

Relaxant effect of *Ent-7 α -hydroxytrachyloban-18-oic acid*, a trachylobane diterpene from *Xylopi*a *langsdorfiana* A. St-Hil. & Tul., on tracheal smooth muscle

Italo R. R. Martins¹, Rosimeire F. dos Santos², Ana C. de C. Correia¹, Gislaïne A. de Oliveira¹, Cibério L. Macêdo¹, Fabio de S. Monteiro¹, Paula F. dos Santos¹, Fabiana de A. Cavalcante³, Josean F. Tavares⁴ and Bagnólia A. da Silva⁴

¹Centro de Ciências da Saúde, Universidade Federal da Paraíba (UFPB), João Pessoa, Paraíba, Brazil

²Departamento de Bioquímica e Farmacologia, Universidade Federal do Piauí (UFPI), Teresina, Piauí, Brazil

³Departamento de Fisiologia e Patologia, Universidade Federal da Paraíba (UFPB), João Pessoa, Paraíba, Brazil

⁴Departamento de Ciências Farmacêuticas, Universidade Federal da Paraíba (UFPB), João Pessoa, Paraíba, Brazil

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Abstract

Ent-7 α -hydroxytrachyloban-18-oic acid, a trachylobane diterpene from *Xylopi*a *langsdorfiana*, has previously been shown to relax the guinea-pig trachea in a concentration-dependent manner. In this study we aimed to elucidate the mechanisms underlying this action and so contribute to the discovery of natural products with therapeutic potential. A possible interaction between diterpene and the Ca²⁺-calmodulin complex was eliminated as chlorpromazine (10⁻⁶ M), a calmodulin inhibitor, did not significantly alter the diterpene-induced relaxation (pD₂ = 4.38 ± 0.07 and 4.25 ± 0.07; mean ± S.E.M., n=5). Trachylobane-318 showed a higher relaxant potency when the trachea was contracted by 18 mM KCl than it did with 60 mM KCl (pD₂ = 4.90 ± 0.25 and 3.88 ± 0.01, n=5), suggesting the possible activation of K⁺ channels. This was confirmed, as in the presence of 10 mM TEA⁺ (a non-selective K⁺ channel blocker), diterpene relaxation potency was significantly reduced (pD₂ = 4.38 ± 0.07 to 4.01 ± 0.06, n=5). Furthermore, K⁺ channel subtypes K_{ATP}, K_V, SK_{Ca} and BK_{Ca} seem to be modulated positively by trachylobane-318 (pD₂ = 3.91 ± 0.003, 4.00 ± 0.06, 3.45 ± 0.14 and 3.80 ± 0.05, n=5) but not the K_{ir} subtype channel (pD₂ = 4.15 ± 0.10, n=5). Cyclic nucleotides were not involved as the relaxation due to aminophylline (pD₂ = 4.27 ± 0.09, n=5) was not altered in the presence of 3 × 10⁻⁵ M trachylobane-318 (pD₂ = 4.46 ± 0.08, n=5). Thus, at a functional level, trachylobane-318 seems to relax the guinea-pig trachea by positive modulation of K⁺ channels, particularly the K_{ATP}, K_V, SK_{Ca} and BK_{Ca} subtypes.

Key words: trachylobane, *Xylopi*a *langsdorfiana*, airway smooth muscle, potassium channels

Introduction

Xylopi *langsdorfiana* A. St-Hil. & Tul. (Annonaceae) is a tree measuring between 5 and 7 meters in height popularly known in Northeast Brazil as “pimenteira da terra” (1). *Xylopi* species are cited in folk medicine as carminative, and used to treat stomach disorders, bronchitis and dysentery (2). Pharmacologically it has been reported to be a diuretic and is used to treat skin conditions (3), as well as being an antibiotic (4) and presenting hypotensive activity (5). Additionally, several natural products have been obtained from *Xylopi* species including diterpenes (6), sesquiterpenes (7), flavonoids (8) and alkaloids (9).

X. langsdorfiana has also been the source of secondary metabolites such as alkaloids, flavonoids and diterpenes (10, 11). In particular, two trachylobanes, a rare type of diterpene, have been extracted from the hexane phase of the stem bark. They have been structurally identified as *ent-7 α -acetoxytrachyloban-18-oic acid* and *ent-7 α -hydroxytrachyloban-18-oic acid* (10), but referred to as trachylobane-360 and trachylobane-318, respectively (Fig. 1). The trachylobanes are poorly studied substances biologically, but have been cited as being cytotoxic to tumor cell lines (12) and to have antimicrobial (13), antituberculosis (14) and vasorelaxant (15, 16) actions.

In a previous pharmacological screening, our group observed that both trachylobane-360 and trachylobane-318 had a spasmolytic effect on the guinea-pig ileum, but that only trachylobane-318 caused relaxation of the guinea-pig trachea in a concentration-dependent manner (17).

In the search for new substances with medicinal interest to treat and expand the current therapeutic arsenal used to treat smooth muscle conditions such as asthma, chronic obstructive pulmonary disease (COPD) and bronchitis, we aimed to elucidate the mechanisms underlying the relaxant effect of trachylobane-318 on the guinea-pig trachea.

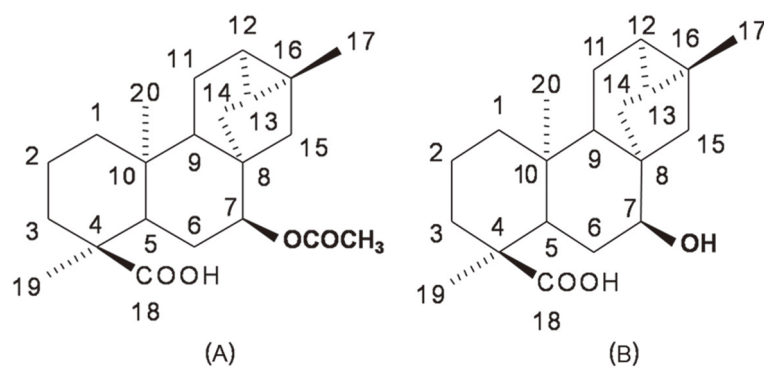


Fig. 1. Chemical structures of *ent-7 α -acetoxytrachyloban-18-oic acid* (trachylobane-360) (1) and *ent-7 α -hydroxytrachyloban-18-oic acid* (trachylobane-318) (2).

Materials and Methods

Animals

Guinea-pigs (*Cavia porcellus*) of both sexes (300–500 g) were provided by the animal facility Prof. Thomas George of Centro de Biotecnologia (CBiotec)/Universidade Federal da Paraíba (UFPB). The animals had free access to food and water, were housed at a constant temperature (21 ± 1 °C) and submitted to a 12 h light-

dark cycle. Care was taken to reduce pain, stress and any suffering in accordance with the internal ethical guidelines for animal usage. All experimental procedures were previously approved and performed in accordance with the Animal Research Ethics Committee of CBIotec/UFPB guidelines (n° 0203/11).

Isolation and preparation of trachylobane-318

The stems of *X. langsdorfiana* were collected in Cruz do Espírito Santo, Paraíba, Brazil, in July 2002. The plant material was identified by Prof. Maria de Fátima Agra (PhD), head of the botany section of CBIotec. A voucher specimen (AGRA 5541) is deposited at the herbarium Prof. Lauro Pires Xavier (JPB) of the UFPB. The extraction and identification of trachylobane-318 (purity $\geq 98.0\%$) was performed as previously described (10). Briefly, dried stems of *X. langsdorfiana* were exhaustively extracted with 95% EtOH. The hexane fraction was subjected to column chromatographic separation, using both 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 into 12 fractions (F1–F12). Fraction F-4 was purified by preparative TLC using AcOEt-hexane (9 : 1) as developer to obtain trachylobane-318, and identified according to ^1H and ^{13}C NMR data. Trachylobane-318 was dissolved in Cremophor[®] plus 7% dimethyl sulfoxide (DMSO) and diluted in distilled water. DMSO never exceeds 0.01% in organ bath and in this concentration it had no effect on the test organ preparation (data not shown).

Drugs and chemicals

Carbamylcholine hydrochloride (CCh), potassium chloride (KCl), apamin, barium chloride (BaCl_2), tetraethylammonium chloride (TEA^+), 4-aminopyridine (4-AP), iberiotoxin (IbTx), aminophylline and chlorpromazine (CPZ) were dissolved and diluted in distilled water, while arachidonic acid (AA) and glibenclamide were dissolved in ethanol and diluted in distilled water. All substances were purchased from Vetec (Brazil) or Sigma-Aldrich Chemicals Co (USA).

Organ preparation and measurement of isometric force

Guinea-pigs were euthanized by cervical dislocation. The trachea was immediately removed, cleaned of connective tissue, cut into transverse strips containing three adjacent cartilage rings (3–4 mm wide) and suspended under 1g load in organ baths at 37°C containing Krebs solution [in mM: NaCl (118.0), KCl (4.6), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (5.7), $\text{KH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (1.1), $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ (2.5), NaHCO_3 (25.0) and glucose (11.0)]. The organs were continuously gassed with carbogen (95% O_2 and 5% CO_2). Tissues were allowed to stabilize for 60 min. An isometric transducer coupled to an amplifier (World Precision Instruments, USA) connected to an analog/digital converter board (Biodata – Brazil) was used to record isometric contractions. Before each experiment, the tracheal epithelium integrity was verified by addition of AA 10^{-4} M (18) in the sustained tonic phase of the contraction induced by CCh 10^{-6} M and if the tracheal rings relaxed by 50% or more, they were considered to have a functional epithelium. The tracheal epithelium was removed using gentle friction using a steel rod wrapped with cotton wool and were used if the relaxation observed after AA addition was less than 10%. All experiments were performed in the absence of functional epithelium since in a previous study the trachylobane-318 relaxant effect on guinea pig trachea was independent of epithelium-derived relaxing factors (EDRF) (17). The trachylobane-318 relaxant effect was reversible within 60 min following its removal and the tracheal responsiveness was not altered (data not shown).

Effect of trachylobane-318 on CCh-induced contraction in the presence and absence of a calmodulin inhibitor

To evaluate a possible action of trachylobane-318 via the calmodulin-Ca²⁺ complex (CaM-Ca²⁺) we used chlorpromazine (CPZ) (10⁻⁶ M) a calmodulin (CaM) inhibitor (19). Preparations were incubated for 20 minutes in the presence of CPZ before a contraction was induced by CCh (10⁻⁶ M), after which trachylobane-318 was added cumulatively. The diterpene relaxation potency was evaluated by comparing pD₂ (negative logarithm of molar concentration of an agonist that produces 50% of its maximal effect) values in the absence or presence of the inhibitor.

Effect of trachylobane-318 on the guinea-pig trachea contracted by a moderated or elevated increase in the extracellular K⁺ concentration ([K⁺]_e)

To analyze if the diterpene had activity as a K⁺ channel activator or as a Ca²⁺ channel blocker, trachylobane-318-induced relaxation was observed in guinea-pig tracheal preparations contracted by two altered Krebs solutions: Krebs solution with KCl 18 mM and Krebs solution with KCl 60 mM (20). In this protocol NaCl was replaced by KCl in equimolar amounts. Diterpene relaxation potency was evaluated by comparing pD₂ values in each situation.

Effect of trachylobane-318 on CCh-induced tonic contractions in the presence and absence of K⁺ channel blockers

To assess the participation of K⁺ channels in the trachylobane-318 relaxant activity we used TEA⁺ 10 mM, a non-selective K⁺ channel blocker (21) in addition to selective K⁺ channel blockers such as apamin (10⁻⁶ M; blocker of small conductance calcium-activated K⁺ channels; SK_{ca}) (22), BaCl₂ (10⁻⁴ M; blocker of inward rectifier K⁺ channels; K_{ir}) (23), glibenclamide (3 × 10⁻⁶ M; blocker of ATP-sensitive potassium channels; K_{ATP}) (24), 4-AP (2 × 10⁻³ M; blocker of voltage-activated K⁺ channels) (24) and IbTx (10⁻⁷ M; blocker of big conductance calcium-activated K⁺ channels; BK_{ca}) (25). The blockers were added to different tracheal preparations for 20 minutes, after which a contraction was induced using CCh (10⁻⁶ M) before trachylobane-318 was added cumulatively. Diterpene relaxation potency was evaluated by comparing pD₂ values in the absence or presence of each blocker.

Effect of trachylobane-318 in the relaxation induced by a non-selective phosphodiesterase inhibitor

To investigate the influence of cyclic nucleotides on diterpene relaxation, aminophylline (10⁻¹⁰ – 10⁻³ M) a non-selective phosphodiesterase inhibitor (26) was used to obtain a relaxation curve (control). Preparations were pre-incubated in trachylobane-318 (3 × 10⁻⁵ M) for 20 minutes, then a contraction induced by CCh (10⁻⁶ M) and a new aminophylline-relaxation curve obtained in the presence of the diterpene. Aminophylline relaxation potency was evaluated by comparing pD₂ values in both absence and presence of trachylobane-318.

Statistical analysis

Data are expressed as means ± S.E.M. The pD₂ values were determined by nonlinear regression. Differences between means were statistically compared using the Student's *t*-test. A probability of less than or equal to 0.05 was considered to be significant in all tests. All values were obtained using Graph-Pad Prism[®] 5.01 software (GraphPad Software Inc., USA).

Results

Effect of trachylobane-318 on CCh-induced contraction in the presence and absence of a calmodulin inhibitor

The relaxant effect of trachylobane-318 (10^{-8} – 3×10^{-4} M; $pD_2=4.38 \pm 0.07$, $n=5$) was not significantly altered in the presence of the calmodulin inhibitor CPZ (10^{-6} M; $pD_2=4.15 \pm 0.07$, $n=5$) (Fig. 2).

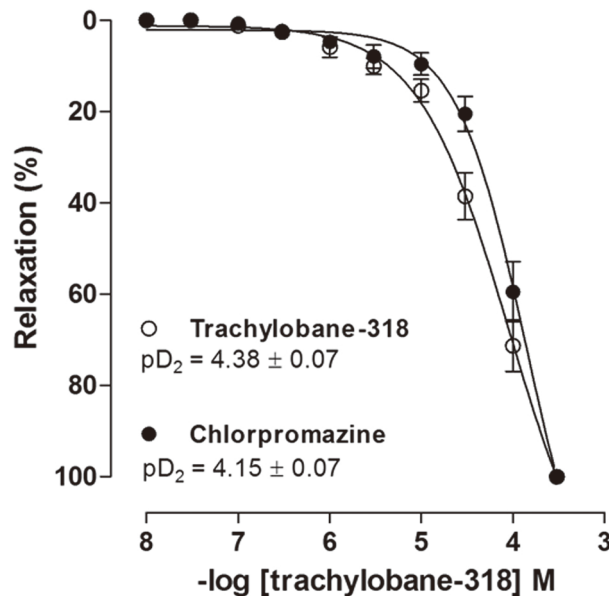


Fig. 2. Effect of trachylobane-318 on the tonic contractions induced by CCh 10^{-6} M in the absence (○) and presence (●) of CPZ 10^{-6} M, $n=5$. Symbols and vertical bars represent the mean and S.E.M., respectively.

Effect of trachylobane-318 on the guinea-pig trachea contracted by a moderated or elevated increase in the extracellular K^+ concentration ($[K^+]_e$)

Trachylobane-318 (10^{-8} – 3×10^{-4} M) showed an approximately ten-fold greater relaxation when the guinea-pig trachea was contracted by 18 mM KCl ($pD_2=4.90 \pm 0.25$, $n=5$) than when the contraction was induced by 60 mM KCl ($pD_2= 3.88 \pm 0.01$, $n=5$) (Fig. 3).

Effect of trachylobane-318 on CCh-induced tonic contractions in the presence and absence of K^+ channel blockers

The trachylobane-318 (10^{-8} – 3×10^{-4} M) relaxation curve was shifted to the right in the presence of TEA⁺ 10 mM, a non-selective K^+ channel blocker, according to the pD_2 value which in the absence of the blocker had a $pD_2=4.38 \pm 0.07$ ($n=5$) and in the presence of the blocker had a $pD_2=4.01 \pm 0.06$ ($n=5$). In the presence of selective K^+ blockers, the trachylobane-318 (10^{-8} – 3×10^{-4} M) relaxant effect ($pD_2=4.38 \pm 0.07$, $n=5$) was significantly attenuated in the presence of glibenclamide, a K_{ATP} blocker, ($pD_2=3.91 \pm 0.003$, $n=5$); 4-AP, a K_V blocker, ($pD_2=4.00 \pm 0.06$, $n=5$); apamin, a SK_{Ca} blocker, ($pD_2=3.45 \pm 0.14$, $n=5$) and IbTx, a BK_{Ca} blocker, ($pD_2=3.80 \pm 0.05$, $n=5$) but not in the presence of BaCl₂, a K_{ir} blocker, ($pD_2=4.15 \pm 0.10$, $n=5$) (Fig. 4).

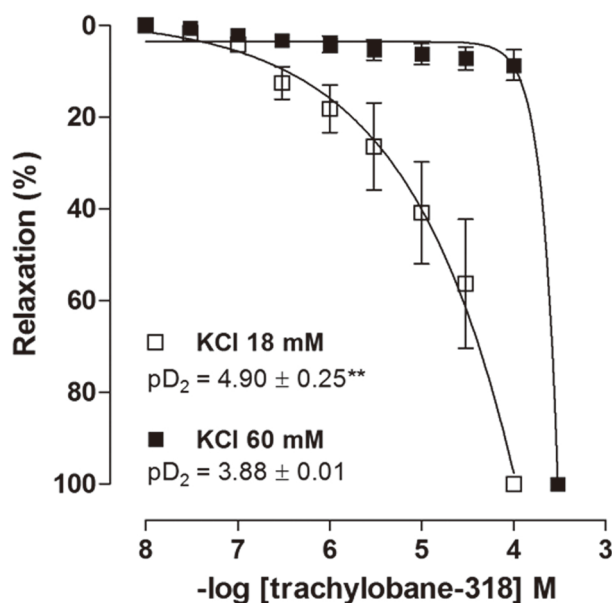


Fig. 3. Effect of trachylobane-318 on the tonic contractions induced by KCl 18 mM (□) and KCl 60 mM (■), $n=5$. Symbols and vertical bars represent the mean and S.E.M., respectively. $**P<0.01$.

Effect of trachylobane-318 on the relaxation induced by a non-selective phosphodiesterase inhibitor

The relaxation curve induced by aminophylline (10^{-10} – 10^{-3} M), a non-selective phosphodiesterase inhibitor, on guinea-pig trachea was not significantly altered in the presence of trachylobane-318 (3×10^{-5} M) as observed by pD_2 values that were $pD_2=4.27 \pm 0.09$ ($n=5$) and 4.46 ± 0.08 ($n=5$), respectively (Fig. 5).

Discussion

The trachylobane diterpenes are a group of secondary metabolites with interesting medicinal potential which have been poorly studied biologically. In this study we have elucidated the relaxant action mechanism of trachylobane-318 isolated from *Xylopija langsdorfiana* on preparations of the guinea-pig trachea, and shown evidence of a positive modulation of K^+ channels that hyperpolarizes the plasma membrane leading to airway smooth muscle relaxation.

Previously, we have demonstrated that trachylobane-318 relaxed the guinea-pig trachea in a concentration-dependent manner independently of the presence of a functional epithelium (17). The classical signaling pathway of smooth muscle cell contraction is evoked by the entrance of Ca^{2+} into the cells, followed by formation of a Ca^{2+} -calmodulin complex resulting in myosin light chain kinase (MLCK) activation and, finally, phosphorylation of light chain kinase leading to contraction (19). Thus we decided to evaluate a possible action of trachylobane-318 within the Ca^{2+} -calmodulin complex. Since trachylobane-318-induced relaxation was not altered in the presence of CPZ, a calmodulin inhibitor, the concept of a possible interaction between the diterpene and Ca^{2+} -calmodulin complex was discarded.

The elevation of cytosolic calcium concentration ($[Ca^{2+}]_c$) is a requisite for the initiation of smooth muscle contraction (27). Both Ca_v and K^+ channels ion channels are involved in this process, with the first of these being mainly responsible for calcium influx and $[Ca^{2+}]_c$ elevation and the second responsible for the control of

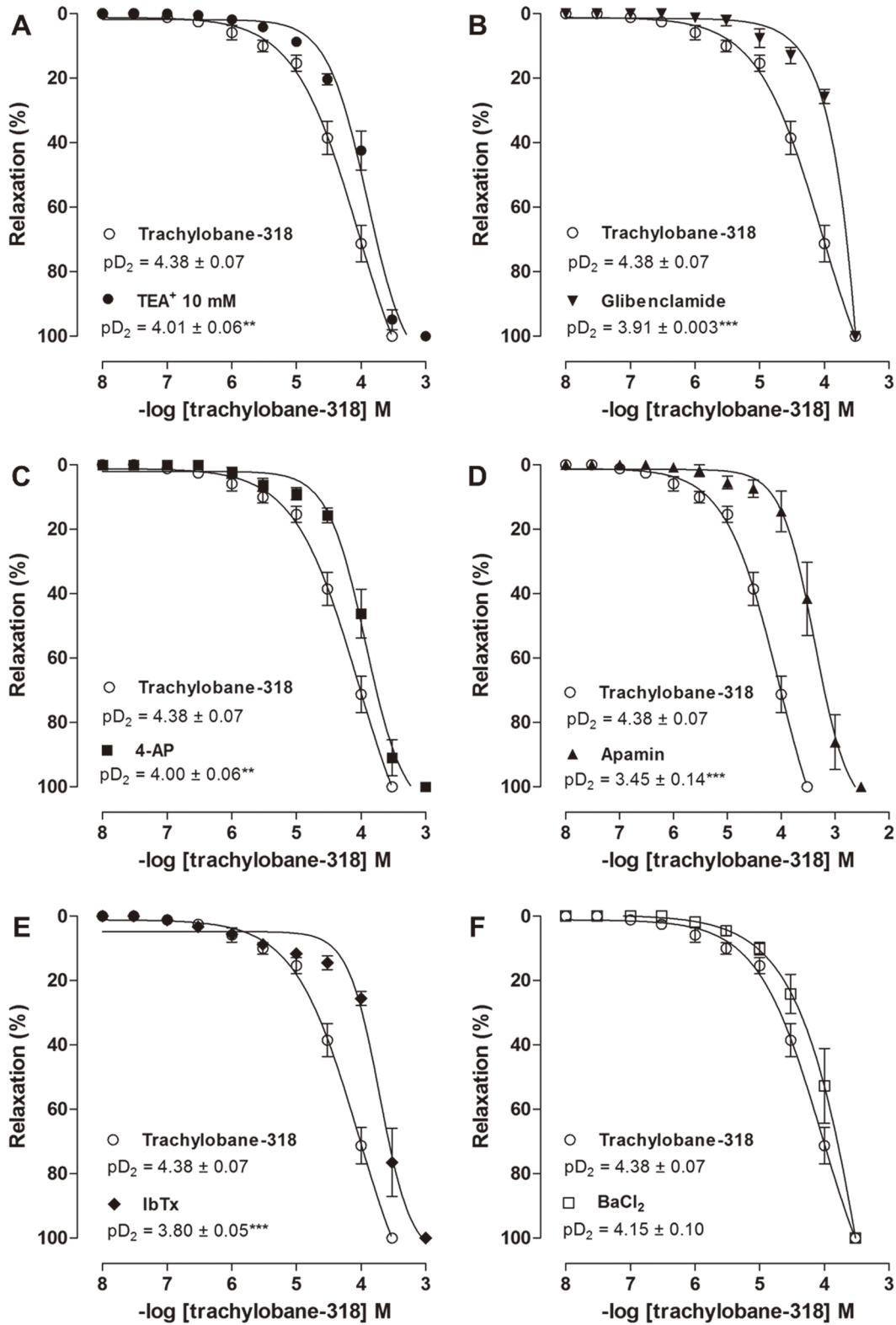


Fig. 4. Effect of trachylobane-318 on the tonic contractions induced by CCh 10^{-6} M in the absence (○) and presence of TEA⁺ 10 mM (●) (A), glibenclamide 3×10^{-6} M (▼) (B), 4-AP 2×10^{-3} M (■) (C), apamin 10^{-6} M (▲) (D), IbT $\times 10^{-7}$ M (◆) (E) and BaCl₂ 10^{-4} M (□) (F), $n=5$. Symbols and vertical bars represent the mean and S.E.M., respectively. $^{**}P<0.01$ and $^{***}P<0.001$.

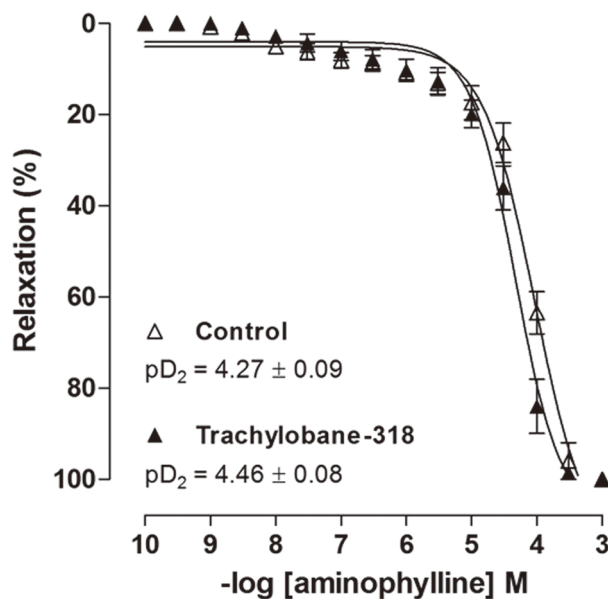


Fig. 5. Effect of aminophylline on the tonic contractions induced by CCh 10^{-6} M in the absence (control) (Δ) and presence (\blacktriangle) of trachylobane-318 3×10^{-5} M, $n=5$. Symbols and vertical bars represent the mean and S.E.M., respectively.

the membrane potential and cellular excitability, thus regulating the smooth muscle contraction or relaxation. By altering the extracellular K^+ concentration ($[K^+]_e$) we can determinate if a drug has activity as either a K^+ activator or a Ca_v blocker, since with a moderate increase of $[K^+]_e$ (KCl 18 mM) the first possibility has a higher relaxant potency, but with an elevated increase of $[K^+]_e$ (KCl 60 mM) the second possibility is more effective (28). Figure 3 shows that trachylobane-318 had a greater relaxant potency in the guinea-pig trachea contracted with a moderate increase of $[K^+]_e$ (18 mM) suggesting that this diterpene positively modulates the K^+ channels, because in elevated $[K^+]_e$ K^+ channels activators present a reduced relaxant potency due to decrease of K^+ efflux (28). The fact that trachylobane-318 caused relaxation of preparations of the guinea-pig trachea contracted with 60 mM KCl could be attributed to a possible inhibition of the Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) or extracellular signal-regulated kinase (ERK), since these kinases are known to be activated in the mechanism of KCl-induced contraction of smooth muscle (29). However, the fact that this effect did not occur in a concentration-dependent manner and was only observed at the highest concentration tested makes the hypothesis of positive modulation of K^+ channels more plausible. To confirm or refute this possibility, experiments were carried out in the presence of TEA $^+$ 10 mM, a non-selective K^+ channels blocker. The observation that the diterpene-induced relaxation was reduced approximately 3-fold confirms the involvement of K^+ channels.

K^+ channels play a key role in the regulation of membrane potential and cellular excitability since the contraction of smooth muscle is dependent on K^+ ion conductance, with an increase leading to hyperpolarization/repolarization and a reduction causing a depolarization (30). K^+ channels are a family divided into many subtypes and several of these are expressed in airway smooth muscle such as K_{ATP} (31), K_v (32), SK_{Ca} , BK_{Ca} (33) and K_{ir} (34). To investigate which K^+ channel subtypes could be involved in the trachylobane-318 relaxant effect we used specific blockers, and in the presence of glibenclamide, 4-AP, apamin and IbTx the trachylobane-318-relaxation curve was shifted to the right indicating that K_{ATP} , K_v , SK_{Ca} and BK_{Ca} are modulated positively by the diterpene (Fig. 4), and comparing the relaxant potencies it is plausible to suggest that trachylobane-318

is mainly acting in via the calcium-activated K⁺ channels (SK_{Ca} and BK_{Ca}), but additional studies are necessary to characterize this hypothesis. These findings are similar to a previous report that trachylobane-318 positively modulated K⁺ channels to reduce the [Ca²⁺]_c and relax the guinea-pig ileum (35).

Phosphodiesterases (PDEs) are enzymes that hydrolyze cyclic nucleotides (cAMP or cGMP) and substances that are capable of raising the cAMP or cGMP content show a potentiated relaxation in the presence of a PDE inhibitor, increasing their effect on smooth muscle relaxation (26). So in order to verify if trachylobane-318 could be acting in this manner, a relaxation-curve was obtained using aminophylline, a non-selective PDE inhibitor. As shown in Fig. 5, trachylobane-318 did not modify the aminophylline relaxation-curve in a significant manner, suggesting that the cyclic nucleotide pathway is not contributing to the diterpene relaxation. Additionally, as the trachylobane-318 relaxant action on guinea-pig tracheal preparations was not totally abolished by the pharmacological tools used in this study, other mechanisms such as the involvement of either of the Rho pathway or the MAPK pathway cannot be discounted.

In conclusion, the present study shows that trachylobane-318, isolated from *X. langsdorfiana*, relaxes guinea-pig tracheal preparations by positive modulation of K⁺ channels, particularly via the K_{ATP}, K_V, SK_{Ca} and BK_{Ca} subtypes. Since *Xylopi*a species are cited in folk medicine for the treatment of bronchitis together with the current findings, we suggest that diterpene trachylobane-318 shows promise as a compound with the potential to be developed into an effective drug for the treatment of airway disorders such as asthma, COPD or bronchitis.

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