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Effects of Linalool on Glutamate Release and Uptake in Mouse Cortical Synaptosomes

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Linalool, a monoterpene compound prevalent in essential oil of plant species traditionally used as sedatives, has been characterized as anticonvulsant in several experimental models. Linalool inhibits the binding of [^{3}H]glutamate and [^{3}H]dizocilpine to brain cortical membranes, indicating a participation of the glutamatergic transmission its mechanism of action. In this study, we investigated the effects of linalool on [^{3}H]glutamate release (basal and potassium-stimulated) and [^{3}H]glutamate uptake in mice cortical synaptosomes. Linalool significantly reduced potassium-stimulated glutamate release as well as glutamate uptake, not interfering with basal glutamate release. The data indicates that linalool may interfere with several relevant elements of the glutamatergic transmission, including detriment of the K^+-stimulated glutamate release.

KEY WORDS: Linalool; anticonvulsants; glutamate; uptake; release; mice.

INTRODUCTION

Linalool, a monoterpene commonly found in essential oils, is present in several traditional medical remedies used for sedative and anticonvulsant purposes (1). It possesses a broad spectrum of action in mice experimental epilepsy models, including protection against pentylenetetrazol, picrotoxin, and electroshock-induced convulsions (1,2). We also reported that linalool interferes with glutamate function in vivo, delaying subcutaneous *N*-methyl-D-aspartate(NMDA)-induced convulsions and blocking intracerebroventricular quinolinic acid-induced convulsions (3). Relevant to its mechanism of action, linalool behaves as a competitive antagonist of [^{3}H]glutamate binding (increasing linalool concentrations increases K_d not changing B_{max}), and as a non-competitive antagonist of [^{3}H]dizocilpine (NMDA antagonist) binding (increasing linalool concentrations decreased B_{max} with no significant alteration in K_d) in brain cortical membranes, pointing to a modulation of glutamatergic transmission (3,4).

The excitatory glutamatergic system has long been postulated to be involved in the etiology of at least some forms of human and experimental epilepsy (5). In fact, the NMDA receptor complex is thought to play key roles on epileptic phenomena, including genesis (6), initiation and propagation of seizure activity (7,8). Considering that seizures in experimental models can be induced by activation and suppressed by inhibition of glutamate receptors, particularly NMDA (9,10), it has been proposed that a high extracellular glutamate level subsequent to excessive release and/or altered glutamate uptake is epileptogenic (11).

In order to further clarify the pharmacodynamic basis of linalool anticonvulsant properties, we investigated the effects of linalool on both release (basal and

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potassium-stimulated) and on high affinity uptake of [^{3}H]glutamate by synaptosomes obtained from mice cerebral cortex.

EXPERIMENTAL PROCEDURE

Drugs and Reagents. (±) Linalool (DL-3,7-dimethyls-3-hydroxy-1,6-octadiene, 95–97% purity) was purchased from Aldrich Chemical Co. (catalog number L260-2) and solubilized in Tween (80 polysorbate, 25%). Glutamate was purchased from Merck and L-[^{3}H]glutamate from Amersham. All other reagents were of analytical grade.

Animals. Male adult albino mice, strain CF-1, from Instituto de Pesquisas Biológicas (Porto Alegre, Brazil) were used throughout the study. The animals were kept on a 12 h light/dark cycle, at 22 ± 1°C, with free access to food (Nuvilab CR1) and water. All procedures were carried out according to institutional policies on experimental animals handling.

[^{3}H]Glutamate Release. Cortical synaptosomes were prepared on a discontinuous Percoll gradient according to Nagy and Delgado-Escueta (12). Protein concentration was measured according to the method of Lowry et al. (13). Glutamate release was evaluated as previously described by Migues et al. (14). Briefly, the synaptosomal preparation was incubated in HBSS [N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (Hepes) buffered salt solution, mM composition: HEPES 27, NaCl 133, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 12, $CaCl_2$ 1.0] for 15 min at 37°C in the presence of [^{3}H]glutamate (specific activity 53 PBq/mol, final concentration 5×10^{-7} M). Aliquots of labeled synaptosomes (1.4 mg protein) were centrifuged at 16,000 *g* for 1 min at 4°C. Supernatants were discarded and the pellets were washed four times in HBSS, and centrifuged at 16,000 *g* for 1 min at 4°C. The final pellets were resuspended in HBSS and incubated for 1 min at 37°C in the following conditions: absence of linalool (control), presence of tween (25%) or linalool (1.0 or 3.0 mM in tween 25%), for the assessment of the basal [^{3}H]glutamate release. For potassium-stimulated glutamate release, synaptosomes were incubated in a medium similar to that described above, except that 40 mM KCl was added to induce depolarization. Incubation was completed by centrifugation (16,000 *g*, 1 min at 4°C). The radioactivity present in the supernatants and in the pellets was separately determined in a WALLAC scintillation counter. The released [^{3}H]glutamate was calculated as a percentage of the total amount of radio label present at start of the incubation period.

[^{3}H]Glutamate Uptake. Cortical synaptosomes were prepared on a discontinuous Percoll gradient according to Nagy and Delgado-Escueta (12). Protein concentration was measured according to the method of Lowry et al. (13). The synaptosomal fraction was washed in 0.3 M sucrose containing 15 mM Tris(hydroxymethyl)aminomethane Tris/acetate buffer (pH 7.4), and centrifuged at 27,000 *g* for 20 min. The final pellet was resuspended in 0.3 M sucrose containing 15 mM Tris/acetate buffer (pH 7.4), and incubated for 1 min at 37°C in HBSS (Hepes buffered salt solution, mM composition: HEPES 24, NaCl 119, KCl 2.1, $MgSO_4$ 1.08, KH_2PO_4 1.08, glucose 10.8, $CaCl_2$ 0.9) loaded with [^{3}H]glutamate (final concentration 1 μM) in the following conditions: absence of linalool (control), presence of tween (25%) or linalool (0.1–3.0 mM in tween 25%). The reaction was stopped by filtration through GF/B filters, followed by 3 washes with 3 ml of ice-cold 155 mM ammonium acetate containing 15 mM Tris/acetate buffer (pH 7.4). Radioactivity retained on the filters was measured in a WALLAC scintillation counter. Specific high affinity glutamate uptake was calculated as the difference between the uptake performed in the incubation medium described above and the uptake performed in a similar incubation medium without Na^+.

Measurement of the Lactate Dehydrogenase (LDH) Activity. In order to evaluate the integrity of the synaptosomes after incubation with linalool (1.0 and 3.0 mM) for 1 min, the lactate dehydrogenase activity in the incubation medium was assessed spectrophotometrically using an assay kit (Doles Reagentes, Brazil).

Statistical Analysis. Statistical significance was assessed by ANOVA followed by Duncan's multiple range test. IC_{50} was calculated by linear regression.

RESULTS

Fig. 1 shows the effects of linalool on [^{3}H]glutamate release. Linalool (1.0 and 3.0 mM) had no effect on the basal [^{3}H]glutamate release, but significantly inhibited (28% and 31% respectively) the potassium-stimulated [^{3}H]glutamate release. Tween (linalool vehicle) was found devoid of effects both on basal and potassium-stimulated [^{3}H]glutamate release. Linalool (0.1–3.0 mM) significantly decreased the [^{3}H]glutamate uptake by mice cortical synaptosomes in a dose-dependent manner, as shown in Fig. 2 (IC_{50} = 1.50 ± 0.08 mM). Synaptosomes incubated with linalool (3.0 mM) for up to 1 min showed no significant leakage of the cytosolic marker lactate dehydrogenase (data not shown).

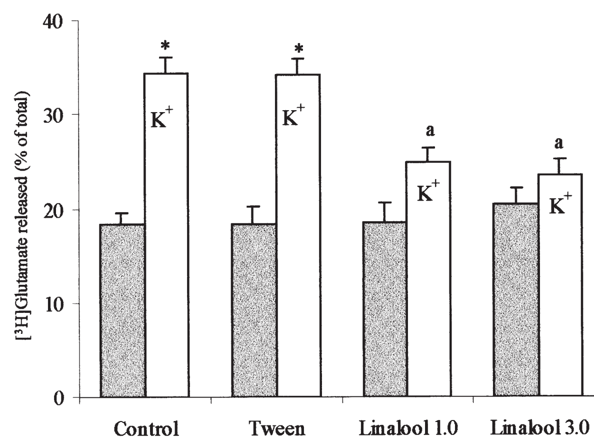

Fig. 1. Effects of linalool on the release of [^{3}H]glutamate from mice cortical synaptosomes. Glutamate released is expressed as a percentage of synaptosomal total radioactivity content. Data are expressed as mean ± sem from 4 independent experiments performed in triplicate. * = $p < 0.01$ compared with basal and a = $p < 0.01$ compared with potassium-stimulated in the absence of linalool (ANOVA/Duncan).

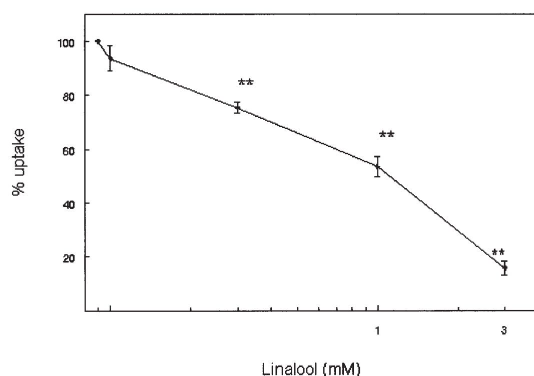

Fig. 2. Effects of linalool on [^{3}H]glutamate uptake. Synaptosomes were incubated in the absence or presence of linalool (0.1, 0.3, 1.0 and 3.0 mM). Data are expressed as mean ± sem from 4 independent experiments performed in triplicate. * = $p < 0.01$, ANOVA/Duncan.

DISCUSSION

The essential role of glutamate transmission in epileptic phenomena has been thoroughly documented (5,8), with several sites at which glutamate excitability could be modulated to attenuate seizures (10,15,16). Nevertheless, in practice only two approaches appear to be of consequence to suppress seizures: decreasing glutamate-induced excitability by acting at post-synaptic receptors, and decreasing extracellular glutamate availability (by inhibiting its release or incrementing its uptake) (17).

Linalool markedly inhibited (90%) glutamate uptake by mice cortical synaptosomes. Considering that in our assay conditions we have measured Na^+-dependent uptake at low glutamate concentrations (1 uM) (see experimental procedure) the transport system probably involved is the high affinity glutamate uptake system (18). Further experiments are required to determine if linalool affects the affinity or the maximal velocity of this uptake system. The actual impact of increases of extracellular glutamate levels on glutamate transmission has been debated. Massieu et al. (19) showed that inhibition of glutamate uptake is not sufficient to induce neuronal damage in vivo. Moreover, the in vivo anticonvulsant profile established for linalool in mice and the claims from traditional therapeutic use in humans (1,3), suggest that the linalool-induced inhibition of neuronal uptake observed in this study does not translate into pro-convulsant activity in vivo. Accordingly, it is accepted that the glutamate transporters primarily responsible for removing glutamate from the synaptic cleft are glial instead of neuronal (20,21). Nevertheless, it is of relevance to evaluate the effects of linalool on glial uptake, in order to conclusively rule out a pro-convulsant effect.

Linalool significantly reduced K^+-stimulated glutamate release. We have recently demonstrated that in our incubation conditions, K^+-induced glutamate release is partially prevented by removing calcium from the medium (22), indicating a Ca^{+2}-dependent component; this component is likely to be related to the vesicular release of glutamate (23). A calcium-independent component of the K^+-stimulated glutamate release has been attributed to the reversal of glutamate uptake system by the high extracellular K^+ concentration (24). Considering that linalool completely prevented K^+-stimulated glutamate release (with no effect on basal release), we propose that both components of the release process were affected by linalool. However, additional experiments are required to determine the precise involvement of calcium on linalool inhibition of glutamate release, to detail the nature of the transport system(s) affected, as well as whether linalool modifies the affinity and/or maximal velocity of glutamate uptake.

Interfering with multiple mechanisms that underlie seizures may be necessary to effectively counteract epileptic phenomena (10,25), and developing drugs that differently affect normal and hyperexcitable neurons has been postulated to spare normal excitatory function (10). Therefore, the fact that anticonvulsant effects of linalool can be attributed to both an inhibition of potassium-stimulated (but not basal) glutamate release, and antagonism of NMDA receptors (4), deserves further investigation as strategy for antiepileptic drug development.

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