Chemistry and Physics of Lipids 198 (2016) 39–45

Contents lists available at ScienceDirect

Chemistry and Physics of Lipids

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

Effects of bioactive monoterpenic ketones on membrane organization. A langmuir film study

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ARTICLE INFO

Article history:
Received 12 February 2016
Received in revised form 13 April 2016
Accepted 3 May 2016
Available online 9 May 2016

Keywords:
Thujone
Dihydrocarvone
Compression isotherm
Membrane elasticity
Brewster angle microscopy
$GABA_A$ receptor modulation

ABSTRACT

The cyclic ketones, thujone and dihydrocarvone, are lipophilic components of essential oils extracted from different plants, which have proven insecticidal activity. The $GABA_A$ receptor is activated by the neurotransmitter GABA and is the action site of widely used neurotoxic pesticides. Many compounds that regulate $GABA_A$ receptor function interact with membrane lipids, causing changes in their physical properties and consequently, in the membrane dynamic characteristics that modulate receptor macromolecules. In the present study, the biophysical effects of thujone (a gabaergic reference compound) and dihydrocarvone (structurally very similar) were explored by using monomolecular films of DPPC as a model membrane system, to gain insight into membrane-drug interaction. The compression isotherms showed that both ketones expand the DPPC isotherms and increase membrane elasticity. They penetrate the monolayer but their permanence depends on the possibility of establishing molecular interactions with the film component, favored by defects present in the membrane at the phase transition. Finally, by using Brewster angle microscopy (BAM) as a complementary technique for direct visualization of the study films, we found that incorporating ketone seems to reduce molecular repulsion among phospholipid headgroups. Our results reinforce the notion that changes in membrane mechanics may be occurring in the presence of the assayed ketones, suggesting that their interaction with the receptor's surrounding membrane may modulate or affect its functionality, possibly as part of the mechanism of the bioactivity described for thujone and DHC.

1. Introduction

Different essential oils and their components extracted from plants are known to possess insecticidal activities, among their other effects. The cyclic ketone, thujone, is an active ingredient of wormwood oil and some other herbal medicines and is reported to have antinociceptive, insecticidal, and anthelmintic activity (Höld et al., 2000). Dihydrocarvone (DHC) is present in oils extracted from the caraway plant and is used for its fragrance as flavoring and for medicinal purposes (Tripathi et al., 2003). Both naturally occurring ketones are highly lipophilic compounds, whose insecticidal activity was demonstrated in previous reports (Tripathi et al., 2003; Grainge, 1988).

$GABA_A$ is the major inhibitory receptor of the brain, and belongs to a superfamily of pentameric ligand-gated ion channels. It is operated by binding of GABA and is also recognized as a molecular target for many drugs (e.g. barbiturates, benzodiazepines, neuroactive steroids, anesthetics) and alcohol (Suzdak and Paul, 1987). In recent years, it has been shown that $GABA_A$ receptors are also targets for several insecticides and other toxicants (Eldefrawi and Eldefrawi, 1987), which act by recognition of the picrotoxinin or noncompetitive antagonist site to block $GABA_A$ (Chen et al., 2011). It is known that thujone is specifically a receptor antagonist and, by inhibiting GABA receptor activation, may make neurons fire more easily, causing muscle spasms and convulsions (Höld et al., 2000; Reiner et al., 2013a).

Although there is an increasing body of information, more study is required of the activities and molecular mechanisms responsible for the activity of some lipophilic compounds, which may be potential insecticides. Perturbation of physical membrane characteristics could be one of their modes of actions, as was suggested

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http://dx.doi.org/10.1016/j.chemphyslip.2016.05.002
0009-3084/

for some antimicrobial agents (Lopes et al., 2009; Sirk et al., 2009) and anesthetics (Cantor, 1998).

In this work, thujone and DHC were selected due to their similarities at molecular structure level (Fig. 1). While thujone presents a bicyclic ring structure, DHC exhibits an aromatic hydrocarbon ring with a comparable substituent to thujone. The inclusion of thujone, as a reference compound acting on the $GABA_A$ receptor (Sanchez-Borzone et al., 2014), and DHC, which recently was found to act as a negative allosteric modulator of this receptor (unpublished results), allows us to gain insight into their effects on membranes, as part of the mechanism of action involved in receptor modulation. Such studies could be significant, taking into account that subtle structural differences may underlie the results observed in lipid interactions and changes of membrane properties.

The interaction between surface active compounds and phospholipids has been extensively studied in artificial model membrane systems, including liposomes and Langmuir monolayers (Dynarowicz-Latka et al., 2001; Peetla et al., 2009). Many crucial phenomena that take place in bilayers, as correspond to biological membranes, can be elucidated by using monolayers at the air-water interface (Feng, 1999), since plasma membranes are the first contact of lipophilic compounds with cells, and the drug-membrane interaction, being a potential regulatory point, is a fundamental condition for their function. Phospholipid monolayers constitute simple models to study intermolecular interactions (Demel, 1974; Bohm et al., 1993), given that the lipid interface can be easily modulated by changing the interfacial composition or lateral packing (Imbenotte and Verger, 1973; De Tullio et al., 2013; Daniele et al., 1996; Scott et al., 1990; Miyamoto and Kollman, 1992). It has been successfully employed to study the characteristics of membrane structure and the interaction between lipids and amphiphilic molecules, peptides, proteins (Brockman, 1999) and more recently with polysaccharides, such as chitosan (Krajewska, 2004). Furthermore, this technique makes it possible to study the effect of compounds on lipid and surrounding molecules, taking into consideration several physical changes (for example, changes in order parameters of monomolecular films, molecular areas, surface potential, surface tension, etc.) (Brockman, 1999; Nowotarska et al., 2014; Pathirana et al., 1998). Moreover, the partition of the receptor-containing membrane itself can cause changes in the physical environment of the receptor and it is known that the membrane fluidity in living organisms is highly regulated ((Søgaard et al., 2006; Hansen et al., 2013) and ref. therein).

The aim of the present work was to emphasize the differences in the membrane interactions of DHC and thujone using DPPC monolayers, which are widely used to perform membrane interaction studies of lipophilic compounds (Pathirana et al., 1998; Hansen et al., 2013; Amador Kane and Floyd, 2000; Reiner et al., 2013b). This approach allowed us to focus on the interaction between ketones and phospholipids, by recording π-area isotherms and calculating different physical parameters. Moreover, Brewster angle microscopy (BAM) of Langmuir monolayers was applied as a complementary technique for visualizing the effect of ketones on the bidimensional phase state of the films, since DPPC domains exhibit remarkable shapes.

2. Materials and methods

2.1. Materials

1,2-dipalmitoyl-sn-*glycero*-3-phosphocholine (DPPC) was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL, USA). Thujone (1-isopropyl-4-methylbicyclo [3.1.0] hexan-3-one) and (+)-dihydrocarvone (2-methyl-5-prop-1-en-2-ylcyclohexan-1-one) (DHC) were purchased from Sigma Chem Co. (St. Louis, MO, USA). All other reagents were of the highest analytical grade. All ketones were prepared as 5 M stock solutions in pure DMSO, light-protected, and stored at 4 °C. Stock solutions were diluted before each experiment in DMSO and finally in phosphate buffer pH 7.4 (135 mM NaCl, 7.5 mM Na_2HPO_4 and 1.5 mM K_2HPO_4) maintaining a 0.25% (v/v) DMSO final concentration. Solutions were prepared with double-deionized water.

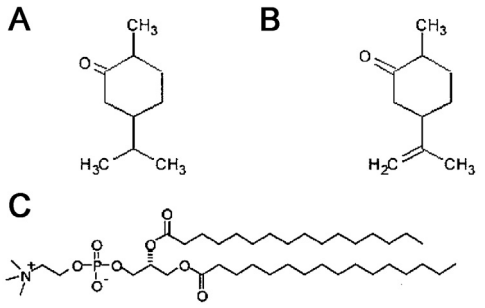

Fig. 1. Molecular structures of thujone (A), dihydrocarvone (B) and DPPC (C).

2.2. Lateral surface pressure (π) – mean molecular area (MMA) isotherms

π (mN/m) vs. MMA (Å^2/molecule) isotherms were performed by the compression of monolayers containing DPPC using a Minitrough II (KSV, Finland). Lipid monolayers on the air-water interface were prepared by spreading pure DPPC dissolved in chloroform/methanol (2:1) on the aqueous surface of a Teflon™ trough filled with phosphate buffer pH 7.4 as subphase. After 5 min solvent evaporation, the film was compressed isometrically at a constant rate of 4 ± 1 Å^2/min/molecule until reaching the target pressure. π was measured with a platinum plate by the Wilhelmy method (Verger and De Haas, 1973), at different MMA of the phospholipid, in the absence or presence of each ketone (20, 250 and 500 μM final concentration), mixed previously with the subphase. Control isotherms obtained in the presence of DMSO 0.25% (v/v) were not different from those at 0% DMSO (data not shown). The collapse point in monolayers (π_c) was characterized either by reaching a rapid decrease in the surface pressure or as a horizontal break in the isotherm. All assays were performed in duplicate at 25 ± 1 °C. Graphics were made by using Sigmaplot 12.5 (Systat Software Inc., USA).

2.3. Compressibility analysis of DPPC films

In order to analyze the elastic behavior of the film, the *compressibility modulus* (Cs^{-1}) was determined. The onset of phase transition points was identified from a minimum and π_c from a maximum in the variation of Cs^{-1} vs. MMA plot. Cs^{-1} values represent the reciprocal of the compressibility and were calculated directly from the slope of π-MMA isotherms applying Eq. (1):

$$Cs^{-1} = -(A_\pi)\,(d\pi/dA)_\pi$$

where A_π is the MMA at the indicated surface pressure (Kodama et al., 2004). All calculations were made by using the program PeakFit v4.12 (Systat Software Inc., USA). The maximal error of this parameter did not exceed 1%.

2.4. Penetration of ketones into the monolayer

To study the penetration of the ketones into DPPC lipid monolayers, the experiments were performed in a homemade