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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).

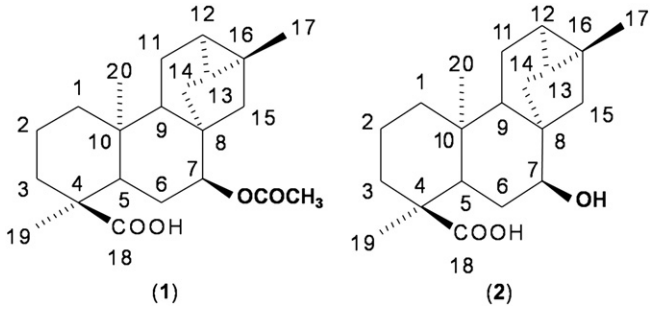

Fig. 1. Chemical structures of *ent*-7α-acetoxytrachyloban-18-oic acid (trachylobane-360) (**1**) and *ent*-7α-hidroxitrachyloban-18-oic acid (trachylobane-318) (**2**).

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