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LINALOOL MODIFIES THE NICOTINIC RECEPTOR–ION CHANNEL KINETICS AT THE MOUSE NEUROMUSCULAR JUNCTION

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Linalool is a monoterpene compound reported to be a major component of essential oils in various aromatic species. Several linalool-producing species are used in traditional medical systems. Among these is *Aeolanthus suaveolens G. Dom (Labiatae)* which is used as an anticonvulsant in the Brazilian Amazon. Psychopharmacological *in vivo* evaluation of linalool showed that this compound has dose-dependent marked sedative effects at the central nervous system (CNS), including hypnotic, anticonvulsant and hypothermic properties. It has been suggested that these neurochemical effects might be ascribed to the local anaesthetic activity of linalool. The present study reports an inhibitory effect of linalool on the acetylcholine (ACh) release and on the channel open time in the mouse neuromuscular junction. These findings could provide a rational basis to confirm the traditional medical use of linalool-producing plant species. Indeed, our data demonstrate some interactions in the modulation of the ACh release at the mouse neuromuscular junction, which are well correlated with the suggested molecular mechanisms. Linalool induced a reduction of the ACh-evoked release. The possibility that this effect could be ascribed to some interaction with pre-synaptic function is noteworthy. Moreover, the inhibitory effect induced on the kinetics of the miniature end-plate current decay demonstrates a local anaesthetic action, either on the voltage or on the receptor-activated channels.

KEY WORDS: loose patch clamp, neuromuscular junction, natural extracts, calcium.

INTRODUCTION

Following our basic research in evaluating the therapeutic potential of some ethnobotanical species we submit linalool to a electrophysiological evaluation. Indeed, even though many papers have dealt with linalool biological actions, its molecular mechanism still remains unknown.

Jirovetz *et al.* (1991) demonstrated that the inhalation of some monoterpenses in concentrations of 5 mg l^{-1} air leads to a significant reduction of motility in mice [1]. The molecular hypotheses of such action could be a block of the voltage-dependent sodium channel or an alteration of the lipids surrounding the channel. The possible anaesthetic effect of these compounds is strengthened by other data in which a significant decrease in the motility of laboratory animals under standardized experimental conditions was found to be closely dependent on the exposure time to the drugs [2]. Nevertheless, the same authors reported that the hyperactivity produced in mice after an injection of caffeine was counteracted to nearly normal motility only by inhalation of these fragrance drugs.

Moreover, some effects of linalool in the rat cerebral cortex [3] indicate a possible role in excitable tissues which could be assessed well with this electrophysiological technique.

In the same year, more papers reported data in relation to a possible antimicrobial activity of some components derived from essential oils, which reinforced the scientific interest in the better definition of the linalool action. Carson and Riley [4] demonstrated the linalool activity against *Candida albicans*, *Escherichia coli* and *Staphylococcus aureus* with the exception of *Pseudomonas aeruginosa*. Other authors proved a therapeutic efficacy of linalool in some parasitic diseases [5]. In these cases, linalool was the most effective treatment in terms of antibacterial activity against Gram-positive bacteria and fungi [6] when compared to five aromatic constituents of essential oils.

Among the effects reported for some terpenes and terpenoids, the most noteworthy is the possibility that linalool could enhance the transdermal penetration

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of different drugs. A study carried out with propranolol, known for its extensive first-pass metabolism and short elimination half-life, showed an increase of permeability to this drug [7]. A similar study showed higher permeation-enhancing ratio with respect to the anti-inflammatory drug ketoprofen [8].

This electrophysiological approach could be useful for the interpretation of the above reported biological actions of linalool.

MATERIALS AND METHODS

Preparation

Left hemidiaphragm of mouse was prepared as described previously [9,10]. Briefly, Charles River male mice, 30–40 days old, were killed and the left hemidiaphragm was dissected with a short length of phrenic nerve. The preparation was bathed in Kreb's solution of the following composition (mM): NaCl (133), KCl (4.7), $MgCl_2$ (1.2), $CaCl_2$ (7.2), NaH_2PO_4 (1.3), $NaHCO_3$ (16.3), glucose (7.8), pH 7.4, gassed with 95% O_2, 5% CO_2 and maintained at room temperature (18–22 °C). Concentrations of $MgCl_2$ (5–15 mM) and $CaCl_2$ (0.9–2 mM) were adjusted in order to abolish the twitch of the muscle fiber.

The muscle was pinned on Sylgard resin, and placed on the stage of a Leitz inverted microscope. End-plates were visible by transillumination of the preparation with an optic fiber system. The preparation was equilibrated in saline for 30 min before starting the experiments.

End-plate signals

Spontaneous and evoked end-plate currents were recorded with a focal extracellular pipette pressed against the edge of an end-plate. The pipettes from Drummond comprised 100 μl measuring pipettes of soft glass (1.4 mm) that were pulled with a Kopf 700C Puller, fire polished with a Narishighe MF83 Microforge, and had a final tip diameter ranging from 3 to 15 μm. Following filling with physiological solution, the electrode was connected to a List LM EPC7 current-to-voltage converter. Pipette resistances were of 100–300 KΩ and seal resistances, measured after pressing the pipette against the sarcolemma, ranged between 300–600 KΩ. The Loose Patch Clamp method [11] enables a good control of the series resistance throughout the experiment [12]. Furthermore, the voltage of the muscle fiber is well monitored with the same technique and virtually clamped at the resting value. Indirect stimulation of the muscle was achieved by means of a suction electrode. Supramaximal square wave pulses of 0.1 ms duration at 2 Hz were applied via a DA analogic output of a PCL 818 card (Advantech). The signals were visualized on a Tektronix 5113 dual-beam storage oscilloscope and fed to the input stage of the PCL 818 analogue-to-digital converter mounted on a computer system (PC IBM) enabling a fully automated analysis of the data [13]. The decay phase of the mepc was analysed to calculate the decay time constant. The elaboration was carried out on the mepc's decay part that fell within 10–90% of its peak amplitude. The function used was:

$$I_t = I_0 \exp^{-t/\tau}$$

where I_t is the current at time t, I_0 is the current at time zero (i.e. the peak current) and τ is the time constant of decay. Marquardt's least squares method was used for the fitting.

The parameters were obtained with the protocol described previously [10]. The technique led to the automatic evaluation of the mean τ value of the mepc's decay, the mean peak value of the miniature events (mepc), the spontaneous release frequency (f) and the mean epc's peak value (epc). The resting membrane potential (RMP) was measured during the experiment by conventional intracellular microelectrodes (3M KCl) led to a P16 differential amplifier (GRASS).

Statistical analysis

Given data are expressed as the means $\pm$ standard deviations of the means. The statistical significance was assessed by Student's t-test. P values <0.05 were considered as significant differences.

Extracts

The crude extract was provided by Professor Silverstrini B, Pharmacology DPT., University La Sapienza, Rome.

RESULTS

Table I reports the effects on the analysed parameters obtained with linalool.

Linalool did not modify the amplitude of the mepc, showing a lack of any antagonistic effect on the postsynaptic receptor. However, it significantly modified the decay time of the spontaneous signals. In Fig. 1 some spontaneous events are shown. The data demonstrated well the striking inhibitory action on the receptor lifetime after its activation by the mediator ACh.

The signals in Figs 2 and 3 show the effect obtained on the evoked ACh release with increasing linalool concentrations.

The epc's amplitudes were decreased by linalool, confirming a possible presynaptic effect.

The membrane potential (RMP) of the postsynaptic muscle fibers was not modified by linalool (control values, 78.2 $\pm$ 3.8 mV from nine fibers; Linalool 80 μg ml^{-1}, 76.5 $\pm$ 6.2 mV from eight fibers).

The pH of the solutions was not changed when measured in the presence of higher assayed extract concentration.

Table 1
Effects of linalool at the mouse neuromuscular junction. Values show the effects of the indicated linalool concentrations on four parameters related to the function of the mouse neuromuscular junction compared to the control and to the final wash out. The amplitudes of the evoked (epc) and spontaneous (mepc) release, the frequency of the quantal release (f) and the mepc's decay time constant (τ) have been analysed. Data are expressed as the mean percentage variations with respect to the mean control values. Standard deviations and number of experiments are indicated in brackets

	Control values	*linalool 2 μg ml^{-1}*	*linalool 20 μg ml^{-1}*	*linalool 80 μg ml^{-1}*	*Wash out*
epc (nA)	2.66(±1.18; 6)	−12.2(±8.26; 4)*	−39.7(±8.90; 3)	−81.2(±11.3; 5)	−60.7(±12.2; 5)
mepc (nA)	0.16(±0.08; 6)	−8.6(±0.60; 3)*	+6.2(±2.89; 3)*	− 4.2(±21.7; 4)*	9.5(±13.18; 4)*
f(Hz)	5.44(±5.54; 6)*	+56.4(±49.1; 3)*	+61.6(±38.3; 3)*	+116.5(±107; 5)*	+66.7(±24.2; 5)*
τ(ms)	1.17(±0.3; 6)	−14.1(±3.7; 4)	−24.1(±9.1; 3)	−50.4(±17.7; 6)	−28.2(±13.2; 5)*

*No significant differences. Bold values indicate significant mean with $P < 0.05$.

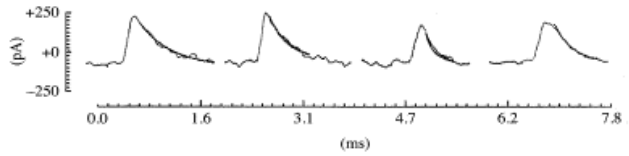

Fig. 1. Spontaneous miniature end-plate currents recorded at the mouse neuromuscular junction. The figure shows digitized raw data related to the control (Left), to the treatment with linalool 20 μg ml^{-1} (left center), linalool 82 μg ml^{-1} (right center) and to the final wash out (right), respectively.

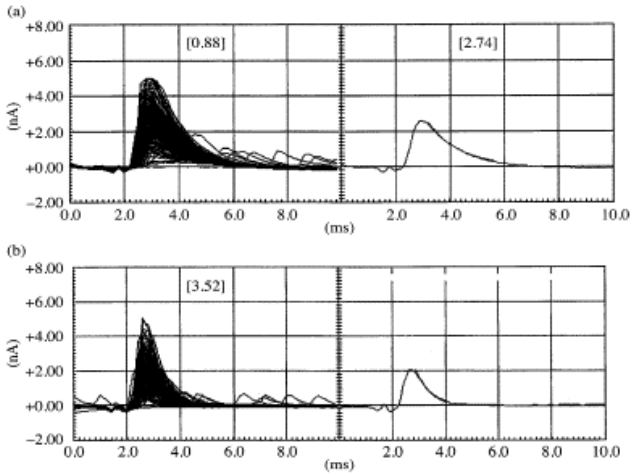

Fig. 2. Averaged signals obtained by 200 evoked end-plate currents recorded at the mouse neuromuscular junction. The figure shows digitized raw data related to the control (a), and to the treatment with linalool 20 μg ml^{-1} (b), respectively. In the left panel the superimposition of the total signal is shown. The resulting averaged mean is shown in the right panel.

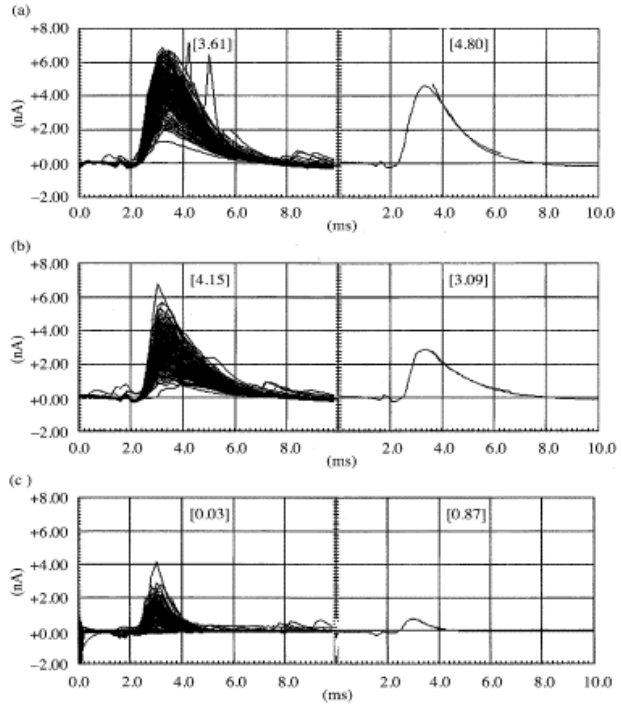

Fig. 3. Averaged signals obtained by 200 evoked end-plate currents recorded at the mouse neuromuscular junction. The figure shows digitized raw data related to the control (top) and to the treatment with linalool 20 μg ml^{-1} (middle) and 80 μg ml^{-1} (bottom), respectively. The dose dependence of the linalool effect should be noted. In the left panel the superimposition of the total signals is shown. The resulting averaged mean is shown in the right panel.

DISCUSSION

The results of the present study demonstrate that in this preparation linalool reduces the efficacy of the nerve impulse in ACh release. It should be noted that the constancy of the mepc amplitude is indicative of the prevalent presynaptic action.

Moreover, the effect observed on the mean open channel time is indicative of some interactions with the nicotinic ACh receptor, i.e. with the molecular complex responsible for the gating action. The decay phase of the synaptic current normally arises from the rate constant of the conformational change, reflection the closing of the ACh-sensitive channels [14,15], i.e. a postsynaptic event.

The effect induced by linalool on the mepc decay time is correlated with some action on the receptor–ion channel complex which could be partially blocked by the linalool molecule. Similar data have been obtained with local anaesthetic drugs, which could induce flickering of the nicotinic ion channel receptor [16].

As stated by other authors [17], the underlying mechanism of a presynaptic effect on the ACh release must be related to a possible reduction of the influx of calcium in the presynaptic terminal or to inhibition of the voltage-gated sodium and potassium channels.

The gating of the presynaptic voltage-dependent channels could be a target of the linalool action. Indeed, the main sites of action of local anesthetic drugs on nervous tissue are sodium [18] and potassium [19] voltage-gated channels.

Finally, it is worth noting the observed increase of mepcs frequency which apparently is in the opposite direction of that producing decrease of the evoked epcs. Our opinion is that any action at presynaptic level on

the function controlling the synchronous release of ACh vesicles, i.e. the action potential, could lead to a decrease of the evoked ACh release. Conversely, any effect on the membrane permeation in terms of lipid-surrounding rearrangement [7,8] could be the cause of the increased spontaneous release. Experiments are in progress to better define the quantal analysis of synaptic transmission and the single-channel properties in the presence of linalool

In conclusion, such as for other natural derivative substances, we proved a specific effect of linalool which could now be considered as potential tool in the pharmacological field.

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