BIOCHEMICAL PHARMACOLOGY 71 (2006) 1248–1254

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journal homepage: www.elsevier.com/locate/biochempharm

Pharmacological characterisation of the plant sesquiterpenes polygodial and drimanial as vanilloid receptor agonists

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ARTICLE INFO

Article history:
Received 7 November 2005
Accepted 21 December 2005

Keywords:
Polygodial
Drimanial
TRPV1
Trigeminal ganglion neuron

ABSTRACT

This study was designed to assess the participation of transient receptor potential vanilloid 1 (TRPV1) in the biological effects induced by the plant-derived sesquiterpenes polygodial and drimanial. In rat isolated urinary bladder, polygodial and drimanial produced a tachykinin-mediated contraction that was inhibited by combination of NK_1 and NK_2 tachykinin receptor antagonists, SR 140333 and SR 48968. Furthermore, two different TRPV1 antagonists, capsazepine and ruthenium red prevented the contraction induced by both compounds. In addition, capsaicin, polygodial and drimanial displaced in a concentration-dependent manner the specific binding sites of $[^3H]$-resiniferatoxin to rat spinal cord membranes, with a IC_{50} values of 0.48, 4.2 and 3.2 μM, respectively. Likewise, capsaicin, polygodial and drimanial promoted an increase of $[^{45}Ca^{2+}]$ uptake in rat spinal cord synaptosomes. In cultured rat trigeminal neurons, polygodial, drimanial and capsaicin were also able to significantly increase the intracellular Ca^{2+} levels, effect that was significantly prevented by capsazepine. Together, the present results strongly suggest that the pharmacological actions of plant-derived sesquiterpenes polygodial and drimanial, seem to be partially mediated by activation of TRPV1. Additional investigations are needed to completely define the pharmacodynamic properties of these sesquiterpenes.

1. Introduction

Capsaicin, the pungent component of hot chilli pepper, excites a subset of small-diameter sensory neurons activating the transient receptor potential vanilloid 1 (TRPV1) [1]. TRPV1 is a membrane protein with six transmembrane spanning segments with a pore loop between domains five and six and a predicted molecular mass of 95 kDa [2]. TRPV1 functions as a non-selective cation channel and can be activated by exogenous (capsaicin, resiniferatoxin and ethanol) and endogenous (low pH, noxious heat, and some fatty acid-derived products) stimuli [1–3]. Recent findings have shown that mice

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0006-2952/$ – see front matter
doi:10.1016/j.bcp.2005.12.030

lacking the TRPV1 gene do not respond to vanilloid agonists and exhibit reduced sensitivity to low pH and noxious heat, especially under inflammatory conditions [4].

Under resting conditions, the channel pore of TRPV1 is closed. When activated, TRPV1 allows the influx of monovalent and divalent cations, predominantly Ca^{2+}, through the cell membrane [2]. This initiates the generation of action potentials perceived as burning pain. In addition, repeated brief applications of vanilloids induce Ca^{2+}-dependent desensitization whereas prolonged applications may induce cell death [5]. In this way, capsaicin and some other vanilloid agonists not only cause pain, but also exhibit anti-nociceptive properties. Thus, capsaicin preparations are clinically used in the management of neuropathic pain and rheumatoid arthritis, and disorders of the urinary bladder in which abnormal afferent sensory information converted by capsaicin-sensitive nerves is a major factor in the etiology (see for review; [6]). Collectively, these data suggest a relevant role played by TRPV1 as a molecular integrator of multiple pain-producing stimuli and suggest that this ion channel may represent a useful and promissing target for the discovery of new clinically relevant analgesics.

Besides capsaicin, other naturally occurring activators of TRPV1 are terpenoids, phenolic ketones, triprenyl phenols and unsaturated dialdehydes products [7–10]. Polygodial and drimanial are unsaturated 1,4-dialdehyde sesquiterpenes isolated from the bark of *Drymis winteri* (Winteraceae), a native Brazilian medicinal plant used in folk medicinal for the treatment of various inflammatory diseases, as well as for substitution of pepper for culinary [11,12]. Similar to capsaicin, systemic administration of polygodial and drimanial produces marked anti-nociceptive, anti-inflammatory and anti-allergic effects [13–16]. Our recent data demonstrate that neonatal treatment of rat with polygodial or drimanial induced pronounced anti-nociceptive capsaicin-like effect in adult animals. Persistent anti-nociception was associated with TRPV1 down-regulation in the spinal cord, but not in dorsal root ganglion [17]. Taken these findings in mind, the aim of the present study was to further investigate the role played by TRPV1 in the pharmacological actions of the sesquiterpenes polygodial and drimanial, using functional and biochemical approaches.

2. Materials and methods

2.1. Isolation and identification of compounds

Polygodial and drimanial were isolated from the bark of *D. winteri* (Winteraceae). Part of the barks extract of *D. winteri* (23 g) was subjected to column chromatography (θ_i 4.5 cm), packed with silica gel 60–230 mesh (220 g), and eluted with hexane gradually enriched in ethyl acetate and ethanol. Seventy-five fractions of 100 ml each were collected. Polygodial was obtained from fractions 28–32, [elution solvent Hex-AcOEt (9:1)] and was further purified by repeated chromatographies, giving 3.1 g yield (0.22% in relation to dry plant). From sub-fraction 30–42, eluted with hexane:ethyl acetate 6:4, drimanial was obtained (3.3 g yield, 0.23% in relation to dry plant). Both compounds were identified on basis of their spectral data in comparison with to those of literature and direct comparison with authentic samples [18].

2.2. Animals

Newborn and adult Sprague–Dawley rats (University of Santa Catarina and Morini, Italy) weighing 220–250 g were used throughout the experiments. All animals were housed in a room maintained at a constant temperature of $22 \pm 2\ ^{\circ}C$ on a 12/12 h light/dark cycle with food and water available ad libitum. The experiments were carried out in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigations of experiments in conscious animals [19]. In addition, the protocols employed had been approved by the local ethics committee (process numbers 262/CEUA and 23080.035334/2003-16/UFSC).

2.3. Isolated urinary bladder strips

Rats weighing 220–250 g were sacrificed by cervical dislocation and exsanguinated. The urinary bladder was rapidly removed, and vertical halves were excised and cleaned from connective tissue and adherent fat. Bladder strips were placed in a Petri dish containing Krebs solution (composition mM: NaCl 119.0; KCl 4.7; $MgSO_4$ 1.5; $CaCl_2$ 2.5; $NaHCO_3$ 25.0; $KHPO_4$ 1.2 and Glucose 11.0; pH 7.2). The bladder strips were carefully mounted in 5 ml organ baths maintained at 37 °C continuously aerated with 95% O_2 and 5% CO_2. Isotonic tension changes were recorded by a personal computer. Preparations were submitted to a basal tension of 1 g, followed by equilibration period of at least 60 min. During the equilibrium period, the Krebs solution was changed each 15 min. In all experiments the tissues were first contracted with carbachol (0.3 μM) and this contraction was expressed as percentage of maximal contractile response produced by carbachol. After reaching stability of the tonic responses, the Krebs solution was changed three times followed by a new equilibration period of at least 30 min. Complete cumulative concentration–response curves for polygodial (0.001–100 μM) or drimanial (0.001–100 μM) were performed. Each new concentration of the cumulative curve was added when the effect of the preceding one had reached its maximum. To explore further the mechanisms by which polygodial and drimanial induce contraction in the urinary bladder, complete cumulative concentration–response curves for polygodial and drimanial were obtained in the absence or presence of the TRPV1 receptor antagonists capsazepine (10 μM), and ruthenium red (30 μM) or with a combination of NK1 and NK_2 receptor antagonists SR 140333 (1 μM) and SR 48968 (1 μM), respectively. All antagonists were left in contact with the tissue for at least 15 min prior contraction with either sesquiterpenes.

To avoid desensitization of the preparation, only one complete cumulative concentration–response curves for polygodial or drimanial was obtained in each preparation. Separate control and test tissues were studied simultaneously in adjacent baths. Only one concentration of each antagonist was tested per preparation. The choice of drug concentration and period of pre-incubation were selected based in previous publications [20,21].

2.4. Extracellular [$^{45}Ca^{2+}$] uptake in the synaptosomes from rat spinal cord

The experiments were carried out as described previously with minor modifications [22]. Rat spinal cord was removed and disrupted with a glass potter in 20 volumes of ice-cold buffer A (pH 7.4) containing 0.32 M sucrose and 10 mM Tris at 4 °C. The homogenate was centrifuged for 15 min at 1000 × *g* at 4 °C. Supernatant was centrifuged for 30 min at 18,000 × *g* at 4 °C. The resulting pellet was re-suspended in buffer B (pH 7.4) containing (mM) NaCl 136; $MgCl_2$ 1.3; KCl 5; $CaCl_2$ 1.2; glucose 10; Tris 20 and centrifuged for 30 min at 18,000 × *g* at 4 °C. The final resulting pellet was re-suspended in buffer B lacking-calcium and used in the experiments. To assess the [$^{45}Ca^{2+}$] uptake the experiments were carried out in duplicate. The aliquots of 250 μl of membrane were added to tubes containing buffer B lacking-calcium and incubated at 37 °C for 12 min. After pre-incubation, [$^{45}Ca^{2+}$] (0.9 μCi) was added in the tube simultaneously with KCl (77 mM), capsaicin (1 μM), polygodial (5 μM) or drimanial (5 μM) and incubated for 1 min at 37 °C. Immediately after the incubation, 500 μl of EGTA/stop buffer (NaCl 136 mM; $MgCl_2$ 1.3 mM; KCl 5 mM; $CaCl_2$ 1.2; glucose 10 mM; Tris 20 mM and EGTA 30 mM; pH 7.4) was added in the tubes and the preparations were filtered three times in GF/B filters pre-washed with EGTA/stop buffer. The filters were dried and then transferred to scintillation counting. Uptake of [$^{45}Ca^{2+}$] for capsaicin, polygodial and drimanial was calculated as percentage of values obtained by [$^{45}Ca^{2+}$] uptake in baseline values.

2.5. Binding of [^3H]-resiniferatoxin in the rat spinal cord

Binding assays were carried out as described previously [23]. To obtain membranes for the binding studies, spinal cords of rats were removed and disrupted with the aid of a tissue homogenizer in ice-cold buffer A (pH 7.4 containing (in mM) KCl 5; NaCl 5.8; $MgCl_2$ 2; $CaCl_2$ 0.75; glucose 12; sucrose 137 and HEPES 10. The homogenate was first centrifuged for 10 min at 1000 × *g* at 4 °C; the low speed pellets were discarded; the supernatants were further centrifuged for 30 min at 35,000 × *g* at 4 °C; and the resulting high speed pellets, re-suspended in buffer A, were stored at −70 °C until assayed.

Binding assays were carried out in duplicate with a final volume of 500 μl, containing buffer A, supplemented with 0.25 mg/ml bovine serum albumin (included to stabilize ligands in aqueous solution), membranes (100 μg/protein), 25 pM of [^3H]-resiniferatoxin and increasing concentrations (0.01–10 μM) of capsaicin, polygodial (0.1–10 μM) or drimanial (0.1–10 μM). Non-radioactive resiniferatoxin (100 nM) was included in some tubes to offer the measurement of non-specific binding.

Assay mixtures were set up on ice and the binding reaction was then initiated by transferring the assay tubes to a 37 °C water bath. Following a 60 min incubation period, binding reaction was stopped by cooling the tubes on ice. After that, 100 μg of bovine α_1-acid glycoprotein was added to each tube to reduce non-specific binding. Finally, separation of bound and free membranes [^3H]-resiniferatoxin was done by centrifuging for 15 min at 20,000 × *g* at 4 °C. The pellet was quantified by scintillation counting. Specific binding was calculated as the difference of the total and non-specific binding. In these conditions the specific binding was greater than 70% of the total binding.

2.6. Intracellular Ca^{2+} fluorescence measurements in cultured rat trigeminal ganglion

Newborn rats (2–3 days old) were terminally anaesthetized and decapitated. Trigeminal ganglion were removed and rapidly placed in cold PBS before being transferred to collagenase/dispase (1 mg/ml, dissolved in Ca^{2+}-Mg^{2+}-free PBS) for 35 min at 37 °C. Enrichment of the nociceptive neurons fraction was obtained following the methods reported previously [24]. After the enzymatic treatment, ganglion were rinsed three times with Ca^{2+}-Mg^{2+}-free PBS and then placed in 2 ml of cold Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS, heat inactivated), 2 mM L-glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin. The ganglions were then dissociated into single cells by several passages through a series of syringe needles (23 G down to 25 G). Finally, the complex of medium and ganglion cells were sieved through a 40 μm filter to remove debris and topped up with 8 ml of DMEM medium and centrifuged (200 × *g* for 5 min). The final cell pellet was re-suspended in DMEM medium, supplemented with 100 ng/ml mouse Nerve Growth Factor (mouse-NGF-7S) and cytosine-b-D-arabino-furanoside free base (ARA-C) 2.5 μM. Cells were plated on poly-L-lysine (8.3 μM) and laminin (5 μM) coated 25 mm glass cover slips and kept for 2–5 days at 37 °C in a humidified incubator gassed with 5% CO_2 and air. Cells were fed on the second day (and subsequent alternate days) with DMEM medium (with 1% FBS instead of 10% FBS).

Plated neurons were loaded with Fura-2-AM-ester (3 μM) in Ca^{2+} buffer solution of the following composition (mM): $CaCl_2$ 1.4; KCl 5.4; $MgSO_4$ 0.4; NaCl 135; D-glucose 5; HEPES 10 with BSA 0.1%, at pH 7.4, for 40 min at 37 °C, washed twice with the Ca^{2+} buffer solution and transferred to a chamber on the stage of Nikon eclipse TE300 microscope. The dye was excited at 340 and 380 nm to indicate relative $[Ca^{2+}]_i$ changes by the F340/F380 ratio recorded with a dynamic image analysis system (Laboratory Automation 2.0, RCS, Florence, Italy). Polygodial (100 μM), drimanial (30 μM), capsaicin (0.1 μM) or their respective vehicles were added to the chamber in the absence or presence of capsazepine (10 μM).

In the end of each experiment ionomycin (5 μM) was added to the chamber and variation in $[Ca^{2+}]_i$ was expressed as the increase in baseline values. A calibration curve using a buffer containing Fura-2-AM-ester and determinant concentrations of free Ca^{2+} [25] was used to convert the data obtained from F340/F380 ratio to $[Ca^{2+}]_i$ (nM).

2.7. Chemicals

The following substances were used: capsaicin, resiniferatoxin, capsazepine, ruthenium red, laminin, ionomycin and poly-L-lysine (all from Sigma Chemical Company St. Louis, USA). [^3H]-resiniferatoxin (37 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Washington, USA). (S)-N-methyl-N-[4-acetylamino-4-phenylpiperidino)-2-(3,4 dichlorophenyl)-butyl]benzamide (SR 48968) and [(S)1-(2-[3-(3,4-dichlorophe-

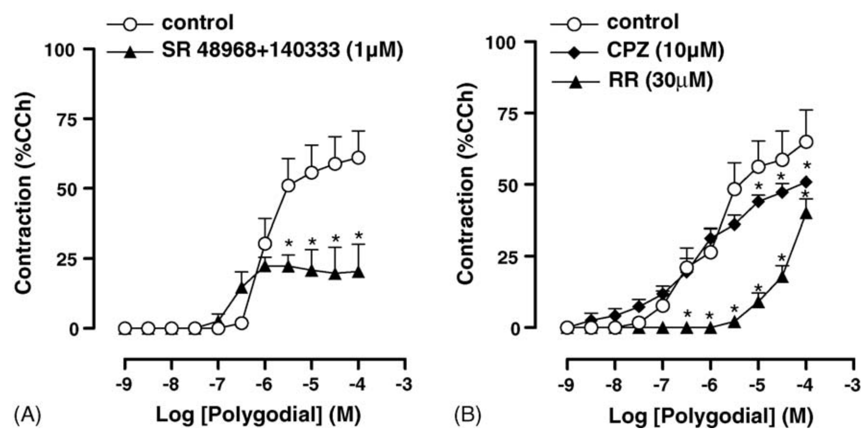

Fig. 1 – Mean contraction concentration–response curves for polygodial (0.001–100 μM) in rat urinary bladder obtained in the absence or presence of (A) SR 140333 plus SR48968 (1 μM); (B) capsazepine (CPZ, 10 μM) or ruthenium red (RR, 30 μM). Each point represents the mean of 8–10 experiments and the vertical lines indicate the S.E.M. Differs significantly from control value $^{*}P < 0.05$ (Student's *t*-test). Carbachol (CCh).

nyl)-1-(3-iso-propoxyphenylacetyl)piperidin-3-yl]etyl)-4-phenyl-1-azoniabicyclo[2.2.2]octane-chloride] (SR 140333) were a kind gift from Dr. Xavier Emonds-Alt, Sanofi-Synthelabo, Montpellier, France. [$^{45}Ca^{2+}$] (from Amersham Pharmacia Biotech, USA). Mouse NGF-7S and collagenase/dispase (Roche Diagnostics, Italy); Dulbecco's Modified Eagle's medium, fetal bovine serum (FBS) heat inactivated, L-glutamine (200 mM), penicillin/streptomycin (10,000 IU/ml–10,000 UG/ml), Ca^{2+}-Mg^{2+}-free PBS (Gibco, Italy); Fura-2-AM-ester (Societa' Italiana Chimici, Italy). The stock solutions of polygodial (100 mM), drimanial (100 mM), capsaicin (10 mM), capsazepine (10 mM) and resiniferatoxin (10 mM) were prepared in 100% ethanol. Fura-2-AM-ester and ionomycin were dissolved in 100% DMSO and stored at 4 °C and diluted in Krebs solution as necessary for the experiments. Polygodial and drimanial were diluted in the day of experiment just before the use. The final concentration of ethanol did not exceed 0.1% and had no effect per se on the basal tonus of the preparations or agonist-mediated responses. Appropriate parallel control experiments were carried out in the presence of the vehicle used to dilute these drugs.

2.8. *Statistical analysis*

The arithmetic mean and standard error of the mean (S.E.M.) were calculated throughout. Contractile responses are expressed as a percentage (%) of the response to carbachol (0.3 μM). Statistical analysis was performed by means of the Student's *t*-test. The concentrations of compounds that produced 50% of the maximal effect (EC_{50} or IC_{50}) were obtained with an iterative curve fitting package (GraphPad Prism Software, San Diego, CA, USA). The data of [^{3}H]-resiniferatoxin and [$^{45}Ca^{2+}$] uptake is represented as mean of four experiments in duplicate. The specific [^{3}H]-resiniferatoxin binding was calculated as the difference of the total and non-specific binding and the uptake of [$^{45}Ca^{2+}$] for capsaicin,

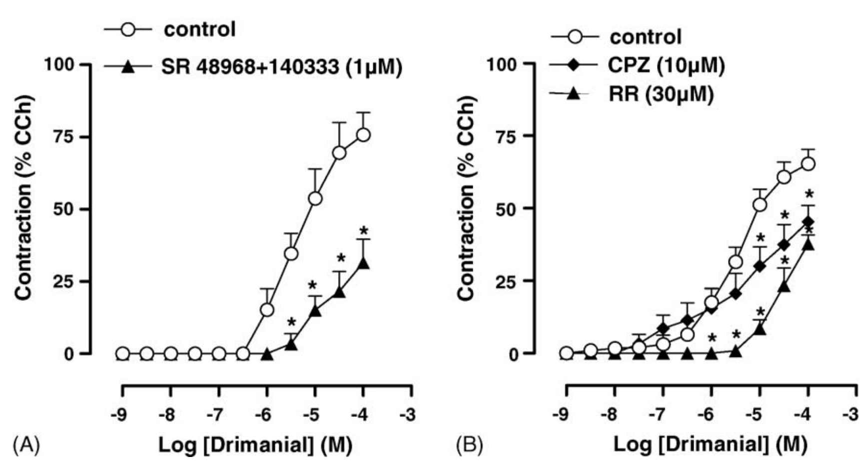

Fig. 2 – Mean contraction concentration–response curves for drimanial (0.001–100 μM) in rat urinary bladder obtained in the absence or presence of (A) SR 140333 plus SR 48968 (1 μM); (B) capsazepine (CPZ, 10 μM) or ruthenium red (RR, 30 μM). Each point represents the mean of 8–10 experiments and the vertical lines indicate the S.E.M. Differ significantly from control value $^{*}P < 0.05$ (Student's *t*-test). Carbachol (CCh).

polygodial and drimanial was calculated as percentage of values obtained by [$^{45}Ca^{2+}$] uptake basal. The intracellular calcium concentration was obtained by program Origin Software (Microcal Software, Northampton, MA, USA). Statistical comparison of the data was performed by analysis of variance (ANOVA) followed by Dunnett's test. Differences between groups were considered to be significant at $P < 0.05$ or less.

3. Results

3.1. *Contraction of rat urinary bladder induced by polygodial and drimanial*

The cumulative addition of polygodial or drimanial produced CCRC in rat urinary bladder strips (Figs. 1 and 2). The calculated mean EC_{50} values and respective 95% confidence limits are: 1.5 (0.004–2) μM and 2.8 (0.005–17) μM, respectively, for polygodial and drimanial. The CCRC induced by polygodial and drimanial was significantly inhibited by the pre-incubation of the preparations with NK_1 (SR 140333, 1 μM) plus NK_2 receptor antagonist (SR 48968, 1 μM) (66 ± 9% and 58 ± 7%, respectively) or with ruthenium red (30 μM, TRPV1 receptor non-selective antagonist) (38 ± 7% and 57 ± 4%, respectively) (Figs. 1 and 2A, B). In addition, capsazepine (10 μM), TRPV1 receptor antagonist, partially inhibited the contraction induced by polygodial and drimanial (Figs. 1 and 2B). On the other hand, capsaicin, but not polygodial and drimanial, caused marked tachyphilaxis in the contractile response (not shown). However, the contractile response induced polygodial and drimanial did not generate cross-desensitization with capsaicin (results not shown).

3.2. *Effect of capsaicin, polygodial and drimanial on the extracellular [$^{45}Ca^{2+}$] uptake in rat spinal cord synaptosomes*

As expected, capsaicin (1 μM) promoted marked increase of [$^{45}Ca^{2+}$] influx in rat spinal cord synaptosomes with efficacy of 63 ± 10% in relation to KCl response (77 mM) (Fig. 3). Furthermore, polygodial (5 μM) and drimanial (5 μM) also induced significant [$^{45}Ca^{2+}$] influx in spinal cord synaptosomes, with efficacy of 70 ± 15% and 47 ± 16%, respectively, when compared to that produced by KCl (77 mM) (Fig. 3).

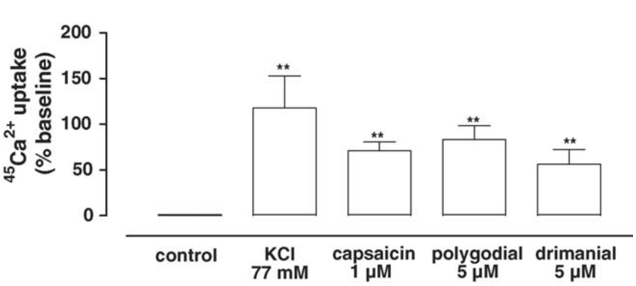

Fig. 3 – Effect of capsaicin (1 μM), polygodial (5 μM) and drimanial (5 μM) on the [$^{45}Ca^{2+}$] uptake in synaptosomes of rat spinal cord. Each column represents the mean ± S.E.M. of four experiments conduced in duplicate. $^{**}P < 0.01$, significantly different when compared with values basal (one-way ANOVA followed by Dunnett's).

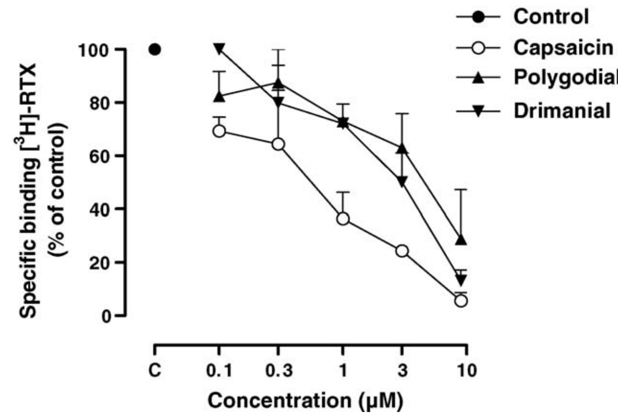

Fig. 4 – Assay of competition for specific [^{3}H]-resiniferatoxin binding in spinal cord membranes of rats induced by capsaicin (0.001–10 μM), polygodial (0.01–10 μM) and drimanial (0.01–10 μM). Each column represents the mean ± S.E.M. of four experiments conduced in duplicate. Results are plotted as percentage of control.

3.3. *Effect of capsaicin, polygodial and drimanial on specific [^{3}H]-resiniferatoxin binding in rat spinal cord membrane*

Fig. 4 shows that capsaicin (0.1–10 μM) caused concentration-dependent displacement of the specific binding for [^{3}H]-resiniferatoxin in rat spinal cord membranes, with maximum inhibition of 94 ± 3% and the calculated mean IC_{50} value of 0.48 (0.08–19) μM. Likewise, polygodial and drimanial produced concentration-dependent displacement of the specific [^{3}H]-resiniferatoxin binding, with maximum inhibition of 71 ± 18% and 86 ± 4% and with calculated mean IC_{50} values of 4.2 (0.3–10) μM and 3.2 (0.1–15) μM, respectively (Fig. 4).

3.4. *Effect of capsaicin, polygodial and drimanial in the intracellular Ca^{2+} levels in cultured rat trigeminal neurons*

The sesquiterpenes polygodial and drimanial caused a concentration-dependent increase in the intracellular Ca^{2+} levels when evaluated in cultured rat trigeminal neurons (Fig. 5A). Potencies of polygodial and drimanial (EC_{50} = 40.76 μM and 73.36 μM, respectively) were 1500 and 2500 folds, respectively, lower if compared to that of capsaicin (EC_{50} = 0.027 μM, not shown). The calcium mobilization induced by the maximal tested concentration of polygodial (100 μM), drimanial (100 μM) or capsaicin (0.1 μM) was significantly inhibited by capsazepine (10 μM) (Fig. 5B). Furthermore, the increase in the intracellular calcium levels produced by capsaicin was not altered by previous incubation of cultured neurons with neither sesquiterpenes (results not shown). In addition, polygodial and drimanial were effective in all the tested capsaicin-sensitive neurons and vice versa.

4. Discussion

There is now great amount of evidence suggesting that the TRPV1 activation is involved in several pathological conditions

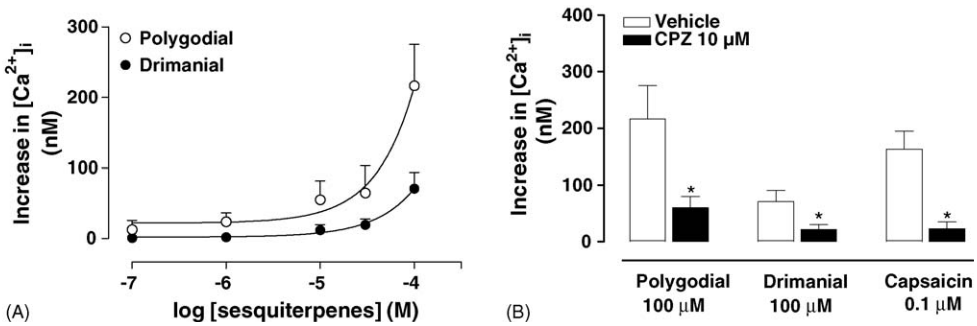

Fig. 5 – (A) Concentrations response curves of polygodial (open circle) and drimanial (closed circle) and (B) inhibitory effect of capsazepine (10 μM) on the intracellular calcium mobilization induced by polygodial (100 μM), drimanial (100 μM) or capsaicin (0.1 μM) in cultured newborn rat trigeminal neurons. Each column represents the mean ± S.E.M. of at least 24 cells. $^{*}P < 0.05$, significantly different when compared with vehicle (Student's *t*-test).

that have been successfully related with the pungent natural substance capsaicin, isolated from plants belonging to the *Capsicum Genera*. Recent data also indicate that several plant-derived compounds, besides capsaicin, are known to selectively activate TRPV1 see for review [10].

In a previous study, we have shown that neonatal treatment of rats with polygodial or drimanial, as well as with capsaicin, produced pronounced anti-nociceptive effect associated with a marked inhibition of specific $[^3H]$-resiniferatoxin binding sites in spinal cord [17]. In the present study, we have further assessed whether or not the two sesquiterpens polygodial and drimanial isolated from the plant *D. winteri* produce their pharmacological actions by interacting with TRPV1. Extending our earlier findings, we have found that similarly to capsaicin, the naturally occurring sesquiterpenes polygodial and drimanial caused concentration-dependent contraction of rat urinary bladder strips, being their action antagonized by the TRPV1 antagonist capsazepine. There is a substantial amount of experimental evidence showing that large proportion of sensory fibres innervating the urinary bladder is sensitive to capsaicin. In fact, intravesical capsaicin application induces acute burning pain, detrusor contractions and reduction of bladder volume threshold to reflex micturition in rats and humans (see for review; [6]). Since capsaicin application produces degeneration of afferent fibres, this treatment is able to induce a long lasting increase in the volume threshold of micturition reflex in the spinal cord-injured patients who suffer from overactive bladder [26]. The important role of TRPV1 in urinary bladder function was also recently confirmed with the use of TRPV1 knockout mice [27].

TRPV1 is highly expressed by neurons in dorsal root ganglion at the origin of C and Aδ fibres being then transported to peripheral and spinal cord terminals [5]. Capsaicin gates TRPV1 channel to induce Ca^{2+} influx into the nerve endings with subsequent release of neuropeptides, including calcitonin gene-related peptide and tachykinins namely, substance P and neurokinin A (see for review; [28]). Thus, the stimulation of tachykinin receptors is directly related to several actions of capsaicin, including the smooth muscle contraction of the urinary bladder (see for review; [28]). Similarly to capsaicin, polygodial and drimanial-mediated contractions in rat urinary bladder were also significantly inhibited by the selective NK_1 and NK_2 tachykinin receptor antagonists or with ruthenium red. On the other hand, the pre-incubation of preparations with the selective TRPV1 antagonist, capsazepine, partially inhibited the contraction induced by polygodial and drimanial. Thus, these findings strongly suggest the view that the sesquiterpenes produce contractile response through activation of a ruthenium red-sensitive channel and release of substance P or neurokinin A. However, in contrast to capsaicin, polygodial and drimanial, failed to produce marked tachyphilaxis. Of note, our results also show that poligodial and drimanial failed to exhibit cross-desensitization with capsaicin. Such results suggest that although some of the actions of polygodial and drimanial in the rat urinary bladder seem to be quite similar to that caused by capsaicin, there are some apparent differences regarding their precise site of action. In addition, the more efficient inhibitory activity induced by ruthenium red, if compared to that produced by capsazepine, suggests that polygodial- or drimanial-induced responses may involve more than one type of TRPV receptors. In fact, ruthenium red is a non-selective TRPV family antagonist since it possesses antagonistic activities against all the receptors belonging to the transient receptor potential vanilloid family (TRPV1, 2, 3, 4, 5 and 6).

In this study, we also investigated if these two sesquiterpenes could interact directly with specific binding of $[^3H]$-resiniferatoxin in rat spinal cord membranes. Our results clearly demonstrate that, with an efficacy similar to capsaicin, both polygodial and drimanial concentration-dependently displaced the specific binding of $[^3H]$-resiniferatoxin sites in rat spinal cord membranes. However, at the IC_{50} level, polygodial and drimanial were about 6–9-fold less potent than capsaicin. These results obtained for polygodial are in agreement with the presented by Szallasi et al. [9]. They demonstrated that polygodial was effective in inhibiting the specific $[^3H]$-resiniferatoxin binding in rat spinal cord with a IC_{50} value of 7.6 ± 0.9 μM. In addition, polygodial and drimanial like capsaicin were also active in promoting $[^{45}Ca^{2+}]$ uptake in the spinal cord synaptosomes. Confirming and extending these results, both sesquiterpenes were also able to increase the intracellular Ca^{2+} concentration in cultured rat trigeminal neurons, an effect that was significantly inhibited by capsazepine. The aforementioned results further reinforce the notion that the pharmacological actions of both sesquiterpenes seem to be likely associated with their abilities to interact with TRPV1.

Collectively, the results of the present study in conjugation with of our earlier data [10,13–17] suggest that both sesquiterpenes polygodial and drimanial, at the tested concentrations, may directly and/or indirectly activate TRPV1. Thus, the activation of TRPV1 could explain, most of the in vivo pharmacological actions of the two sesquiterpenes and/or those observed previously by the extract of the plant *D. winteri*, especially their anti-inflammatory and anti-nociceptive properties. Additional studies are required to investigate the precise mechanisms through which these naturally occurring plant sesquiterpenes exert their pharmacological actions at the TRPV1.

Acknowledgements

This study was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Apoio a Ciência e Tecnologia de Santa Catarina (FAPESC), by Programa de Apoio aos Grupos de Excelência (PRONEX) (Brazil). E. André, J. Ferreira and A. Malheiros are Ph.D. students in Pharmacology or Chemistry and they thank CNPq and CAPES (Brazil) for the fellowship support.

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