Borneol inhibits TRPA1, a proinflammatory and noxious pain-sensing cation channel

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ABSTRACT: Borneol, a natural product isolated from several species of *Artemisia*, *Blumea* and *Kaempferia,* has a widespread use in traditional medicine. TRP ion channels are a class of nonselective cation channel proteins involved in a variety of physiological and pathological processes in mammals. TRPA1, a member of TRP family of cation channels, is involved in plethora of processes including noxious-cold, noxious-pain sensations, inflammation and the detection of irritant chemicals. Borneol is chemically related to camphor (a known inhibitor of TRPA1 ion channels); therefore, it is beneficial to investigate the effects of borneol on TRPA1. In the present investigation it was found that borneol inhibits TRPA1 mediated cationic currents in low millimolar range (IC_{50} 0.3mM) in heterologous expression systems like *Xenopus* oocytes and in neurons cultured from trigeminal ganglia. Effects of nicotine, a known chemical irritant and agonist of TRPA1 are also inhibited by borneol in both systems. It is concluded that borneol, being an inhibitor of TRPA1, could be a safer therapeutic-combination in clinical situations where TRPA1 channelopathies like neuropathic-pain, trigeminal neuralgia or nicotine withdrawal treatments are involved.

Keywords: TRPA1, borneol, cultured neurons, oocytes, calcium imaging.

INTRODUCTION

Naturally occurring d-(+)-borneol is an optically active compound found in several species of *Artemisia*, *Blumea* and *Kaempferia.* It is used in traditional Eastern medicine. Borneol is a monoterpene like camphor and menthol. Terpenes are extensively used in pharmaceutical, medical and cosmetic industry. These uses range from anaesthetic, analgesic (Galeotti *et al*., 2001, Galeotti *et al*., 2002, Xu *et al*., 2005), anti-inflammatory (Santos and Rao, 2001) to antipruritic medications (Anand, 2003). Monoterpenes have been shown to activate, inactivate or modulate ion channels. For example borneol, thymol, α-thujone and menthol modulate γ-aminobutyric acid-A (GABA-A) receptors (Abdullah and Zhang, 2013, Granger *et al*., 2005). Camphor and borneol non-competitively inhibit nicotinic acetylcholine receptors (Park *et al*., 2003).

Transient Receptor Potential Ankyrin-1 channel (TRPA1) belongs to a large-family of cation channel proteins. TRPA1 functions as a Ca^{2+}-permeable non-selective cation channel in many different cellular processes. TRPA1 is extensively expressed both in neuronal and non-neuronal cells (Jaquemar *et al*., 1999, Asima Hameed, 2013). The TRPA1 channel protein comprises of 14-19 amino-terminal ankyrin repeats as an exclusive structural feature among the members of TRPs super-family of cation channels (Sotomayor *et al*., 2005). Noxious cold ($<17^0$C) and a whole range of chemical substances are known to activate the TRPA1 ion channels. The activation agents include free-radical oxidants that alter nucleophilic cysteine residues in the N-terminus of the channel protein. Compounds that do not bind covalently to the channel proteins (e.g. the physiological agonist mustard oil, menthol and nifedipine) also activate TRPA1 (Bandell *et al*., 2004, Bautista *et al*., 2005, Hinman *et al*., 2006, Klionsky *et al*., 2007, Nagata *et al*., 2005, Wang *et al*., 2011, Macpherson *et al*., 2007, Alpizar *et al*., 2012).

TRPA1 is assumed to be a key player in acute-, chronic-neuropathic pain and inflammation exclusively because of its expression patterns and functional attributes (Nilius *et al*., 2012). High density of TRPA1 and TRPV1 co-expression in Trigeminal (TG) and dorsal root ganglion (DRG) neurons is reported (Akopian *et al*., 2007, Kobayashi *et al*., 2005, Salas *et al*., 2009). Behavioural studies in knockout mice have explicitly delineated the role of TRP channels in nociception (Kwan *et al*., 2006). TRPA1 knockout mice exhibit impaired behavioural responses to a cold-plate maintained at 0°C (Kwan *et al*., 2006). Ablation of TRPA1 channels that are linked to TRPV1 lineage result in a complete loss of thermal sensitivity (Mishra *et al*., 2011).

The preceding discussion substantially augments the postulation that TRPA1 is a critical nociceptor. Therefore potent pharmacological antagonists of TRPA1 could be potential analgesic drugs (Andrade *et al*., 2012). In this context variety of monoterpenes like menthol and its derivatives, camphor and its chemical analogue, thymol and borneol were investigated to target TRPA1 inhibition

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or desensitization in heterologous expression systems like *Xenopus* oocytes. Borneol is found to be a very useful natural inhibitor of TRPA1 channel activity both in heterologous expression system and in cultured trigeminal neurons.

MATERIALS AND METHODS

Expression of cDNA in Xenopus laevis oocytes

Plasmids containing cDNA inserts of TRPA1 ion channels were designed in a similar fashion as published earlier (Sherkheli *et al*., 2010, Sherkheli *et al*., 2008, Sherkheli, 2012, Sherkheli, 2007). For *in vitro* transcription, plasmids were linearized and cRNAs were synthesized in the presence of the capping analog m7G (5`) ppp (5`) G and T7 polymerase using a Ampli Cap T7 High Yield Message Marker Kit (Epicentre). RNA concentration in-water after re-dissolution was 1µg/µl. Gel electrophoresis was used to ensure a very high quality of cRNA and this also helps in excluding degradation processes for cRNA. Oocytes were harvested surgically from female *Xenopus laevis* frogs. Frogs were anesthetized with 0.06% (w/v) ethyl-2-aminobenzoic acid (methanesulfonate salt; Sigma-Aldrich, Germany) for 30 minutes. Ovarian tissue was removed and placed in Barth's solution (88mM NaCl, 1 mM KCl, 0.82mM $MgSO_4$, 0.33mM Ca $(NO_3)_2$, 0.42mM $CaCl_2$, 2.4mM $NaHCO_3$, 5mM Tris-HCl, pH 7.4, 100 U/ml penicillin, and 50µg/ml streptomycin). Oocytes were treated with collagenase (2mg/ml Type 1) in Ca^{2+} free Barth's solution for durations of 1.5 to 2h at room temperature. Healthy stage IV-VI oocytes were selected for cytoplasmic injection of cRNA. Before injection, oocytes were stored at 18°C overnight. The cRNA was injected using nanoliter injector 2000 (WPI). Each oocyte was injected with 5-10ng cRNA. Thereafter, injected oocytes were kept again in fresh Barth's solution maintained at 18°C. Oocytes were probed after 3-5 days of cRNA injections for TRPA1 mediated cationic currents.

Two-electrode voltage-clamp recordings

Electrophysiological recordings were performed as described elsewhere (Sherkheli *et al*., 2013, Vogt-Eisele *et al*., 2007). Agonist induced currents were recorded by the use of voltage-clamp mode of two electrode voltage-clamp technique (Turbo Tec-3x npi, Tamm, Germany). Voltage-clamp electrodes were fabricated from borosilicate glass (1.17x1.50 x 100mm, Science Products, Hofheim, Germany). Electrode resistance was 0.5-1.5 MΩ when filled with 3mM KCl. Oocytes were kept submerged in Normal Frog Ringer's (NFR) solution through the entire length of experiment(s). NFR solution contained: 115mM NaCl, 2.5mM KCl, 1.8mM $CaCl_2$, 10mM HEPES, pH 7.2 (NaOH/HCl). All ligands and tested substances were diluted in NFR to the stated concentration and delivered to the oocyte (100µl) using an automatic pipette (research pro, Eppendorf, Germany). Pulse 8.4 software (HEKA, Germany) was used to register the evoked currents.

Primary culture of trigeminal ganglia neurons

Trigeminal ganglia neurons were harvested from CD1 mice. European Union Community Council guidelines for animal handling were strictly adhered to for all experiments involving animals.

Mice were sacrificed by decapitation at postnatal day 2 to 5. Trigeminal ganglia (*Ganglion gasseri*) were excised from brains harvested from scarified mice. Ganglia were washed in $PBS^{+/+}$ (Invitrogen) and collected in Leibovitz Medium (L15, Invitrogen), which was constantly kept on ice. The ganglia were transferred to Dulbecco's modified essential medium (DMEM) containing 0.025% collagenase (type IA, Sigma-Aldrich). Minced ganglia were incubated for 45 minutes in a humidified atmosphere (37°C, 95% air humidity, 6% CO_2). The tissue was triturated to isolate cells. Cell suspension was centrifuged at 1000rpm for 4 minutes at room temperature and the pellet was re-suspended in F-12 medium (Invitrogen; 31331-D-MEM/F-12, GlutaMax™) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 200U/ml penicillin and 200 U/ml streptomycin. Afterwards, the cell suspension (50µl) was plated on poly L-lysine (0.01%) coated glass coverslips (30mm), which were kept in cell culture dishes (35mm). The dishes were kept in a humidified atmosphere for one hour. Subsequently, F-12 medium (2ml) supplemented with FBS (10%), 200U/ml penicillin and 200 U/ml streptomycin was added to each dish. Until use, cells were incubated at 37°C (95% air humidity, 6% CO_2).

Calcium-imaging experiments

Calcium imaging experiments were carried out as described earlier (Sherkheli *et al*., 2009). Thirty to forty five minutes before the start of the experiment, neurons were incubated with the Ca^{2+}-sensitive reporter dye Fura-2/AM (Invitrogen, final concentration 3mM). Cover-slips were placed under an inverted microscope (Axiovert 200, Zeiss) equipped with a fluorescence-optimized 20-fold Olympus (20x/0.75) objective. Fura-2 loaded neurons were excited at wavelengths of 340nm and 380nm, alternatively. CCD camera was used to detect emitted light. Changes of intracellular calcium levels were calculated as a ratio; (E (510) at 340nm illumination)/ (E(510) at 380 nm illumination). During an experiment neurons were constantly super fused with extra cellular solution. The extra cellular solution could be exchanged quickly by the desired test solution through a custom-made 7-in-1 miniaturized metal tube application system. Viability and neuronal character of the neurons were verified by a short pulse of high potassium solution at the end of each experiment. The composition of extra cellular solution was; NaCl 140mM, KCl 5mM, $CaCl_2$ 2 mM, $MgCl_2$ 1mM, EGTA 5mM, HEPES 10mM; pH 7.4. High potassium solution consisted of; NaCl 100mM, KCl 45mM, $MgCl_2$ 1mM, EGTA 5mM, HEPES 10mM; pH 7.4.

Chemicals for electrophysiology and calcium-imaging experiments

Stock solutions of substances used were prepared in *aqua dest* or dimethyl sulfoxide (DMSO). The stock solutions were diluted to their final concentration (DMSO concentration was kept 0.1%) with standard extra cellular solution. All chemicals including nonspecific transient receptor potential channel blocker ruthenium red were purchased from Sigma-Aldrich (Germany), unless stated otherwise. Fura-2/AM, used for calcium detection in imaging experiments was supplied by Invitrogen (Darmstadt, Germany). Borneol in general means (-) borneol isomer unless stated otherwise.

Data analysis

Currents were measured using pulse software from HEKA (Lambrecht, Germany). Concentration response data currents were fitted and EC_{50} values calculated with the 3-parameter Hill equation using Sigma Plot 8.0 software. Student's t-test was used for statistical analysis of the data. Calcium-imaging data were analyzed with the Slide book software from Intelligent Imaging Innovations (Denver, USA), further processed by IGOR Pro software from Wave Metrics (Portland, USA). fig. design was performed with Corel DRAW X6 (Corel Corporation).

RESULTS

Expression of hTRPA1 in Xenopus oocytes and its inhibition by borneol

TRPA1 is modulated by pro-inflammatory mediators, neuropeptides and cytokines and hence offers a therapeutic option for targeted pain therapies (Premkumar and Abooj, 2012, Nilius *et al*., 2012). To establish the functional expression of hTRPA1 in oocytes, microinjected oocytes with cRNA of human TRPA1 were analyzed after three to seven days of injection as described elsewhere (Sherkheli, 2012, Sherkheli, 2013). fig. 1 show a typical two-electrode voltage-clamp Registration recorded from an oocyte expressing hTRPA1 response to 100µM mustard oil (MO) in Ca^{2+}-free extra cellular solution. The activation of TRPA1 is nearly completely blocked either by co-application of 10µM ruthenium red (RR) or 1mM borneol. In both cases the MO response was restored after washout of the blocker with extra cellular solution (fig. 1).

To resolve whether hTRPA1 inhibition by borneol is dose-dependent different concentrations (from 10µM to 1000 µM) of the antagonist were used; while the mustard oil (agonist) concentration (100µM) was kept constant. With rising borneol concentrations, a gradual decrease in magnitude of mustard oil-induced currents was observed. The mustard oil-induced current was insignificant in the presence of 1000µM borneol (fig. 2 & 3). The IC_{50} value calculated for inhibition of hTRPA1 by (-) borneol in Ca^{2+}-free solution was 0.3±0.1mM and by (+) borneol was 0.5±0.2mM (fig. 3A). The block of hTRPA1 by borneol could be overcome by using higher mustard oil concentrations (1mM; fig. 3B). The channel was responsive to mustard oil after borneol wash-out.

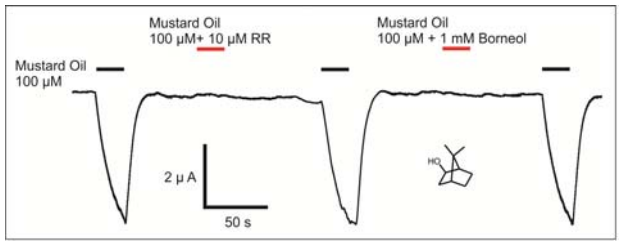

Fig. 1: Mustard oil induced TRPA1 activation and its block by borneol.

A sample trace showing hTRPA1 mediated cationic currents induced in *Xenopus* oocytes in response to application of mustard oil in Ca^{2+}-free solution. TRPA1 activity was blocked by common TRP channel inhibitor ruthenium red (RR). When coupled with 1mM borneol co-application, mustard oil induced currents were drastically reduced. The mustard oil response was restored after washout with extra cellular solution. Ca^{2+}-free bath solution is used to avoid desensitization of TRPA1 channels induced by calcium.

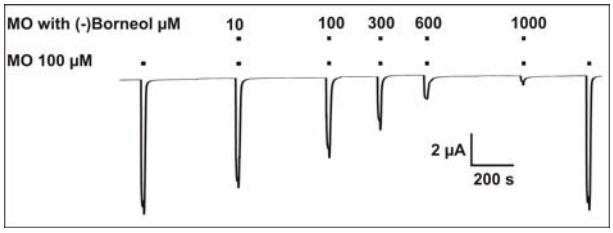

Fig. 2: Dose-dependent inhibition of TRPA1 mediated currents in oocytes

A sample trace showing dose response inhibition curve for hTRPA1 mediated currents in oocytes bathed in Ca^{2+}-free solution. MO stands for mustard oil. The Ca^{2+}-free solution was used to avoid desensitization effects of MO on TRPA1 commonly seen after repeated activation of the channels in calcium containing extra cellular solutions. Normal response to MO at the end of inhibition experiment shows no desensitization of TRPA1 activity.

Subsequently, it was also investigated whether borneol block is voltage-dependent or not. For this set of experiments inhibition of hTRPA1 by borneol was measured at positive (+40mV) holding potentials and contrasted with inhibition at negative (-40 mV) holding potentials. No voltage-dependence of TRPA1 inhibition could be found (fig. 4).

Borneol and camphor are related chemical structures and camphor is a known inhibitor of TRPA1. This makes it interesting to compare the inhibitory potential of borneol with camphor. For this set of experiments a fixed concentration of borneol (2mM) and camphor (2mM) was used to determine the comparative inhibitory effects on mustard oil induced currents mediated through TRPA1 ion channels. Both camphor and borneol caused a significant rightward shift in dose response curve of mustard oil induced responses (fig. 5).

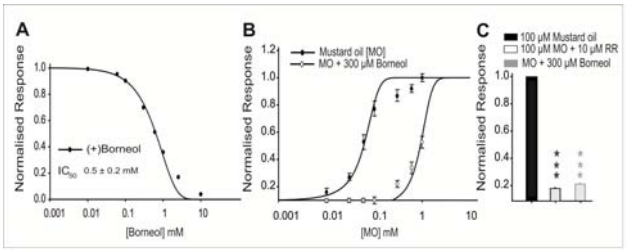

Fig. 3: Dose-dependence and reversibility of TRPA1 inhibition by borneol.

(A) Inhibition curve for (-)borneol and IC_{50} values. (B) Reversibility of inhibition of hTRPA1 by increasing mustard oil concentrations (n=6 in each case). (C) Normalized and quantified current inhibition response for hTRPA1 by ruthenium red and (-)borneol.

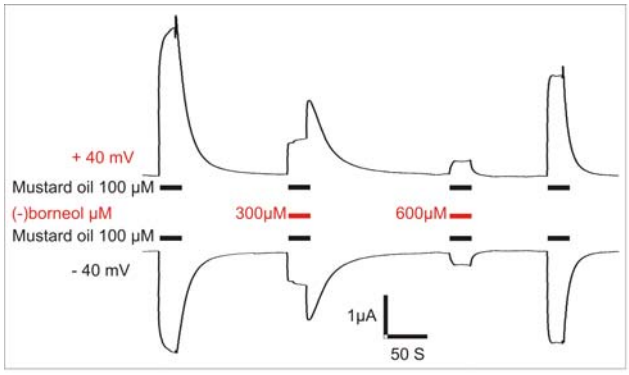

Fig. 4: Voltage-independent Block of TRPA1 by borneol.

Original trace showing inhibition of hTRPA1 by borneol at positive and negative holding potentials. hTRPA1 expressing oocytes were held at constant holding potentials either at +40 mV or at -40 mV. Mustard oil was applied alone or in combination with borneol.

Chemosensory-perception of nicotine is partly contributed by hTRPA1 (Talavera *et al*., 2009). To see whether structures closely related to nicotine also activate hTRPA1, oocytes expressing the ion channels were subjected to benzylnicotinate challenge (fig. 6). Nicotine and benzylnicotinate activate TRPA1 and this activation is effectively inhibited by borneol.

Inhibition of mTRPA1 mediated calcium influx in trigeminal neurons

Nicotine activates TRPA1 (Talavera *et al*., 2009); which is extensively expressed in sensory nerves of airways in lungs (Nassenstein *et al*., 2008, Hameed, 2013). Therefore, it is clinically useful to investigate whether borneol is capable of inhibiting nicotine-induced TRPA1 mediated calcium influx in neurons. For this part of study, calcium imaging was used. TRPA1 responses, induced either by naturally occurring (-)-nicotine or by its stereoisomer (+)-nicotine, were recorded. Borneol (1mM) was able to effectively block (+)- and (-)-nicotine-induced calcium currents in cultured neurons (fig. 7 A and B) but some exclusively (+)-nicotine response neurons were resistant to borneol block. Nicotinic acetylcholine receptors (nACh-R), broadly expressed in trigeminal neurons, were blocked by the specific nACh-R blocker hexamethonimum (1mM) in these experiments.

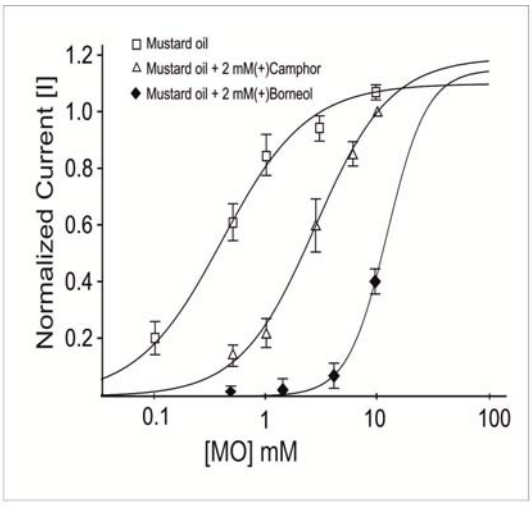

Fig. 5: Comparative inhibitory potential of borneol and camphor.

Fixed concentration (2 mM) either of borneol or camphor was used to find out dose response curve of mustard oil. Both borneol and camphor caused a significant rightward shift. Owing to solubility issues of camphor and mustard oil at higher concentrations, effects at doses higher than 10mM could not be measured.

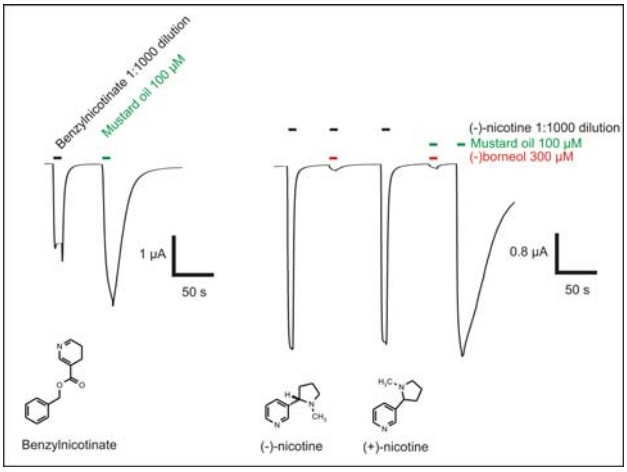

Fig. 6: Borneol inhibits nicotine activation of hTRPA1.

Shown are the traces of original recordings of hTRPA1 activation by nicotine, benzylnicotinate and its inhibition by borneol in oocytes expressing hTRPA1 maintained in calcium free extra cellular solution.

Trigeminal neurons in this investigation were not homogeneous in their responses to nicotine and block by borneol. There were subpopulations within the same culture from single animal.

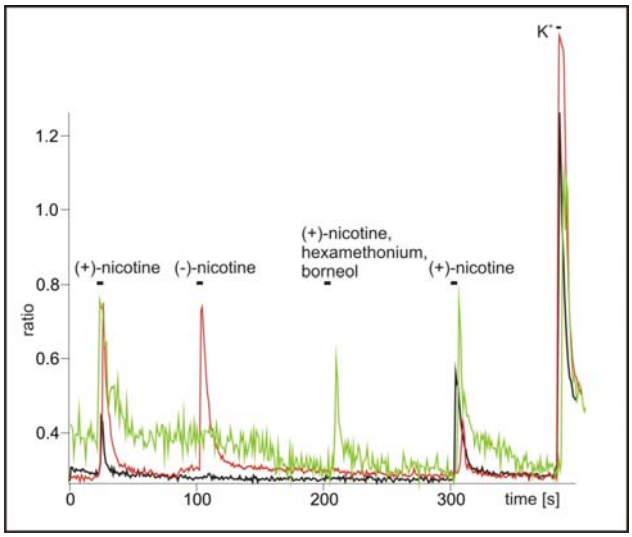

Fig. 7A: Borneol does not inhibit nicotine induced responses in a subpopulation of trigeminal neurons.
Green trace: exclusively (+)-nicotine responsive, not blocked by borneol and hexamethonium. Red trace: (-)- and (+)-nicotine responsive, (+)-nicotine response is blocked by hexamethonium and borneol. Black trace: exclusively (+)-nicotine responsive, borneol and hexamethonium blocked.

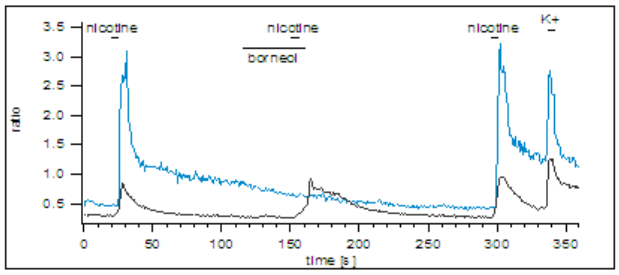

Fig. 7B: Borneol inhibts (-)-nicotine (250µM) induced responses in a subpopulation of trigeminal neurons.
The blue curve shows a neuron responding to (-)-nicotine (250µM) is blocked by borneol (1mM) (n=13), the black curve shows a neuron responding to (-)-nicotine and is not blocked by borneol (n=4). Black bars indicate time of application.

DISCUSSION

Due to wide-ranging expression patterns, decisive role in cellular inflammation, pain and chemosensation TRPA1 ion channel proteins afford a key target for anti-inflammatory and analgesic drugs. TRPA1 (noxious cold sensor) and TRPV1 (a noxious heat sensor) are the best investigated members of TRP superfamily of channel forming proteins (Caterina *et al*., 1999, Jordt *et al*., 2003, Dhaka *et al*., 2006, Hu *et al*., 2006, Levine and Alessandri-Haber, 2007). Borneol is widely used in traditional remedies with the assumption that it induces production of gastric juices, improves circulation, alleviates bronchitis, coughs, rheumatic diseases, swelling and relives stress as a tonic for relaxation (Ehrnhofer-Ressler *et al*., 2013, Song *et al*., 2013, Yu *et al*., 2013). In the present investigation essential oils and their active constituents (terpenes like menthol, thymol, camphor, borneol etc) were analyzed for their effects on TRPA1 in search for a natural compound that does not accelerate pain sensation. Borneol was found to inhibit TRPA1 in expression systems as well as in cultured neurons. Although more potent synthetic TRPA1 antagonists like A-967079 (Chen *et al*., 2011) and HC-030031(Eid *et al*., 2008) are available but borneol has the advantage of a low-cost natural product with proven efficacy and safety profile for human use (Ehrnhofer-Ressler *et al*., 2013). Additionally, borneol (IC_{50} 0.3±0.1mM) is a better antagonist of TRPA1 than camphor, which has an IC_{50} value of 3.65±0.13 in oocytes expression system (fig. 5).

In Ca^{2+}-free extra cellular solutions TRPA1-mediated cationic currents manifest an increase in amplitude with recurring applications of low concentrations of the agonists. The reverse is observed with recurring application of higher agonist concentrations, i.e., tachyphylaxis. The extent of tachyphylaxis is significantly larger in the presence of extra cellular Ca^{2+}. Under physiological conditions the strength of stimulus impinging upon TRPA1 activation site is significantly influenced by the presence of extra cellular calcium because it helps to increase the intracellular calcium which intern activates TRPA1 channels. This initial activation may also underpin the strength of activation by subsequent activation stimuli owing to the influence on intracellular calcium concentrations thus overall strength of responses mediated by TRPA1 whole cell currents are modulated. A low strength stimulus might lead to a phenomenon akin to wind-up and enhance nociceptive transmission, whereas a stimulus of higher magnitude might lead to a run-down of the response. Inhibition of TRPA1 in Ca^{2+}-free solution (figs. 1, 2 & 3) clearly demonstrate that reduction in TRPA1 conduction is calcium-independent and borneol might be interacting directly with the channel protein. Similarly TRPA1-mediated currents were also inhibited at positive holding potentials (fig. 4). Inhibition of TRPA1 activity at positive and negative holding potentials clearly suggests that channel inactivation by borneol is voltage-independent. Voltage and calcium are two independent physiological modulators of TRPA1 channel activity and the influence of these two modalities might be unpredictable under path physiological situations.

Nicotine and its analogue substance like benzylnicotinate (fig. 6) are lethal and toxic for mammals (Forsyth *et al*., 1994, Okamoto *et al*., 1994) and they target hTRPA1 for many of their effects (Talavera *et al*., 2009). In this study it is demonstrated that mTRPA1 activation by nicotine is effectively blocked by borneol in cultured neurons in calcium imaging studies (figs. 7 & 8). The experiments with trigeminal neurons clearly show subpopulations of neurons where the nicotine response could not be blocked

by borneol. Inhibition of MO responses in neurons by borneol is assumed to be the result of TRPA1 inhibition. It is difficult to explain whether the observed blockage of response is the consequence of TRPA1 inhibition or that of acetyl-nicotine receptors. This part of the investigation needs further investigations. However, it may be right to assume that inclusion of borneol in remedies for pain, inflammation and nicotine-withdrawal (antismoking) is useful.

CONCLUSION

Borneol is a natural product with strong inhibitory effects on proinflammatory TRPA1 noxious cold-sensor ion channels. Its potential inclusion in preparations intended for relief of inflammation, pain, cosmetic smoothening and smoking-cessation could be beneficial. (-)borneol (IC_{50} 0.3±0.1mM) and (+) borneol (IC_{50} 0.5±0.3mM) are better antagonists of TRPA1 compared to (+)camphor (IC_{50} for TRPA1 inhibition 3.65±0.13mM). These three have closely related terpenoid chemical structures. Their structure similarity provides the evidence about basic pharmacological concepts that structural modifications in antagonist may lead to improved efficacy of potential therapeutic agents.

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