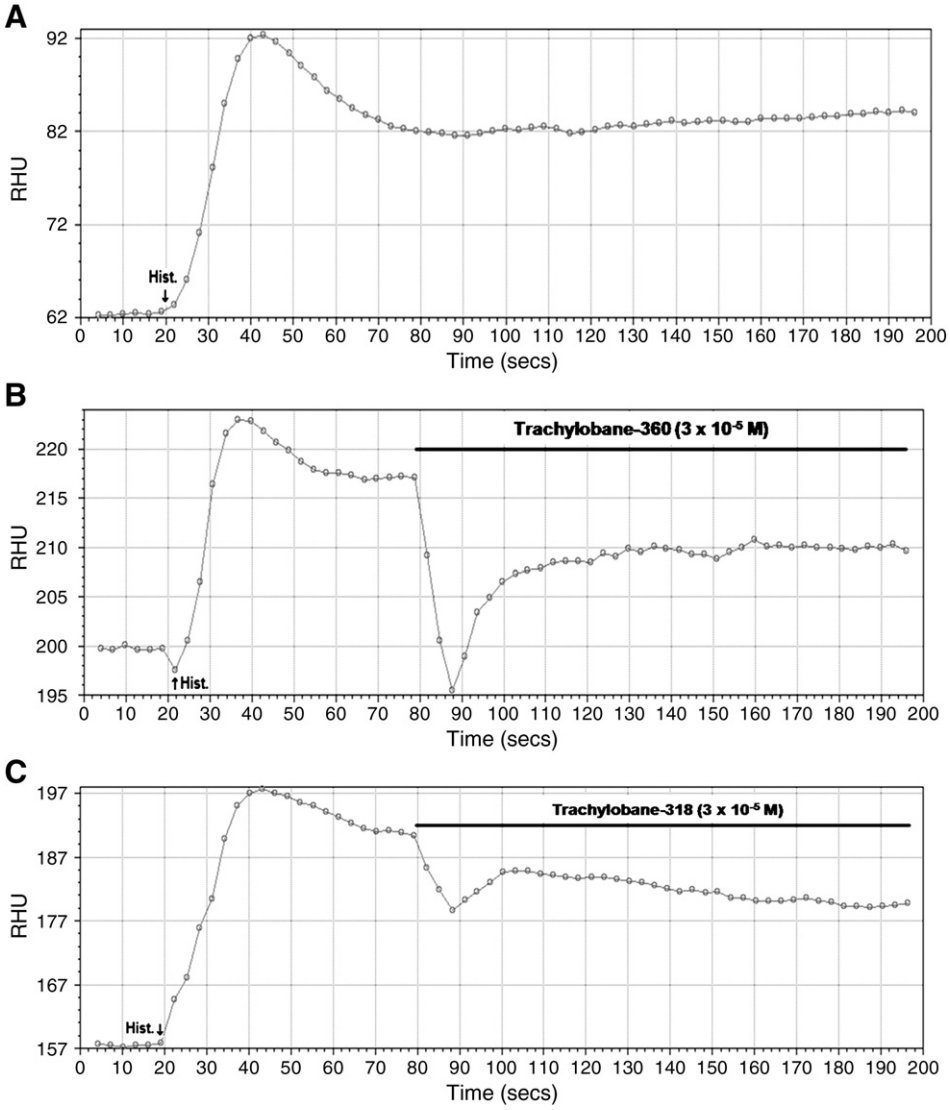

Fig. 8. Representative original records of control (A), trachylobane-360 (B) and trachylobane-318 (C) effects under the sign of calcium in myocytes from the longitudinal layer of guinea pig ileum stimulated with histamine and loaded with Fluo-4.

ion conductance, leading to depolarization (Knot et al., 1996; Lee, 2000), and the K^+ movement across the membrane regulates Ca^{2+} influx through Ca_v (Thorneloe and Nelson, 2005). We observed that both diterpenes relaxed guinea pig ileum by blocking calcium channels, but this may be through an indirect effect, the activation of potassium channels. Hence, the trachylobanes effect on guinea pig ileum was assessed with TEA^+ 5 mM, a non-selective K^+ channels blocker. In the presence of TEA^+, trachylobane-360 and trachylobane-318-induced relaxation curves were shifted to the right (3 and 20 times), indicating the participation of K^+ channels on the spasmolytic effect of both trachylobanes.

Several K^+ channel subtypes are present in smooth muscle, such as K_{ATP} (Teramoto, 2006), K_v (Gordienko et al., 1999), SK_{ca}, IK_{ca}, BK_{ca} (Wei et al., 2005). All of them show regulation and operation particularities but, in general, they act regulating membrane excitability and contribute to the contraction and relaxation processes of smooth muscle. Thus, as a positive modulation of K^+ channels was observed on trachylobane-360 and trachylobane-318 spasmolytic effect, we decided to investigate which K^+ channel subtypes (K_{ATP}, K_v, SK_{ca}, or BK_{ca}) might be involved in this effect. For that, the aforementioned specific K^+ channel blockers were used and, interestingly, different results were found. Trachylobane-360 did not seem to modulate any of the four studied potassium channel subtypes. However, as previously described, we evidenced that the diterpene non-specifically modulated K^+ channels and these diverging results can be explained by the fact that trachylobane-360 may be acting on other not studied K^+ channel subtypes such as inwardly rectifying K^+ channels (K_{ir}) or intermediate-conductance calcium-activated K^+ channels (IK_{Ca}). On the other hand, trachylobane-318 modulated positively K_{ATP}, K_V and SK_{Ca}, but not BK_{Ca}, and that would hyperpolarize/repolarize the plasmatic membrane inactivating Ca^{2+} channels and allowing the ileum to relax. The main reason for the observed differences in modulation manner of both trachylobanes on the studied K^+ channel subtypes is possibly due to the chemical dissimilarity between them. The hydroxyl group being present in position 7 of trachylobane-318 structure and absent in trachylobane-360 might allow this K^+ current modulation and contributes to the K_{ATP}, K_V and SK_{Ca} participation on the spasmolytic effect of this diterpene.

Cyclic nucleotides phosphodiesterases (PDEs) are widely distributed in mammal tissues and are responsible for cAMP and cGMP hydrolysis, resulting in their inactive products 5′-AMP and 5′-GMP, which do not activate PKA and PKG, respectively, thus stopping the cell signaling mechanism dependent on cyclic nucleotides increase (Lugnier, 2006). Substances capable to raise the intracellular content of cAMP or cGMP show a potentiated relaxant effect through PDE inhibition in different tissues due to an increase in the total content of

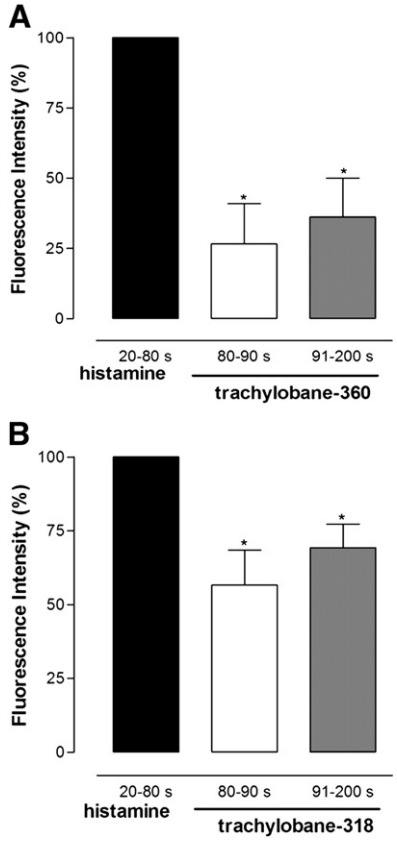

Fig. 9. Effect of trachylobane-360 (A) and trachylobane-318 (B) on the fluorescence induced by histamine in guinea pig ileum myocytes (n = 3). Columns and vertical bars represent the mean and S.E.M., respectively. *t*-test; $^{*}P<0.05$ (histamine *vs.* trachylobane-360 or trachylobane-318).

cyclic nucleotides, exacerbating its effect in smooth muscle relaxation (Bender and Beavo, 2006). Therefore, in order to verify if trachylobane-360 or trachylobane-318 can alter the intracellular content of cyclic nucleotides, the relaxant effect of diterpenes was assayed in the presence of aminophylline, a non-selective phosphodiesterase inhibitor. The presence of aminophylline did not alter trachylobane-360 or trachylobane-318 spasmolytic activity in guinea pig ileum, suggesting that cyclic nucleotides are not involved in their action mechanism.

To relax smooth muscle it required a reduction of $[Ca^{2+}]_c$, since contraction triggers and part of contraction maintenance depends on $[Ca^{2+}]_c$ increase (Somlyo and Somlyo, 1994; Webb, 2003). Nowadays, techniques that use fluorescent indicators allow us to measure the cytosolic calcium concentration in several models of smooth muscles (Wray et al., 2005). Hitherto, the spasmolytic action mechanism of both compounds theoretically reduces $[Ca^{2+}]_c$ availability through Ca^{2+} channel blockade and K^+ channel activation, but no evidence of this has been shown. Thus, we aimed to provide evidence of $[Ca^{2+}]_c$ reduction by trachylobane-360 and trachylobane-318 in myocytes isolated from ileum longitudinal layer and, so, better characterize their action mechanism. Fluo-4, a fluorescent indicator of calcium, was added to the preparations containing ileum myocytes and the resultant fluorescence was quantified in the presence of each diterpene. Both trachylobane-360 and trachylobane-318 similarly reduced the great fluorescence emitted in the presence of histamine, which indicates $[Ca^{2+}]_c$ reduction. These results in cellular experiments support our findings in functional level and evidence that both trachylobanes reduce $[Ca^{2+}]_c$ to play their spasmolytic role. Furthermore, chemical differences between them, or differences in their action mechanisms, did not interfere or alter their ability to reduce $[Ca^{2+}]_c$ since the reduction was equipotent.

In conclusion, this study shows that both trachylobane-360 and trachylobane-318 exert their spasmolytic effect on guinea pig ileum through a blockade of Ca^{2+} channels and that the channel subtype involved is Ca_v 1.2. Furthermore, both diterpenes modulate K^+ channels positively, though in different ways. Trachylobane-360 seems to act in other K^+ channels subtypes not studied in our experiments, while trachylobane-318 seems to activate K_{ATP}, K_V and SK_{Ca} but different subtypes are not discarded of participating in its spasmolytic activity. In addition, the elevation of cyclic nucleotide content is not involved in the effect of any of the studied diterpenes. It is now established that, in cellular experiments, trachylobane-360 and trachylobane-318 decrease $[Ca^{2+}]_c$ to promote the spasmolytic activity observed in tissue experiments.

Acknowledgements

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