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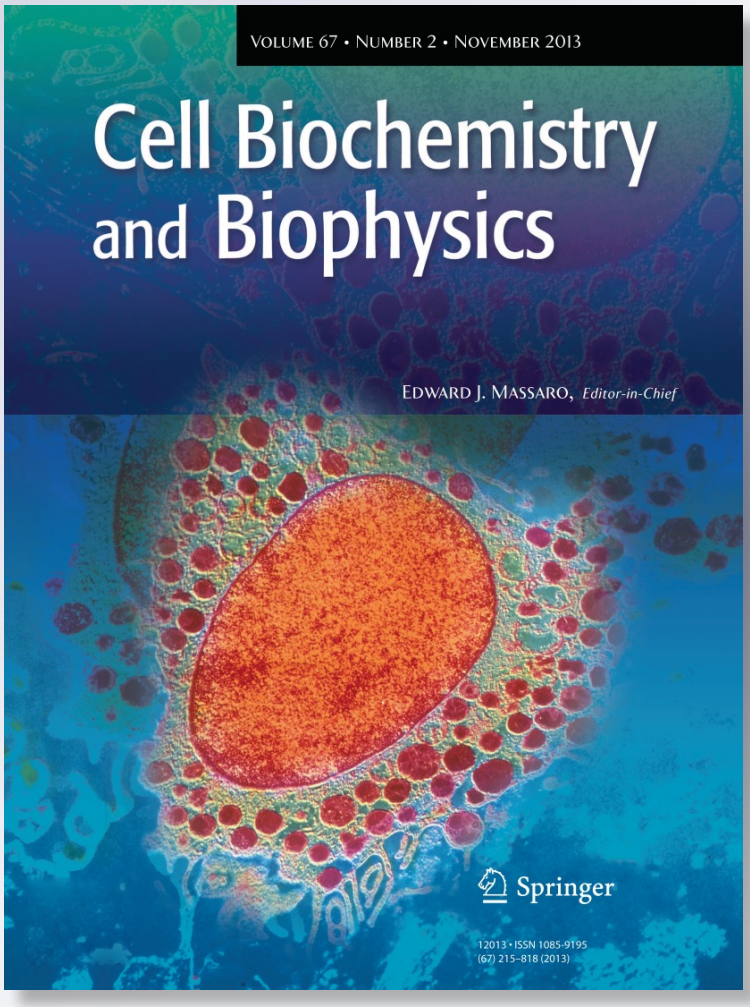

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ORIGINAL PAPER

Gabaergic Pharmacological Activity of Propofol Related Compounds as Possible Enhancers of General Anesthetics and Interaction with Membranes

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Abstract Phenol compounds, such as propofol and thymol, have been shown to act on the $GABA_A$ receptor through interaction with specific sites of this receptor. In addition, considering the high lipophilicity of phenols, it is possible that their pharmacological activity may also be the result of the interaction of phenol molecules with the surrounding lipid molecules, modulating the supramolecular organization of the receptor environment. Thus, in the present study, we study the pharmacological activity of some propofol- and thymol-related phenols on the native $GABA_A$ receptor using primary cultures of cortical neurons and investigate the effects of these compounds on the micro viscosity of artificial membranes by means of fluorescence anisotropy. The phenol compounds analyzed in this article are carvacrol, chlorothymol, and eugenol. All compounds were able to enhance the binding of [^{3}H]flunitrazepam with EC_{50} values in the micromolar range and to increase the GABA-evoked Cl^- influx in a concentration-dependent manner, both effects being inhibited by the competitive $GABA_A$ antagonist bicuculline. These results strongly suggest that the phenols studied are positive allosteric modulators of this receptor. Chlorothymol showed a bell-type effect, reducing its positive effect at concentrations >100 μM. The concentrations necessary to induce positive allosteric modulation of $GABA_A$ receptor were not cytotoxic. Although all compounds were able to decrease the micro viscosity of artificial membranes, chlorothymol displayed a larger effect which could explain its effects on [^{3}H]flunitrazepam binding and on cell viability at high concentrations. Finally, it is suggested that these compounds may exert depressant activity on the central nervous system and potentiate the effects of general anesthetics.

Keywords $GABA_A$ receptor · Chloride uptake · Carvacrol · Eugenol · Chlorothymol · Membrane fluidity

Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the vertebrate central nervous system. Altered GABAergic neurotransmission has been involved in many neurologic and psychiatric disorders such as epilepsy, brain ischemia, schizophrenia, etc. $GABA_A$ receptors ($GABA_A$-Rs) are ligand-gated Cl^- ion channels activated by the neurotransmitter GABA and by their agonists such as muscimol or isoguvacine, and modulated by numerous therapeutically important drugs, including barbiturates, anesthetics, and benzodiazepines. These compounds are allosteric modulators as they bind to sites distinct from the GABA binding sites to potentiate GABA-evoked currents. A wide spectrum of drugs, toxic agents, and metals modify the $GABA_A$-R function by directly interacting either with these binding sites or with other as yet not well-described sites present in the receptor complex [1–4].

G. N. Reiner and L. Delgado-Marín contributed equally to this study.

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General anesthetics are molecules that induce a reversible state of unconsciousness, characterized by amnesia and analgesia. Although general anesthetics have been used since the nineteenth century, their precise mechanism of action remains incompletely elucidated. They were originally believed to act via nonspecific interactions with the lipid bilayers, affecting membrane fluidity. More recently, general anesthetics have been shown to act by modulating ligand-gated ion channels such as the $GABA_A$-R (see references in: [4]). Phenol compounds, such as propofol (widely used as a general anesthetic) and thymol, have been shown to act on this receptor as allosteric modulators at low concentrations or to have a direct effect on the channel opening at higher concentrations. These activities have been described as mediated by their interaction with specific sites at the $GABA_A$-R [5, 6].

However, although specific pharmacological regulation of $GABA_A$-R function can be analyzed using well-described theories of ligand–receptor interactions, it should be considered that many compounds that regulate $GABA_A$-R function are noticeably lipophilic (e.g., benzodiazepines, barbiturates, long-chain alcohols, and anesthetics) [7–9], which may change the physical properties of the lipid bilayer.

Thus, it is possible that the anesthetic activity of lipophilic phenols, as previously mentioned, could be the combined result of the interaction of phenol molecules with specific receptor proteins ($GABA_A$-R) and with the surrounding lipid molecules modulating the supramolecular organization of the receptor environment [9, 10]. Taking into account not only their lipophilicity but also their conspicuous ability to interact with membranes, which has been demonstrated for phenol compounds [3, 11–15], their contact with the membrane component surrounding the receptor and a consequent non-specific effect on receptor modulation cannot be excluded.

In the present study, we further study the pharmacological activity of some propofol- and thymol-related phenols on native $GABA_A$-R by determining their effects on benzodiazepine recognition sites using primary cultures of cortical neurons which express functional $GABA_A$-R [6, 16]. Also, we investigate the effects of phenol compounds on the micro viscosity of artificial membranes (liposomes) in order to deepen the understanding of the interaction and location of these compounds in the membrane by means of fluorescence anisotropy. The phenol compounds analyzed are carvacrol, chlorothymol, and eugenol (Fig. 1), which, together with propofol and thymol, was previously studied by our group as regards their lipophilicity, membrane partition ability, and antioxidant properties [15, 17]. The correlation between their pharmacological activities and these physical properties is also discussed.

Materials and Methods

Materials

Fetal calf serum was obtained from Gibco (Glasgow, UK) and Dulbecco's modified minimum essential medium (DMEM) from Biochrom (Berlin, Germany). [^{3}H]Flunitrazepam (88 Ci/mmol) and $Na^{36}Cl$ (0.2 mCi/mmol) were procured from Amersham Life Sciences (Buckinghamshire, UK) and American Radiolabeled Chemicals Inc. (St. Louis, MO, USA), respectively. Liquid scintillation cocktail Optiphase Hisafe 2 was obtained from Wallace Oy (Turku, Finland). Carvacrol (2-methyl-5-isopropyl-phenol), eugenol (2-methoxy-4-prop-2-enyl-phenol), chlorothymol (5-methyl-4-chloro-2-isopropyl-phenol), GABA, (−)-bicuculline methiodide, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazam (MTT), trypsin, soybean trypsin inhibitor, DNase, amino acids, poly-L-lysine, diphenylhexatriene (DPH), and trimethylammonium–DPH (TMA–DPH) were obtained from Sigma Chemical Co. (St Louis, MO, USA). 1,2-Dipalmitoyl-phosphatidylcholine (dpPC) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). All the other chemicals were of analytical grade. All phenols were prepared as 400 mM stock solutions in DMSO, light protected, and stored at 4 °C. Stock solutions were diluted before each experiment in buffered solution, maintaining a 0.25 % (v/v) DMSO final concentration.

Cell Cultures

Primary cultures of cortical neurons were prepared from the cerebral cortices of 16–17 day-old mouse fetuses, as previously described [18, 19]. Pregnant animals were killed by cervical dislocation and fetuses extracted. Neocortices were dissected with forceps, mechanically minced, with cells then dissociated by mild trypsinization (0.02 % w/v) at 37 °C for 10 min followed by trituration in a DNase solution (0.004 %-w/v) containing a soybean trypsin inhibitor (0.05 %-w/v). The cells were re-suspended in a DMEM (5 mM KCl, 31 mM glucose, and 0.2 mM glutamine) supplemented with insulin, penicillin, and 10 % fetal calf serum. The cell suspension (1.6×10^6 cells/ml) was seeded in 24×-multiwell plates pre-coated with poly-L-lysine, and incubated for 6–7 days in a humidified 5 % CO_2/95 % air atmosphere at 36.8 °C. A mixture of 5 μM 5-fluoro-2-deoxyuridine and 20 μM uridine was added after 48 h in culture to prevent glial proliferation.

[^{3}H]Flunitrazepam Binding

The benzodiazepine binding to intact cultured cortical neurons was determined as previously described [6] using

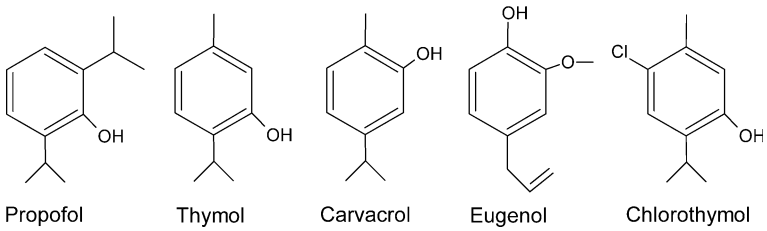

Fig. 1 Chemical structures of the phenolic compounds analyzed. Propofol and thymol are included for comparison

1.3–2.0 nM [^{3}H]flunitrazepam. Prior to incubation with the radioligand, the plates were washed three times with 1 mL/well of HEPES buffer (136 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.4 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 10 mM HEPES, and 9 mM glucose adjusted to pH 7.3), and the binding assay took place in the culture well in the presence of the HEPES buffer, [^{3}H]flunitrazepam, and drug solutions. After 30 min of incubation at 25 °C without shaking, a cold buffer was added and rapidly removed by suction. The cells were rinsed three times with cold buffer, then they were disaggregated in 0.2 M NaOH overnight, and their radioactivity was determined by liquid scintillation counting (with cocktail Optiphase Hisafe2). Basal binding was determined in HEPES solution containing 0.25 % DMSO in the absence of drugs. Nonspecific binding was determined in the presence of 20 μM diazepam. All experiments were run simultaneously with a parallel experiment that determined the increase of [^{3}H]flunitrazepam binding induced by 100 μM GABA, used as a positive assay control. The GABA stimulation was 198 ± 20 % with respect to basal, in agreement with our previous report using this type of culture [6]. Bicuculline (100 μM) was used as a GABA antagonist. Data are expressed as the percentage of basal specific binding.

Chloride Influx

$GABA_A$-R potentiation was determined by measuring the $^{36}Cl^-$ uptake in intact cellular cultures [6, 18]. Briefly, culture medium was replaced with a prewarmed Earle's balanced salt solution (EBSS: 116 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 15.2 mM $NaHCO_3$, and 5.5 mM glucose, adjusted to pH 7.4), and cell cultures were incubated in a humidified 5 % CO_2/95 % air atmosphere at 36.8 °C. Following 30 min incubation, buffer was replaced with new EBSS solution and incubated for an additional 15 min. Cells were then rinsed twice with EBSS solution and preincubated for 10 min at room temperature. Cultures were incubated for 20 s with 225 μl HEPES buffer containing $^{36}Cl^-$ (0.5 μCi/ml), 2 μM GABA, and test agents. After removing the $^{36}Cl^-$ solution, each well was immediately rinsed three times with 1.5 ml cold HEPES solution. Cells were lysed by water-induced hypo-osmotic shock, and radioactivity was determined by liquid scintillation counting. Values are normalized with respect to the $^{36}Cl^-$ uptake evoked by 100 μM GABA, as this concentration produces the maximum response [18].

Cell Viability

Following 7 days in vitro, the cells were exposed to different concentrations of each compound for 30 min or 24 h. Phenols were added after solubilization in 0.2 ml of culture medium previously extracted from each well. Cell viability was determined by measuring the reduction of 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a colored formazan salt by mitochondrial reducing activity, as described previously [6, 20]. Briefly, the cultures were rinsed and incubated for 15 min with a solution of MTT (250 μg/ml) dissolved in a HEPES buffer solution at 37 °C. After washing off the excess MTT, the cells were disaggregated with 5 % SDS, and the colored formazan salt was measured at 560 nm in a spectrophotometer plate reader. Data are expressed as a percentage of non-treated cultures.

Large Unilamellar Vesicles (LUVs) Formation

Multilamellar vesicles (MLVs) were prepared as described elsewhere with modifications [21]. Briefly, a dry film was obtained by evaporating, under a stream of N_2, a chloroformic solution of pure dpPC. The dried lipid was resuspended in bidistilled water by vigorous shaking for 1 min at room temperature. The MLVs obtained were passed 19 times through a 0.1 μm cellulose filter using a Liposofast mini-extruder (Avestin Inc., Ottawa, ON, Canada) in order to obtain LUVs of a homogeneous size with diameters around 100 nm. The LUVs were always used immediately after their preparation.

Steady-State Fluorescence

The fluorescent probes DPH (2 μM) and TMA–DPH (6 μM) were added to the dpPC LUVs suspension prepared as described above and incubated for 1 h at room temperature. The effects of phenol compounds on the DPH and TMA–DPH steady-state fluorescence anisotropy were studied.

Anisotropy values were calculated from the emission fluorescence intensities at λ emission = 430 nm (λ excitation = 356 nm) measured with the excitation and the sample polarizer filters oriented parallel and perpendicularly with

respect to each other, in an L-format FluoroMax-3 spectrofluorometer (Jovin Yvon, Horiba). Steady-state fluorescence anisotropy (A) was calculated as

$$A = (VV - VH.G) / VV + 2.VH.G,\ G = HV/HH \quad (1)$$

where VV, HH, VH, and HV are the values of the different measurements of fluorescence intensity, taken with both polarizers in vertical (VV) and horizontal (HH) orientations or with excitation polarizer vertical and emission polarizer horizontal (VH) or vice versa (HV). *G* is a correction factor for differences in sensitivity of the detection system for vertically and horizontally polarized light [22].

Data Analysis

Data shown represent the mean ± standard error of mean (SEM). Sigmoid curves were fitted to concentration response data and statistical analyses were performed using GraphPad Prism (GraphPad Software Inc, San Diego, CA, USA). A two-tailed Student's *t* test, as well as one-way and two-way analysis of variance (ANOVA), was used.

Results

It has been previously reported that an increase of [^{3}H]flunitrazepam binding corresponds to potentiation of the $GABA_A$-R [6, 23, 24]. Figure 2 shows the concentration–response curve of the effects of phenol compounds on specific [^{3}H]flunitrazepam binding in primary cultures of cortical neurons. Carvacrol and eugenol induced a concentration-dependent increase in specific binding, with EC_{50} values of 235 and 532 μM, and maximum responses (E_{max}) of 195 and 166 % with respect to basal condition (100 %), respectively (Table 1). Chlorothymol exerted an enhancement of the specific binding, but only until around 100 μM, showing a response of 146 % with respect to basal. Higher concentrations of chlorothymol reduced the binding to values lower than those of the basal binding. Therefore, calculation of the EC_{50} value took into account concentrations up to 100 μM (Table 1). Increases in [^{3}H]flunitrazepam binding induced by stimulant concentrations of each phenol were completely inhibited by the competitive $GABA_A$-R antagonist bicuculline (Fig. 3). The non-significant decrease of about 25 % with respect to the control binding induced by bicuculline was in accordance with bicuculline responses reported previously [9, 25]. To confirm that these phenolic compounds act as positive allosteric modulators of the $GABA_A$-R, we tested their effects on GABA-evoked chloride influx. Figure 4 shows that these compounds increased the GABA-evoked Cl^- influx in a concentration-dependent manner. Furthermore, 100 μM bicuculline significantly reversed this increase. When compounds were tested alone in the absence of GABA, they did not induce an influx of Cl^- (data not shown).

Figure 5 shows the effects of phenols on cell viability in primary neuronal cultures determined by the capability of the live cells to reduce the MTT salt. The concentrations assayed for each compound were selected considering ranges that include stimulant concentrations on [^{3}H]flunitrazepam binding. Thus, the ranges used were 100–1,000 μM for carvacrol, 400–2,000 μM for eugenol, and 10–200 μM for chlorothymol. The phenolic compounds assayed did not reduce cell viability after exposure of cortical neurons for 30 min (time period of the binding assay). Upon increasing the exposure time up to 24 h, carvacrol did not affect cell viability, while eugenol and chlorothymol showed negative effects (although non-significant with respect to the control) at the highest concentrations tested (2,000 and 200 μM, respectively).

Anisotropy values for DPH and TMA–DPH in dpPC LUVs were evaluated as a function of temperature (36–45 °C) in the presence of different phenolic compound concentrations (0–200 μM) (Fig. 6). The temperature dependences of DPH and TMA–DPH fluorescence anisotropy on control samples containing dpPC (without any phenol compound) showed an abrupt change at approximately 41.5 °C in agreement with the phase transition of dpPC [26]. All phenol compounds induced changes at this temperature transition, moving it toward lower temperatures, and this effect was concentration-dependent. Comparatively, chlorothymol produced the greater changes according to its higher lipophilicity [15]. Figure 7 shows the fluorescence anisotropy values of both probes (DPH and TMA–DPH), taken from data of Fig. 6 and plotted as a function of compound concentration at constant temperature (≈38.5 °C). These results indicate that, while carvacrol and eugenol affect the fluorescence anisotropy of both probes in the same way, chlorothymol produced a more marked effect on the DPH probe.

Discussion

The phenolic compounds studied here potentiated the specific binding of flunitrazepam, and this effect was saturable, reaching a maximal value (E_{max}) with carvacrol and eugenol. The $GABA_A$ antagonist bicuculline blocked the effect. Thus, the phenolic agents produced their effects on a site of the receptor different from that of flunitrazepam, thereby modifying receptor binding with this ligand. These compounds, therefore, appear to act as allosteric activators of benzodiazepine binding sites on the $GABA_A$-R that indirectly modulate the activity of the receptor [16, 27, 28]. It should be noted that these allosteric sites are the targets of many neuroactive drugs, including benzodiazepines, propofol, barbiturates, inhaled

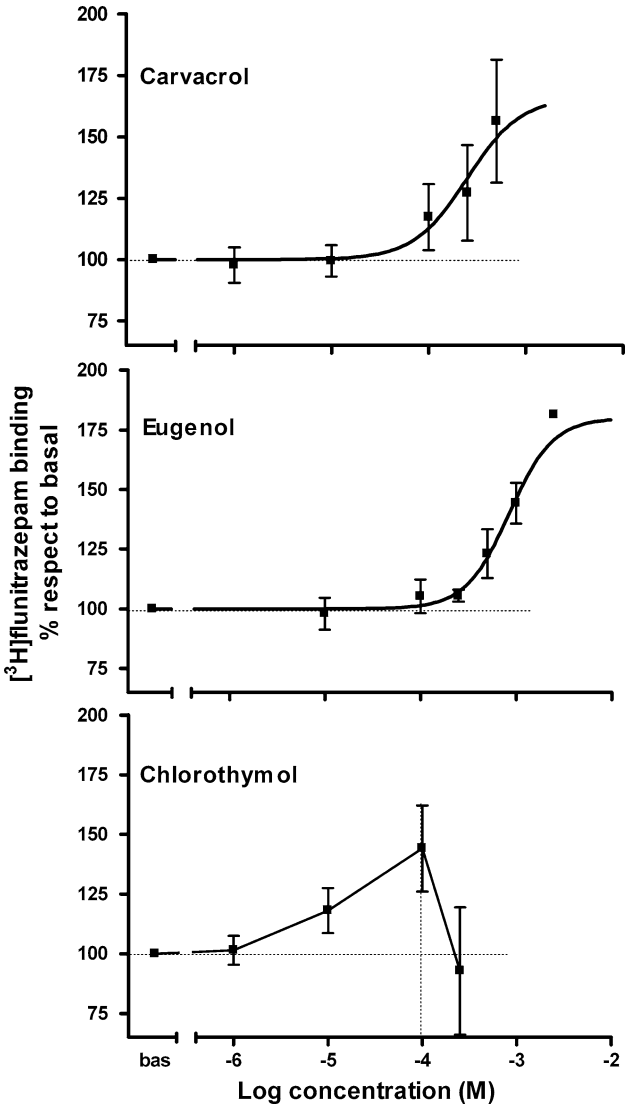

Fig. 2 Concentration–response curves for [^{3}H]flunitrazepam binding in the presence of carvacrol, eugenol, and chlorothymol. [^{3}H]Flunitrazepam binding was performed in intact cultured cortical neurons at 25 °C for 30 min. Results are expressed as the percentage of basal binding (*bas*) (100 %) in the absence of the test agent. Each point represents the mean ± S.E.M. of 3–4 independent experiments, each performed in triplicate

Table 1 Effects of phenol compounds on [^{3}H]flunitrazepam binding in primary cultures of cortical neurons

	Log EC50	EC50 (μM)	E_{max} (%)
Carvacrol	−3.760 ± 0.271	235	195 ± 36
Eugenol	−3,291 ± 0.071	532	166 ± 14
Chlorothymol[a]	−5,128 ± 0.365	19	146 ± 17

The values correspond to the mean ± S.E.M. of 3–4 independent experiments, each performed in triplicate. A minimum of six concentrations were used for each curve. EC_{50} values were calculated as explained in the Materials and Methods section. E_{max} corresponds to the percentage increase with respect to the basal binding

[a] Chlorothymol data were fit to sigmoidal curve up to 100 μM

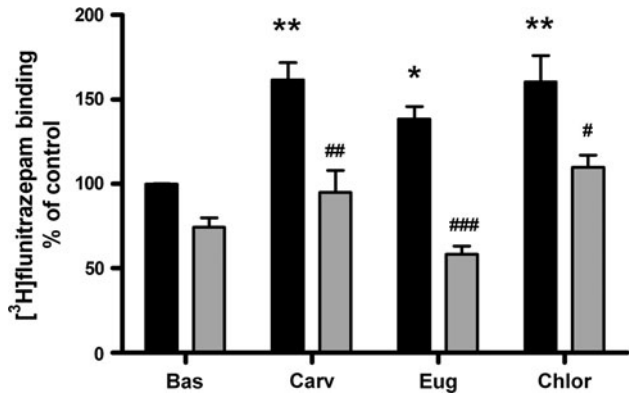

Fig. 3 Effects of bicuculline on the increase of [^{3}H]flunitrazepam binding induced by phenolic compounds. *Bas:* basal samples (in the absence of any phenolic compound), *Carv:* carvacrol (500 μM), *Eug:* eugenol (900 μM), and *Chlor:* chlorothymol (100 μM). The *bars* correspond to mean ± S.E.M. of 2–3 experiments done in triplicate in the absence (*black bars*) or in the presence of 100 μM bicuculline (*gray bars*), and represent the percentage with respect to the basal (100 %). * and **$p < 0.01$ and $p < 0.001$, respectively, versus basal in the absence of bicuculline; $^{\#,\#\#}$ and $^{\#\#\#}p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, versus the same treatment in the absence of bicuculline (two-way ANOVA)

anesthetics, and ethanol, among others. We have previously reported that the release of endogenous GABA from cultured cortical neurons exposed to basal conditions resulted in a GABA concentration in the medium of around 34 nM [6]. This concentration does not elicit $GABA_A$-R-mediated responses [29–31]. For this reason, the stimulations obtained in our experiments are considered to be exerted by each compound specifically.

Carvacrol is a monoterpenic phenol present in numerous aromatic plants, such as oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), and wild bergamot (*Monarda fistulosa*) [32]. It is used on a large scale in the food and cosmetic industries, and is a common ingredient of the human diet [33]. It has been reported that carvacrol has an analgesic action [34], anti-acetylcholinesterase (AChE) activity [35], and an anxiolytic-like effect [36]. The latter report suggests the involvement of the GABAergic system in the anxiolytic effect, demonstrated by means of behavioral tests. Our results support this hypothesis, since carvacrol, like eugenol, was able to induce an increase in the binding of the $GABA_A$-R allosteric modulator flunitrazepam, with this effect also being hindered by the antagonist bicuculline. Furthermore, the EC_{50} obtained for this stimulant effect of carvacrol (235 μM) is comparable to the doses used by Melo et al. [36] in in vivo experiments to induce an anxiolytic effect (25–50 mg/kg ≈ 167–333 μM). Also, carvacrol was more potent as a $GABA_A$-R modulator (EC_{50}: 235 μM; this study) than as an AChE inhibitor (IC_{50}: 400 μM; [35]).

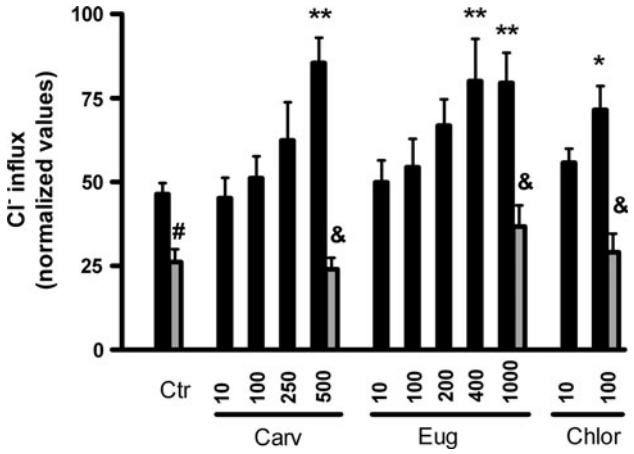

Fig. 4 Effect of carvacrol, eugenol, and chlorothymol on GABA-evoked Cl^- influx. $^{36}Cl^-$ uptake was performed in intact cultured cortical neurons exposed to 2 μM GABA and different concentrations (in μM) of carvacrol, eugenol, and chlorothymol. Control cells (Ctr) were exposed to 2 μM GABA and vehicle. Values were normalized to the maximum Cl^- influx evoked by 100 μM GABA. The *bars* correspond to mean ± SEM ($n = 6$–8 from two independent experiments). 100 μM bicuculline (*gray bars*) reversed the effect of the highest tested concentration of thymol-analogs. * and **$p < 0.05$ and $p < 0.01$, respectively, versus control in the absence of bicuculline; # and &$p < 0.05$ and $p < 0.001$, respectively, versus the same treatment in the absence of bicuculline (two-way ANOVA)

Eugenol is an aromatic compound that is commonly contained in various sorts of plants, especially in spices and medicinal herbs (clove, nutmeg, cinnamon, etc.). It is extensively used in dental procedures for analgesic and antiseptic purposes [37, 38]. Its effects on the nervous system have been widely studied (see refs in [39]). Eugenol is neuroprotective against excitotoxicity, ischemia, and amyloid-β peptide [40–42], induces anesthesia in rats [43], restrains epileptiform field potentials and spreading depression in hippocampus and neocortex [44], inhibits Na^+ currents as a mechanism of analgesia [39], inhibits *N*-methyl-D-aspartate receptors and potentiates $GABA_A$-R in mammals [41, 45]. In the present study, we further demonstrate that eugenol is a positive $GABA_A$-R modulator. The EC_{50} value obtained for this positive effect (530 μM) is very close to the eugenol concentration used by Aoshima and Hamamoto [45], (650 μM) to potentiate $GABA_A$-R responses. The fact that the increase in [^{3}H]flunitrazepam binding induced by eugenol was inhibited by bicuculline corroborates its positive modulation of the $GABA_A$-R. Compared with the known bactericide activity of carvacrol and eugenol at concentrations in the range of 1–10 mM [46–48], our results demonstrate that their active concentrations on $GABA_A$-R (EC_{50} 235 and 532 μM, respectively) for the stimulation of flunitrazepam binding are orders of magnitude lower than those found to be active against bacteria.

The inclusion of chlorothymol in the present study is justified by its phenolic structure, similar to that of thymol,

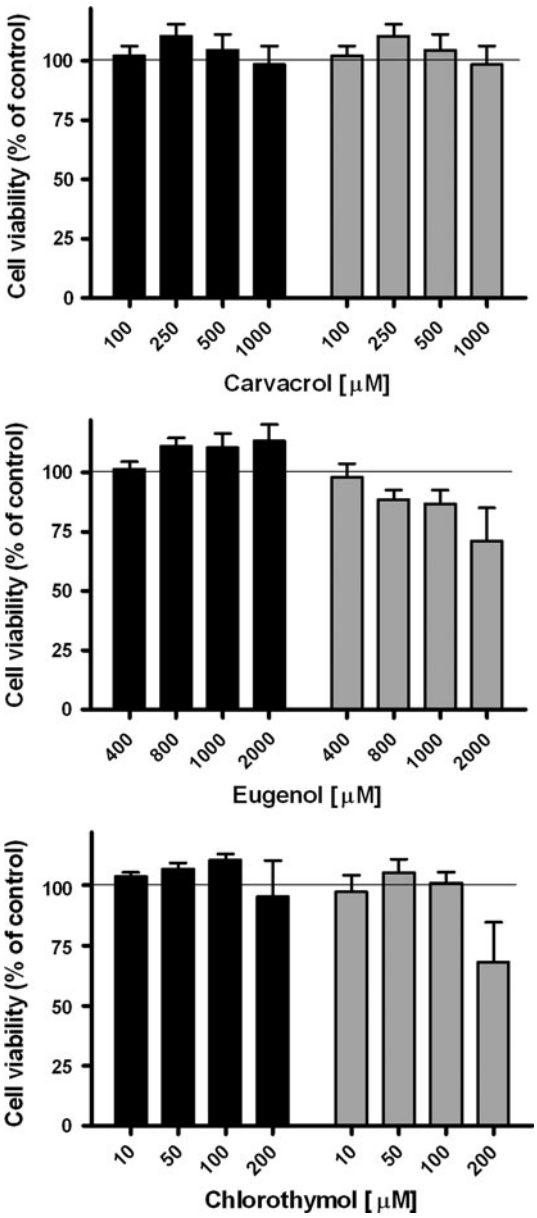

Fig. 5 Cell viability MTT assay in the presence of phenolic compounds. All values are mean S.E.M. of 3–6 independent experiments done in triplicate. The *bars* represent the percentage of cell viability with respect to 100 % control (without any phenol) after 30 min (*black bars*) or 24 h (*gray bars*) of exposure to each compound

which showed important GABAergic activity (see [6, 16] and refs) with a halogen atom (Cl) bound to the phenolic ring. It is used in cosmetics and personal care products. We have no knowledge until now of studies of the effects of chlorothymol on the nervous system. Thus, its effect on $GABA_A$-R reported here and its inhibition by a $GABA_A$ antagonist would be the first description of a possible pharmacological activity in the central nervous system. The shape of the concentration–response curve and the decline in activity at higher concentrations may be

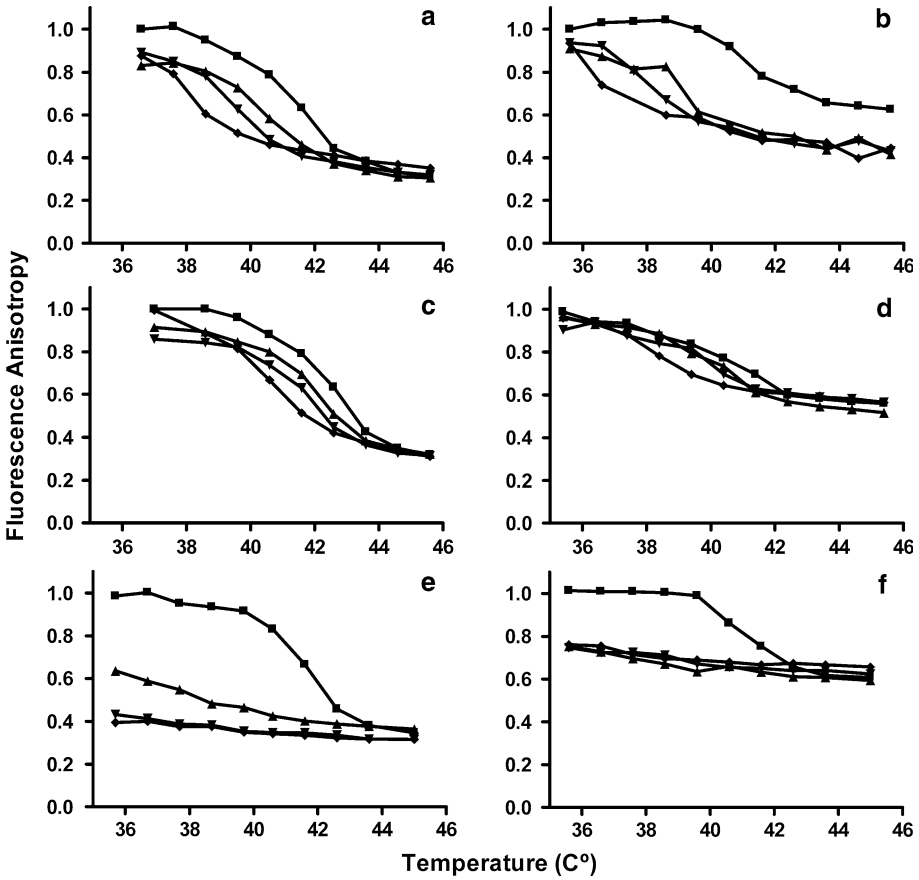

Fig. 6 Effects of phenol compounds on temperature dependence of DPH and TMA–DPH fluorescence anisotropy. Fluorescence anisotropy of DPH (*left*) and TMA–DPH (*right*) as a function of temperature in DPPC LUVs containing phenol compounds at different concentrations: 0 (*filled square*), 50 (*filled triangle*), 100 (*filled inverted triangle*), and 200 μM (*filled diamond*). **a**, **b** Carvacrol, **c**, **d** eugenol, and **e**, **f** chlorothymol. The anisotropy values were normalized, considering the value corresponding to the lower temperature assayed in the absence of any phenol compound as equal to unit

accounted for by the introduction of a Cl atom in the molecule and the increased toxicity of this compound.

Our results showing the effects of carvacrol, eugenol, and chlorothymol on the $GABA_A$-R also have pharmacological implications for drug safety in clinical practice. The widespread use of herbal products and phytomedicines containing the phenolic compounds considered here raises the issue of the potential effects deriving from their concomitant use with commonly prescribed drugs with central nervous system effects.

Carvacrol-containing products are mostly purchased at herbal medicine and health food stores or over the internet [49]. Nowadays, the most commonly advertised so-called natural medicines are preparations of essential oils of oregano with concentrations of carvacrol in the range of 75–85 %. These oils are marketed as either a liquid or as capsules for oral administration, and it can be calculated that, at the usual dosage recommended, the carvacrol dose ranges between 100 and 200 mg/day. Extrapolating from the experimental data at these doses, carvacrol might induce GABAergic effects. The interaction of carvacrol with prescribed psychoactive drugs acting on the $GABA_A$-R might increase the inhibitory activity of benzodiazepines, barbiturates, propofol, etc. The interaction of this agent with perioperative patient care may be of particular importance as has been reported with other herbal medicines [50–52]. Thus, for example, interaction with propofol, the most commonly used parenteral anesthetic, would increase the time of sedation and the operative patient management, given that propofol acts primarily through enhancement of $GABA_A$-R function, as carvacrol seems to do. Moreover, one of the main concerns with these herb–drug interactions is that patients do not usually report to their doctors that they are taking the herbal medicines [50, 53–56].

As regards eugenol, although essential oils of clove (*Syzygium aromaticum*) and other spices are also marketed, they are mostly intended for topical use. However, there are large populations (millions of people) exposed to eugenol through the smoke of specific cigarettes. This is the case of Indonesian clove-flavored cigarettes (kreteks) which contain levels of eugenol in their smoke ranging from 2.4 to 38 mg/cigarette [57]. A similar case is that of Indian bidi cigarettes which consist of tobacco wrapped in tendu leaf plants (*Diospyros malanoxylon*). Kreteks and bidis are gaining popularity among young people in Western countries [58].

We previously developed a pharmacophoric hypothesis for the binding site of propofol-analog phenolic compounds in the $GABA_A$-R [6]. This hypothesis includes three hydrophobic

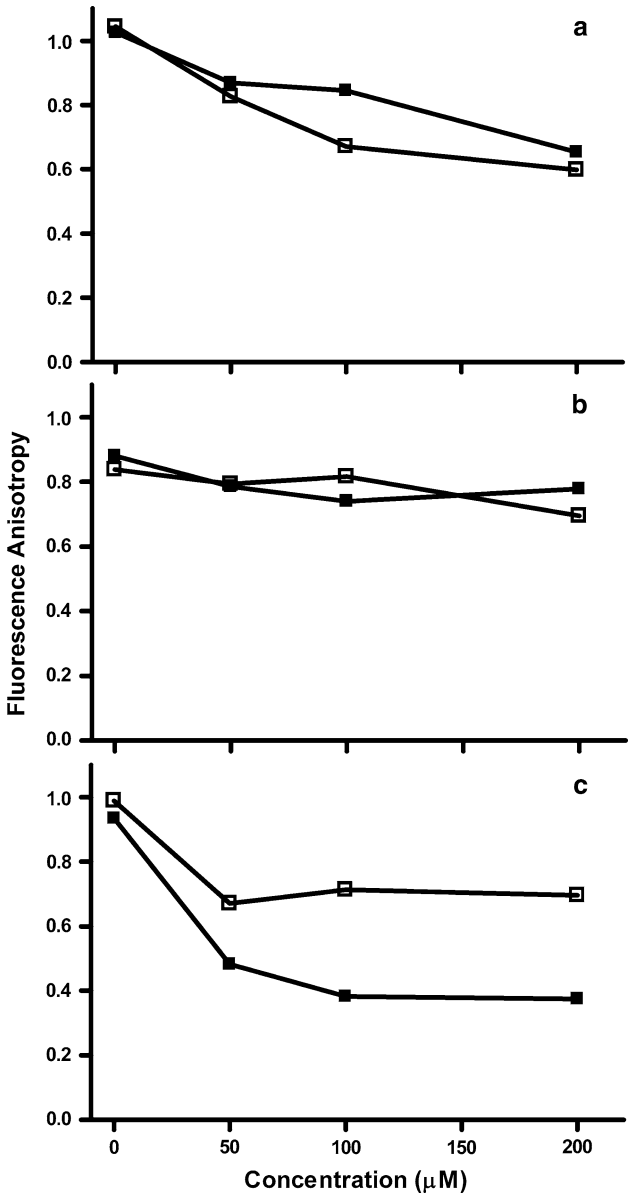

Fig. 7 Effects of phenol compounds on fluorescence anisotropy at constant temperature. Fluorescence anisotropy of DPH (*filled square*) and TMA-DPH (*open square*) in DPPC LUVs containing phenol compounds at different concentrations (0, 50, 100, and 200 μM) at ≈38.5 °C. **a** carvacrol, **b** eugenol, and **c** chlorothymol. Normalized values correspond to Fig. 6

aliphatic features, one hydrogen bond donor group, an exclusion volume, and an aromatic ring. We demonstrated the possible alignments of propofol and thymol using that hypothesis. Carvacrol can clearly accommodate most of the features described in the proposed hypothesis, whereas eugenol would not entirely fit the model due to the substitution of an aliphatic group by the polar methoxy group in position 2. The present results support the previously reported pharmacophoric hypothesis in which the better fit of carvacrol compared to eugenol would justify their different potencies at the $GABA_A$-R found in this study (EC_{50}: 235 and 532 μM, respectively). On the other hand, although chlorothymol showed a significant lower EC_{50} value (19 μM) similar to that of propofol (25 μM) [59], its pharmacological activity could be considered more limited by its inhibitory effects at high concentrations.

An important characteristic of phenol compounds is their hydrophobicity, which enables them to partition in the lipids of the cell membrane [15]. This partition can disturb the cell structures rendering them more permeable, permitting the leakage of ions and other cell contents [47]. However, none of the phenolic compounds was able to significantly induce the release of hemoglobin from red blood cells until 1 mM (chlorothymol) and 2 mM (carvacrol and eugenol) (results not shown). The hemolysis is a suitable method to measure membrane permeability as it was previously reported (see [60] and refs.). In addition, we recently demonstrated that the velocity of penetration of these compounds in phospholipid model membranes was quite similar [61] in spite of the fact that their partition ability was different [15]. Both results would indicate that the compounds do not strongly affect cell permeability and that other different mechanisms would be involved in the loss of pharmacological activity induced by chlorothymol concentrations over 100 μM and in the eventual neurotoxic effects shown by chlorothymol (200 μM) and eugenol (2 mM) at 24 h with the MTT assay.

Fluorescence anisotropy gives information about the organization of the membrane environment around the fluorescent probe. DPH is known to be located within the hydrocarbon chain region of the membrane core, while TMA–DPH, due to its polar ionic portion, is located near the interfacial region in the polar head group region [62]. The complex structural dynamics of the bilayers is governed by temperature-dependent parameters such as the average interfacial area per lipid, thickness of bilayer, and disorder of hydrophobic tails, which determine their phase behavior. For saturated phosphatidylcholines, such as dpPC, the main transition between the liquid-crystalline phase (La) and the gel phase (gel–fluid transition) occurs at 41.5 °C [26, 63]. In the present study, all compounds reduced their transition phase temperature, as revealed by the occurrence, for both fluorescent probes, of an anisotropy reduction at lower temperatures in a concentration-dependent manner. This effect seems to indicate a fluidizing effect on the membrane by decreasing molecular order and mobility throughout the bilayer thickness. However, these results were more marked in the presence of chlorothymol which is to be expected considering its higher lipophilicity. We previously demonstrated a superior partition coefficient (logP) in the dpPC liposome-buffer system for chlorothymol with respect to carvacrol and eugenol (logP values: 3.54, 2.48, and 1.89, respectively) [15]. Furthermore, in spite of the fact that all compounds affected the anisotropy of both probes used,

carvacrol and eugenol showed a similar effect on the mobility of DPH and TMA–DPH, while chlorothymol, more essentially, affected the dynamics at the hydrocarbon chain region, demonstrated by the strong reduction in the DPH anisotropy. This would indicate its special preference for a location in the core of the membrane where the hydrophobicity is much more obvious, causing changes in the physical properties of the cytoplasmic membrane. Thus, the results presented in Figs. 6 and 7 suggest that the comparatively higher ability of chlorothymol to interact with membranes may be responsible for the larger effect against neuron viability since the concentrations of chlorothymol and eugenol needed to reach similar deleterious effects (a loss of cell viability around 25 % after 24 h of exposure) differ by an order of magnitude (0.2 and 2 mM, respectively). Considering that $GABA_A$-R function can be regulated by lipid bilayer elasticity which is affected by amphiphiles partition [3], this hypothesis may also explain the rapid loss of activity described for [^{3}H]flunitrazepam binding at chlorothymol concentrations over 100 μM. With respect to the modulation of the $GABA_A$-R by the physical state of the membrane, it has been reported that the functions of proteins in the membrane might be altered as a result of the bilayer properties like elasticity, fluidity, thinning, etc. Because of the hydrophobic interactions between a membrane protein transmembrane region and the lipid bilayer core, a protein conformational change that involves the protein-bilayer hydrophobic interface can cause a local bilayer deformation [3, 10, 64].

Taking into account: (i) the structural similarity between the compounds assayed with thymol and propofol, a widely proved general anesthetic (see refs. in [2]) and (ii) the effects presented here on the $GABA_A$-R, we may strongly suggest a sedative activity for carvacrol, eugenol, and chlorothymol. This hypothesis is supported for eugenol, the anesthetic effect of which has been already reported in in vivo experiments [43, 65].

In conclusion, we have found that carvacrol, eugenol, and chlorothymol were able to enhance the binding of [^{3}H]flunitrazepam at the $GABA_A$-R and the GABA-evoked Cl^- influx, both effects being inhibited by the competitive $GABA_A$-R antagonist bicuculline. These results strongly suggest that the phenols studied are positive allosteric modulators of the $GABA_A$-R. The concentrations necessary to induce positive allosteric modulation of $GABA_A$-R are not neurotoxic. Furthermore, they are much lower than those reported to elicit bactericide activity. Although all compounds were able to decrease the microviscosity of artificial membranes, chlorothymol displayed a greater effect, which may explain its cytotoxic action at high concentrations. It is suggested that these compounds may exert depressant activity on the central nervous system and could potentiate the effects of general anesthetics.

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