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Putative Mode of Action of the Monoterpenoids Linalool, Methyl Eugenol, Estragole, and Citronellal on Ligand-Gated Ion Channels

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ABSTRACT

Essential oil has been used as sedatives, anticonvulsants, and local anesthetics in traditional medical remedies; as preservatives for food, fruit, vegetable, and grain storage; and as bio-pesticides for food production. Linalool (LL), along with a few other major components such as methyl eugenol (ME), estragole (EG), and citronellal, are the active chemicals in many essential oils such as basil oil. Basil oil and the aforementioned monoterpenoids are potent against insect pests. However, the molecular mechanism of action of these chemical constituents is not well understood. It is well-known that the γ-aminobutyric acid type A receptors ($GABA_A$Rs) and nicotinic acetylcholine receptor (nAChR) are primary molecular targets of the synthetic insecticides used in the market today. Furthermore, the $GABA_A$R-targeted therapeutics have been used in clinics for many decades, including barbiturates and benzodiazepines, to name just a few. In this research, we studied the electrophysiological effects of LL, ME, EG, and citronellal on $GABA_A$R and nAChR to further understand their versatility as therapeutic agents in traditional remedies and as insecticides. Our results revealed that LL inhibits both $GABA_A$R and nAChR, which may explain its insecticidal activity. LL is a concentration-dependent, non-competitive inhibitor on $GABA_A$R, as the half-maximal effective concentration (EC_{50}) values of γ-aminobutyric acid (GABA) for the rat α1β3γ2L $GABA_A$R were not affected by LL: (36.2 ± 7.9) μmol·L^{-1} and (36.1 ± 23.8) μmol·L^{-1} in the absence and presence of 5 mmol·L^{-1} LL, respectively. The half-maximal inhibitory concentration (IC_{50}) of LL on $GABA_A$R was approximately 3.2 mmol·L^{-1}. Considering that multiple monoterpenoids are found within the same essential oil, it is likely that LL has a synergistic effect with ME, which has been previously characterized as both a $GABA_A$R agonist and a positive allosteric modulator, and with other monoterpenoids, which offers a possible explanation for the sedative and anticonvulsant effects and the insecticidal activities of LL.

1. Introduction

Linalool (LL), methyl eugenol (ME), estragole (EG), and citronellal are major compounds found in many plant essential oils such as those extracted from basil, lavender, and lemongrass. Essential oils have been used as sedatives, anticonvulsants, antimicrobials, anxiolytics, and local anesthetics in traditional medical remedies [1,2]. In modern applications, the aforementioned compounds are found in aromatherapy products [3], flavoring agents [4], fragrance products, insecticides [5,6], and various household cleaning agents [7]. Many of the chemical constituents of basil oil display anticonvulsant activities in experimental animal models [8]. Basil oil has also shown anti-hyperalgesic effects in fibromyalgia murine models [9]. In addition, LL (a major constituent in many essential oils, particularly basil oil) is effective in the pentylenetetrazol, picrotoxin, and maximal electroshock seizure models [10,11], and has exhibited sedative effects in murine models [12]. Previous studies have found that these properties are related to the γ-aminobutyric acid (GABA)ergic, cholinergic, dopaminergic, glutaminergic, serotoninergic, and parasympathetic systems [6,8,13–16]. However, the molecular mechanisms of action of those chemical constituents, including LL, ME, EG, and citronellal are not well understood.

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The γ-aminobutyric acid type A receptors ($GABA_AR$s) are the primary inhibitory neurotransmitter receptors in the central nervous system (CNS). The $GABA_AR$s belong to the cys-loop ligand-gated ion channel family, which also includes the nicotinic acetylcholine receptor (nAChR), an excitatory neurotransmitter receptor. Many studies have proven that $GABA_AR$s play essential roles in epilepsy and many other neurodegenerative disorders. A number of clinically prescribed drugs, such as the benzodiazepine family, potentiate $GABA_AR$ inhibitory functions. Interestingly, the potentiation potency of some anticonvulsants on $GABA_AR$ does not correlate well with the drug's anticonvulsive efficacy, and some of them inhibit the Nav1.2 voltage-gated sodium ion (Na^+) channels [17,18]. Furthermore, lamotrigine, an anticonvulsant, was recently reported to inhibit the nAChR [19], in addition to blocking the voltage-gated Na^+ channels [20]. Therefore, since basil oil has been used as sedative, anticonvulsant, bio-pesticide, and food preservative [5,21], here we studied the electrophysiological effects of the major monoterpenoid components of basil oil (Fig. 1) on the nAChR and $GABA_AR$ in order to further elucidate the molecular mechanism of action of those monoterpenoids.

2. Materials and methods

2.1. Chemicals

All chemicals, including GABA, (−)-LL, ME, EG, (*R*)-citronellal ((*R*)-C), (*S*)-citronellal ((*S*)-C), and pentylenetetrazole (PTZ), were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA) unless otherwise specified.

2.2. Cell culture

The WSS-1 cell line (ATCC; CRL-2029™) and the KXα3β4R2 cell line (a generous gift from Dr. Yingxian Xiao, Georgetown University), which stably express the rat α1β3γ2L $GABA_AR$ [22,23] and the rat α3β4 nAChR subtype [24], respectively, were maintained in GlutaMAX™ medium (Thermo Fisher Scientific; Waltham, MA, USA) supplemented with 10% fetal bovine serum, 0.4 mg·mL^{-1} G-418, 100 units·mL^{-1} penicillin, and 0.1 mg·mL^{-1} streptomycin at 37 °C and 5% CO_2. Subcultures were performed twice a week at approximately 75%–90% confluency using a 1:5 dilution ratio split. The cells were used between 24 and 72 h after incubation in tissue culture plates for whole-cell current recordings.

2.3. Whole-cell current recordings

Whole-cell currents were amplified using a computer-controlled patch clamp amplifier (EPC 10 USB) (HEKA Elektronik; Holliston, MA, USA) with a built-in digitizer (LIH 8+8) (HEKA Elektronik; Holliston, MA, USA) and recorded using the data-acquisition software PatchMaster (HEKAElektronik; Holliston, MA, USA). Recording pipettes of 2–3 MΩ were pulled from borosilicate glass capillaries (World Precision Instruments; Sarasota, FL, USA) using a Narishige PC-10 vertical pipette puller (Narishige International USA, Inc.; Amityville, NY, USA). The intracellular electrode buffer consisted of 140 mmol·L^{-1} CsCl, 10 mmol·L^{-1} ethylene glycol tetraacetic acid (EGTA), 2 mmol·L^{-1} $MgCl_2$, 10 mmol·L^{-1} tetraethylammonium chloride (TEA-Cl), and 10 mmol·L^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), adjusted to pH = 7.4 with CsOH. The TEA-Cl was not included in the intracellular buffer when the KXα3β4R2 cell line (expressing nAChR) was used. The extracellular electrode buffer was composed of 145 mmol·L^{-1} NaCl, 5 mmol·L^{-1} KCl, 2 mmol·L^{-1} $CaCl_2$, 1.5 mmol·L^{-1} $MgCl_2$, and 25 mmol·L^{-1} HEPES, adjusted to pH = 7.4 with NaOH. The cells were clamped at −60 mV. Experiments were performed in at least triplicate at room temperature (25 °C).

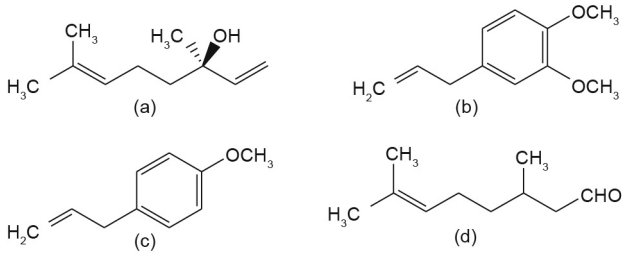

Fig. 1. Structures of (a) (−)-LL, (b) ME, (c) EG, and (d) citronellal.

2.4. Rapid ligand solution delivery

The cell-flow device used for rapid ligand solution delivery to membrane proteins at the cell surface has been described in detail [25]. To summarize, a U-shaped tube with a porthole of diameter of 150 μm in the middle was connected to the inlet and outlet tubing, which were pumped by a Minipuls® 3 peristaltic pump (Glison, Inc.; Middleton, WI, USA). The solution delivery timing and length were automatically triggered and controlled. This device allowed the delivery of ligand solution to flow over the cell and exchange within milliseconds.

2.5. Curve fitting

GraphPad Prism Version 5.04 for Windows (GraphPad Software, Inc.; San Diego, CA, USA) was used to fit the inhibition curves of the receptors by LL and the dose-dependent responses of the $GABA_AR$ in the absence and presence of 5 mmol·L^{-1} LL. Agonist concentration–response relationships were fitted to the equation $I/I_{max} = 1/[1 + (EC_{50}/C_A)^{n_H}]$, where I and I_{max} represent the current at a given agonist concentration (C_A) and the maximal agonist-induced current, respectively. EC_{50} is the half-maximal effective concentration and n_H is the Hill coefficient. PTZ inhibition-response relationships were fitted with the equation $I_A/I_0 = 1/[1 + (IC_{50}/C_{LL})^{n_H}]$, where I_A and I_0 are the current amplitudes in the presence and absence of LL, respectively. IC_{50} is the half-maximal inhibitory concentration, and C_{LL} is the concentration of LL. Data were expressed as the mean ± standard error of mean (SEM).

3. Results

3.1. Dose-dependent inhibition by LL

LL at various concentrations was co-applied with 100 μmol·L^{-1} of GABA, a near-saturating concentration. Compared with the control current induced by 100 μmol·L^{-1} of GABA, it is clear that LL dose-dependently inhibited the α1β3γ2L $GABA_AR$ (Fig. 2(a)). Interestingly, LL also inhibited the α3β4 nAChR with similar potency as on the $GABA_AR$ (Fig. 2(b) versus Fig. 2(a)) when co-applied with carbamoylcholine, even though its efficacy seemed higher. The incomplete inhibition by LL on the $GABA_AR$ is probably due to the solubility limit of LL in aqueous solution, as the highest concentration we were able to obtain was 50 mmol·L^{-1}. The whole-cell currents of the nAChR were induced by a near-saturating concentration of carbamoylcholine, which is a stable analog of the endogenous agonist acetylcholine.

The GABA dose–response curves in the absence and presence of 5 mmol·L^{-1} LL showed that LL inhibited the whole-cell currents

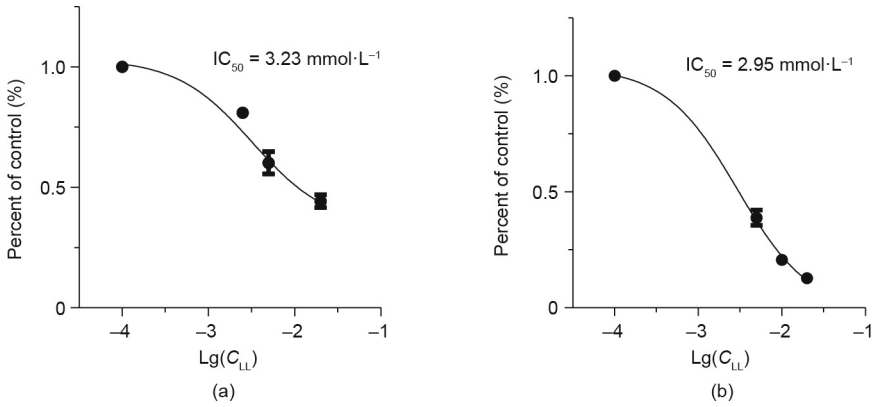

Fig. 2. Dose-dependent inhibition of LL on the whole-cell currents of the (a) α1β3γ2L $GABA_AR$ induced by 100 μmol·L^{-1} GABA and (b) α3β4 nAChR induced by 3 mmol·L^{-1} carbamoylcholine. Rat α1β3γ2L $GABA_AR$ was stably expressed in the WSS-1 cells, while the rat α3β4 nAChR subtype was stably expressed in the KXα3β4R2 cell line. The maximal inhibition by LL on the $GABA_AR$ was 44% of the control and 13% on the nAChR. Carbamoylcholine, a stable analog of acetylcholine, was used as a control for the whole-cell current recordings of the nAChR.

induced by the agonist GABA, while the agonist affinity was not changed (Fig. 3): The EC_{50} values were (36.2 ± 7.9) μmol·L^{-1} and (36.1 ± 23.8) μmol·L^{-1} in the absence and presence of LL, respectively. Therefore, LL is a non-competitive inhibitor of the receptor.

Pentobarbital, an anticonvulsant drug used to treat seizures approved by the US Food and Drug Administration, potentiates $GABA_AR$ function at low concentrations (μmol·L^{-1}), but inhibits $GABA_AR$ function at high concentrations (mmol·L^{-1}) [26,27]. Therefore, we further tested the effect of LL on the $GABA_AR$ at concentrations of 25, 50, 75, 100, 200, 500, and 1000 μmol·L^{-1}. However, none of these low concentrations of LL exhibited any effect on the $GABA_AR$ (data not shown).

3.2. Mode of action of LL on $GABA_AR$

PTZ, also known as metrazol, causes convulsions and has been used in animal models to induce seizure. The PTZ-induced mouse seizure model (i.e., the scMET mouse model) has been used routinely to screen anticonvulsant drugs by programs such as the National Institutes of Health (NIH)'s Epilepsy Therapy Screening Program[†]. We wondered whether LL binds to the same site as PTZ. Our results showed that when LL was co-applied with PTZ, the inhibition on $GABA_AR$ was significantly increased in comparison with LL or PTZ alone (Fig. 4(a)). The PTZ binding site on $GABA_AR$ is characterized as overlapped with the picrotoxin binding site [28,29]. Furthermore, the antagonistic effect of LL on the $GABA_AR$ was reversible (Fig. 4(b)). Therefore, LL exerts its reversible allosteric $GABA_AR$ modulation at a different site from the PTZ/picrotoxin site.

3.3. ME, EG, and citronellal are weak $GABA_AR$ and nAChR inhibitors

ME is a dose-dependent, direct agonist of the $GABA_AR$ at concentrations greater than 100 μmol·L^{-1} (up to 10 mmol·L^{-1}), while sensitizing and potentiating GABA-induced currents at low concentrations of GABA (30 μmol·L^{-1}) [30], which offers a possible explanation for its anticonvulsant activity. We further expanded on this to assess the antagonistic activity of ME at high doses (mmol·L^{-1}), considering that LL also inhibited $GABA_AR$ at high doses. It was apparent that high doses (10 mmol·L^{-1}) of ME weakly antagonized the $GABA_AR$ (Fig. 5). EG also had a similar effect at high doses (Fig. 5). Interestingly, both (*R*)-C and (*S*)-C weakly inhibited the α3β4 nAChR (data not shown); however, (*R*)-C appeared to have no effect on the $GABA_AR$, whereas (*S*)-C had weak inhibitory

[†] https://www.ninds.nih.gov/Current-Research/Focus-Research/Focus-Epilepsy/ETSP.

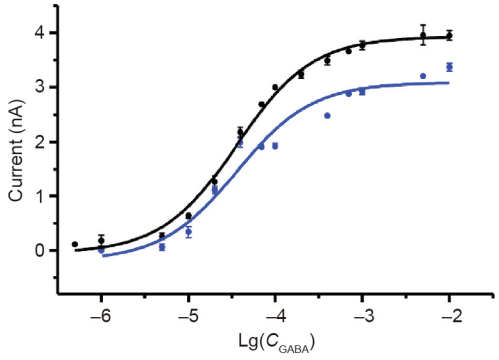

Fig. 3. GABA dose–response curves in the absence (black) and presence (blue) of 5 mmol·L^{-1} LL in WSS-1 cells that expressed the rat α1β3γ2L $GABA_A$ receptor. The EC_{50} values were (36.2 ± 7.9) μmol·L^{-1} and (36.1 ± 23.8) μmol·L^{-1} in the absence and presence of LL, respectively. C_{GABA}: the concentration of GABA.

activity on the $GABA_AR$ (Fig. 5). In a word, ME, EG, and (*S*)-C are comparatively weak antagonists of the $GABA_AR$, and require very high doses to produce any change in GABA-induced currents.

4. Discussion

Based on the data available so far, it is difficult to make a conclusion on the primary target of EG and (*R*)-C/(*S*)-C. (*R*)-C/(*S*)-C has been shown to exert anticonvulsant and antinociceptive effects *in vivo*, which have been inferred to occur via $GABA_AR$ modulation, albeit without a clear mechanism of action [31,32]. However, in the present *in vitro* study, EG and (*S*)-C had weak antagonistic effects on the $GABA_AR$ and nAChR, suggesting that the primary target of action lay elsewhere. ME has been shown to be both a $GABA_AR$ agonist at low concentrations and a positive allosteric modulator at moderate concentrations [30]; however, we have illustrated that it may be a weak $GABA_AR$ antagonist at high doses, which suggests that the effects of ME are very complex.

The results of the present study demonstrate that LL is a concentration-dependent $GABA_AR$ antagonist that modulates its activity outside of the PTZ/picrotoxin site on the $GABA_AR$. The inhibitory effect of LL on the $GABA_AR$ observed in the present study disagrees with previous findings in which LL had anticonvulsant activities in cells and mouse models [8,33], and depressant effects on the CNS, sensory neurons [34], and even peripheral nerves [35]. The inhibitory effect of LL on the $GABA_AR$ suggests neuronal

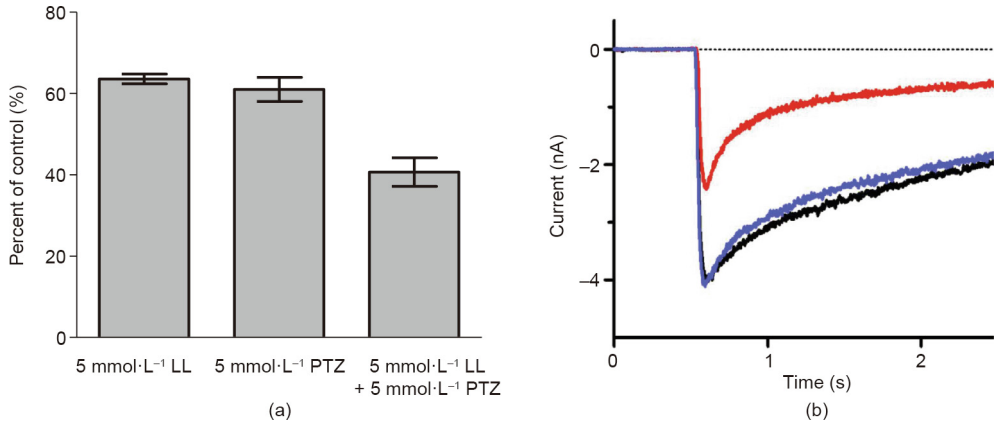

Fig. 4. (a) Significant increase in inhibition on rat α1β3γ2L $GABA_AR$ by the co-application of LL with PTZ relative to LL or PTZ alone. (b) Representative whole-cell current recording induced by 100 μmol·L^{-1} GABA (control, black), followed by 5 mmol·L^{-1} LL + 100 μmol·L^{-1} GABA (red) with subsequent return to the original control current with 100 μmol·L^{-1} GABA only (blue) 1 min after treatment.

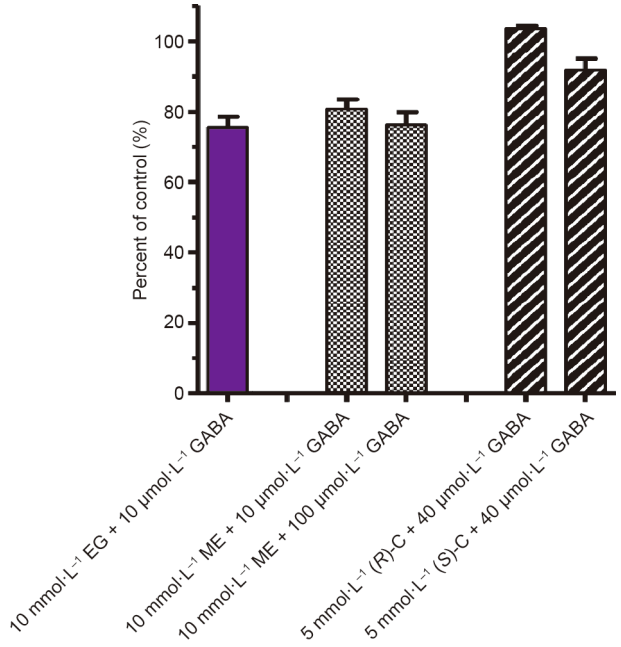

Fig. 5. Co-applications of 10 mmol·L^{-1} ME with 10 μmol·L^{-1} GABA and 100 μmol·L^{-1} GABA, respectively, weakly inhibited $GABA_AR$ functions. Co-applications of 10 mmol·L^{-1} EG with 10 μmol·L^{-1} GABA inhibited $GABA_AR$ functions. 5 mmol·L^{-1} (*R*)-C did not inhibit $GABA_AR$-induced currents when co-applied with 40 μmol·L^{-1} GABA; and 5 mmol·L^{-1} (*S*)-C in the presence of 40 μmol·L^{-1} GABA weakly inhibited the $GABA_AR$.

excitability (e.g., seizure-like activity), which may provide a possible explanation for its use as an insecticide [5,6].

While this finding may seemingly contradict prior studies on LL's anticonvulsant and sedative effects, LL has been described to have multiple targets, including the *N*-methyl-*D*-aspartate (NMDA) receptor [11,33,36] and voltage-gated Na^+ and Ca^{2+} channels [16,35]. LL has also been shown to inhibit acetylcholine release at the neuromuscular junction and the kinetics of the miniature end-plate current decay [37]. Our results reveal that LL is a potent inhibitor of neuronal nAChR. When the homology between the muscle type and neuronal nAChR and the sharing of many allosteric modulators are considered [38], this finding comes as no surprise. At this time, further research is warranted to describe the primary molecular mechanism of action of LL. We hypothesize that the nAChR, NMDA receptors, and/or voltage-gated Na^+ and Ca^{2+} channels may be the primary targets, as suggested by other studies [36], especially considering that NMDA receptors and voltage-gated Na^+ and Ca^{2+} channels are targets of many other anticonvulsant drugs [39].

More importantly, the co-localization and crosstalk among those receptors in the CNS [40,41] make the interaction with small molecules different from the simple recombinant system used here. Mounting evidence has exemplified the co-localization and crosstalk among excitatory and inhibitory neurotransmitter receptors. For example, both potentiation and suppression of the $GABA_AR$ function by NMDA receptor activation have been studied extensively, and vice versa [42–45]. The nAChR and $GABA_AR$ are also co-localized in many areas of the brain, and activation of nAChR has been reported to block GABA-induced currents on hippocampal interneurons [46]. However, their interactions are not well understood.

Essential oils are composed of multiple monoterpenoids (including LL, ME, EG, and citronellal). It is reasonable to hypothesize that LL and other monoterpenoids modulate multiple different receptors and channels with varying potency to achieve synergistic effects [5]. It is also possible that monoterpenoids modulate the interplay among different neurotransmitter receptors. The observed mode of action of the monoterpenoids supports the use of essential oil components as sedatives, anticonvulsants, local anesthetics, and insecticides. The results warrant further research and development of essential oil components as human therapeutics and botanical insecticides, particularly the selectivity of monoterpenoids to various insect neuro-receptors.

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Compliance with ethics guidelines

Amy S. Li, Akimasa Iijima, Junhao Huang, Qing X. Li, and Yongli Chen declare that they have no conflicts of interest or financial conflicts to disclose.

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