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Allosteric positive interaction of thymol with the $GABA_A$ receptor in primary cultures of mouse cortical neurons

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Abstract

Thymol is a naturally occurring phenolic monoterpene known for its anti-microbial and anti-oxidant properties. It is used in dental practice and in anaesthetic halothane preparations. Recent studies have reported enhanced $GABA_A$ receptor-operated chloride channel activity and increased binding affinity of [^{3}H]flunitrazepam in the presence of thymol. In the present work, we more closely examined the pharmacological action of thymol on the native $GABA_A$ receptor by using primary cultures of cortical neurons. Thymol enhanced GABA-induced (5 μM) chloride influx at concentrations lower than those exhibiting direct activity in the absence of GABA (EC_{50} = 12 μM and 135 μM, respectively). This direct effect was inhibited by competitive and non-competitive $GABA_A$ receptor antagonists. Thymol increased [^{3}H]flunitrazepam binding (EC_{50} = 131 μM) and showed a tendency to increase [^{3}H]muscimol binding. These results confirm that thymol is a positive allosteric modulator of the $GABA_A$ receptor. The thymol structural analogues menthol and cymene, which lack an aromatic ring or a hydroxyl group, did not affect [^{3}H]flunitrazepam binding. Using a pharmacophoric model that includes a hydrogen bond donor group as well as an aromatic ring with two aliphatic substituents, we propose to demonstrate the molecular essential features of these compounds to interact with $GABA_A$ receptors. Thymol (0–1 mM) did not affect cellular viability.

Keywords: Thymol; $GABA_A$ receptor; Receptor binding and chloride flux; Cultured cortical neurons; Cell viability; Pharmacophore model

1. Introduction

$GABA_A$ receptors are ligand-gated ion channels that mediate rapid synaptic inhibition in the central nervous system. They are structured in an arrangement of five different individual subunits from seven families with multiple isoforms. The $GABA_A$ receptor possesses binding sites for drugs other than the neurotransmitter GABA, including benzodiazepines, barbiturates, and the convulsant picrotoxinin, which behave as allosteric modulators or channel blockers. A wide spectrum of drugs, toxic agents and metals modify $GABA_A$ receptor function by directly interacting either with these binding sites or with other as yet not well-described sites present in the receptor complex. Known allosteric modulations include the enhanced binding of benzodiazepine agonists by GABA, the enhanced GABA-induced chloride flux by benzodiazepines and barbiturates, and the different modifications of [^{35}S]*t*-butylbicyclophosphorothionate (^{35}S-TBPS, ligand of choice for the picrotoxinin recognition site) binding induced by GABA,

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benzodiazepines and barbiturates (for review see Macdonald and Olsen, 1994; Sieghart, 1995, 2000; Korpi et al., 2002; Smith and Simpson, 2003; Rudolph and Möhler, 2004).

Thymol is a naturally occurring phenolic monoterpene which is found as a component of many essential oils used extensively in fragrances, flavour additives, or scenting products. This compound is a particularly well-known anti-microbial agent commonly incorporated in mouthwashes and used in dental practices for its bactericidal properties against oral bacteria (Shapiro and Guggenheim, 1995; Burt, 2004). As with other phenolic compounds, thymol possesses anti-oxidant properties, which explains its use as anaesthetic stabiliser in halothane preparations (MacPherson, 2001). A thymol-supplemented diet has been reported to increase anti-oxidant status and to safeguard polyunsaturated fatty acid levels in the aging rat brain (Youdim and Deans, 1999, 2000). In addition to its anti-oxidant action in the brain, thymol has been shown to specifically interact with synaptic neural functions. Studies have reported blocking action on neuronal Na^+ channels (Haeseler et al., 2002) as well as enhanced chloride channel activity in oocytes and cell lines expressing $GABA_A$ receptor subunits (Mohammadi et al., 2001; Priestley et al., 2003). Recently, Sánchez et al. (2004) described the ability of thymol to incorporate itself in artificial membranes and to increase the binding affinity of [^{3}H]flunitrazepam to $GABA_A$ receptors in synaptosomal membranes, suggesting a possible role for thymol as a $GABA_A$ receptor agonist/modulator.

Other phenolic compounds, among them the anaesthetic propofol (2,6-isopropyl-phenol), reportedly enhance GABA agonist activity and directly activate the $GABA_A$ receptor (Squires et al., 1999; Trapani et al., 2000; Mohammadi et al., 2001 and references therein). Krasowski et al. (2002), by means of a QSAR analysis of several propofol analogues, have described two main structural conditions for a molecule to exhibit activity on this receptor: (i) an OH group as a hydrogen bond donor and (ii) two hydrophobic groups close to the hydroxyl. Based on this finding, and considering that thymol partially meets such structural requirements, we examined whether the action of thymol on the $GABA_A$ receptor is mimicked by its structural analogues menthol and cymene. These compounds share with thymol the presence of either the OH group (menthol) or the aromatic ring (cymene) (see Fig. 2). Furthermore, both thymol and menthol have been reported to block sodium channels (Haeseler et al., 2002).

In the current study, we more closely examined the pharmacological action of thymol on native $GABA_A$ receptors by determining its effects on GABA and benzodiazepine recognition sites, as well as on chloride influx by using primary cultures of cortical neurons, which express functional $GABA_A$ receptors (Pomés et al., 1994; Vale et al., 1999 and references therein). Moreover, we propose a pharmacophoric model, based on that proposed by Krasowski et al. (2002), addressing the action of thymol and its analogue phenolic compound, propofol, on $GABA_A$ receptors. Finally, we determined a lack of neurotoxic effects exerted by thymol at concentrations relevant to its neuroactive window.

2. Methods

2.1. Materials

Pregnant NMRI mice (16th gestational day) were obtained from Charles River, Iffa Credo (St. Germain-sur-l'Arbreste, France). Plastic culture multiwell plates were purchased from CoStar (Corning Science Products, Acton, MA, USA). Foetal 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 $^{36}Cl^-$ (0.1 Ci/mol) were procured from Amersham Life Sciences (Buckinghamshire, UK); [^{3}H]muscimol (36.5 Ci/mmol) from Perkin–Elmer (Boston, MA, USA). Liquid scintillation cocktail Optiphase Hisafe 2 was obtained from Wallace Oy (Turku, Finland). 2-Isopropyl-5-methyl-phenol (thymol), 2-isopropyl-5-methyl-cyclohexanol (menthol), 1-isopropyl-4-methyl-bencene (cymene), GABA, (−)-bicuculline methiodide, picrotoxinin, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl-formazam (MTT), propidium iodide (PI), trypsin, soybean trypsin inhibitor, DNAse, amino acids and poly-L-lysine were obtained from Sigma Chemical Co. (St Louis, MO, USA). SR95531 was obtained from Research Biochemicals International (One Strathmore Road Natick, MA, USA). All the other chemicals were of analytical grade. Thymol, menthol and cymene 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.

2.2. Cell cultures

Primary cultures of cortical neurons were prepared from the cerebral cortices of 16-day-old mouse foetuses according to the method described by Frandsen and Schousboe (1990). Pregnant animals were killed by cervical dislocation and foetuses extracted. Neocortices were dissected with forceps, mechanically minced, with cells then dissociated by mild trypsinisation (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% foetal 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 6–9 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.

Animals were handled in compliance with protocols of the University of Barcelona, approved by the Generalitat de Catalunya, Spain, in accordance with EU guidelines, and in compliance with the Office of Laboratory Animal Welfare (OLAW)/National Institutes of Health (NIH) (identification number A5224-01).

2.3. [^{3}H]Flunitrazepam binding

Benzodiazepine binding to intact cultured cells was determined according to the method described by Fonfría et al. (2001) with modifications. Briefly, the 24×-multiwell plates were washed four times with 0.5 ml/well of prewarmed (37 °C) HEPES-buffered saline solution (136 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.4 mM MgCl, 1 mM NaH_2PO_4, 10 mM HEPES and 9 mM glucose, adjusted at pH 7.4) and pre-incubated with this solution for 10 min at room temperature. The HEPES solution was then replaced with fresh solutions containing different concentrations of the compounds, and the binding assay was initiated by adding the radioligand (2.5–4 nM [^{3}H]flunitrazepam). Non-specific binding was measured in the presence of 20 μM diazepam. Following 30 min incubation at 25 °C, the medium was aspirated and the cells were quickly rinsed four times with cold HEPES solution. Cells were disaggregated in 0.2 M NaOH overnight and bound radioactivity was determined by liquid scintillation counting (Optiphase Hisafe 2). Proteins were determined by a microtest using the Bradford method, with bovine serum albumin as a standard. Basal binding was determined in HEPES solution containing 0.25% DMSO in the absence of drugs. Data are expressed as the percentage of basal specific binding. Non-specific binding accounted for less than 11% of the total binding.

2.4. [^{3}H]Muscimol binding

Muscimol binding was conducted in intact cultured cells in essentially the same manner as described for benzodiazepine binding in the presence of 20–25 nM [^{3}H]muscimol (similar to previously reported K_d values; Squires et al., 1999; Baur and Sigel, 2003), and incubated for 20 min at 25 °C. Basal binding was determined in the absence of any drug, non-specific binding in the presence of 1 mM GABA. Data are expressed as the percentage of basal-specific binding. Non-specific binding accounted for around 29% of the total binding.

2.5. Chloride influx

Chloride influx was determined as $^{36}Cl^-$ uptake in intact cellular cultures (Vale et al., 2003, with modifications). Briefly, culture medium was replaced by 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 by 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 (22–25 °C). Cultures were incubated for 7 s with 225 μl HEPES solution containing $^{36}Cl^-$ (0.5 μCi/ml) without/with test agents. After removing the $^{36}Cl^-$ solution, each well was immediately rinsed four 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 as previously described. Basal $^{36}Cl^-$ uptake was determined in the absence of drugs.

2.6. GABA determination

The concentration of GABA in the exposure medium was determined by high performance liquid chromatography (Babot et al., 2005). Cell cultures were exposed to basal conditions and to different GABA concentrations for 30 min at 25 °C or for 7 s at room temperature and GABA concentration was determined thereafter.

2.7. Computational methods

Pharmacophoric models were created using the program Catalyst, version 4.9 (Accelrys Inc., Burlington, MA, USA), on a Silicon Graphics Octane2 workstation operating under IRIX 6.5. Molecular properties (molecular volume and AlogP98) were calculated using Cerius 2 (Accelrys Inc.). Within Catalyst, pharmacophore models are represented as a set of features distributed in 3D space. These features can be point-features (i.e. hydrophobic centres), described by a centroid and a tolerance sphere, or vector-features (i.e. hydrogen bond donors and acceptors) described by a centroid and a projection point, each with its respective tolerance sphere. The pharmacophoric hypothesis for the binding site of propofol-analogue phenolic compounds in the $GABA_A$ receptor was generated manually, based on the models described by Krasowski et al. (2002) for direct activation and response potentiation of the $GABA_A$ receptor. The spatial position of the features included in this hypothesis was determined by averaging the position of

analogous pharmacophoric groups in models described in the literature. Aligning the compounds examined in this study to the pharmacophoric model was carried out using Catalyst, after sampling the conformational space of each product within 5 kcal/mol from the lowest energy minimum value.

2.8. Cell viability assays

Following 7 days in vitro, the cells were exposed to different concentrations of thymol (0–1 mM) for 30 min or 24 h. Thymol was added after solubilisation in 0.2 ml of culture medium previously extracted from each well.

Reduction of 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazam (MTT assay; measuring cell viability and mitochondrial activity) was conducted essentially according to the method described by Babot et al. (2005). Following each exposure period, the cells were washed three times with 0.5 ml of pre-warmed HEPES solution (37 °C) and then incubated for 20 min at 37 °C with 0.25 ml MTT reagent solution (0.25 mg/ml). After removing the MTT solution, 0.25 ml/well of solubilisation solution (SDS 5% w/v) was added and the cells were kept overnight at 37 °C in darkness. Absorbance was measured at a wavelength of 560 nm using a spectrophotometer plate reader (iEMS Reader MF; Labsystems, Helsinki, Finland). Data are expressed as percentage of non-treated cultures.

Lactate dehydrogenase (LDH) leakage and propidium iodide (PI) incorporation assays (measuring membrane integrity) were determined simultaneously in cell cultures exposed to 1 mM thymol for 24 h, as previously described (Rosa et al., 1997; with modifications). One hundred percent cell death was achieved by exposing cells to 0.025% (w/v) Triton X-100 for 1 h. PI (0.147 mg/ml) was added 1 h before the end of the exposure period and fluorescence was measured in a fluorometric plate reader (SpectraMax GeminisXS, Molecular Devices, Sunnyvale, CA, USA) set at 535 nm excitation/617 nm emission. The amount of LDH released from the cells was determined by measuring its activity in aliquots of culture media using standard procedures. PI staining and LDH release data are expressed as the percentage of Triton X-100-treated cells. PI staining did not interfere with LDH assays.

2.9. Data analysis

Data shown represent the mean ± standard error of mean (S.E.M.). 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 analysis of variance (ANOVA), was used.

3. Results

3.1. Parameters of [^{3}H]flunitrazepam binding and $^{36}Cl^-$ uptake in intact cultured cortical neurons

In primary cultures of mice cortical neurons [^{3}H]flunitrazepam binding had an apparent K_d of 7.4 ± 1.8 nM and B_{max} of 731 ± 31 fmol/mg protein (Fig. 1A), in agreement with previous reports using this type of cultures (Mehta and Ticku, 1992; Vale et al., 1999). Fig. 1B shows the concentration–response curve for GABA-induced Cl^- flux in intact cultured cortical neurons. The EC_{50} value was 8.4 μM (log EC_{50} = −5.1 ± 0.1), similar to that reported by Hu and Ticku (1994) and Vale et al. (1999). Because primary cultures of cortical neurons are enriched in GABAergic neurons (Sonnewald et al., 2004), we also determined whether

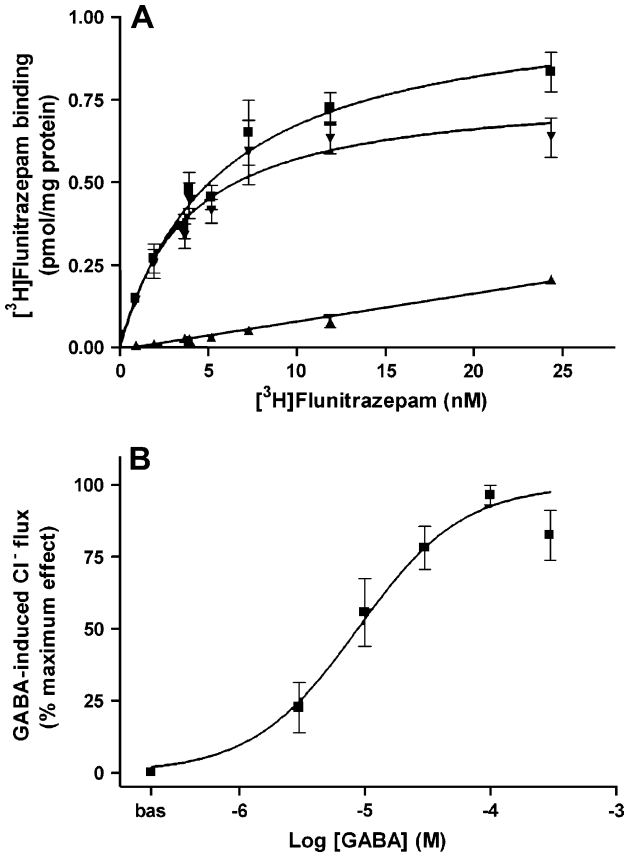

Fig. 1. (A) Binding of [^{3}H]flunitrazepam (0.8–24 nM) to intact cultured cortical neurons. [^{3}H]Flunitrazepam binding was performed at 25 °C for 30 min. Each point represents the mean ± S.E.M. (n = 4) of a representative experiment. ■, total binding; ▲, non-specific binding; ▼, specific binding. (B) Concentration–response curve for GABA-induced $^{36}Cl^-$ uptake in intact cultured cortical neurons. Cultured cells were exposed to different concentrations of GABA for 7 s at room temperature. $^{36}Cl^-$ uptake was normalised to maximum response induced by GABA over basal (bas) $^{36}Cl^-$ uptake. Each point represents the mean ± S.E.M. of four independent experiments, each performed in duplicate–quadruplicate.

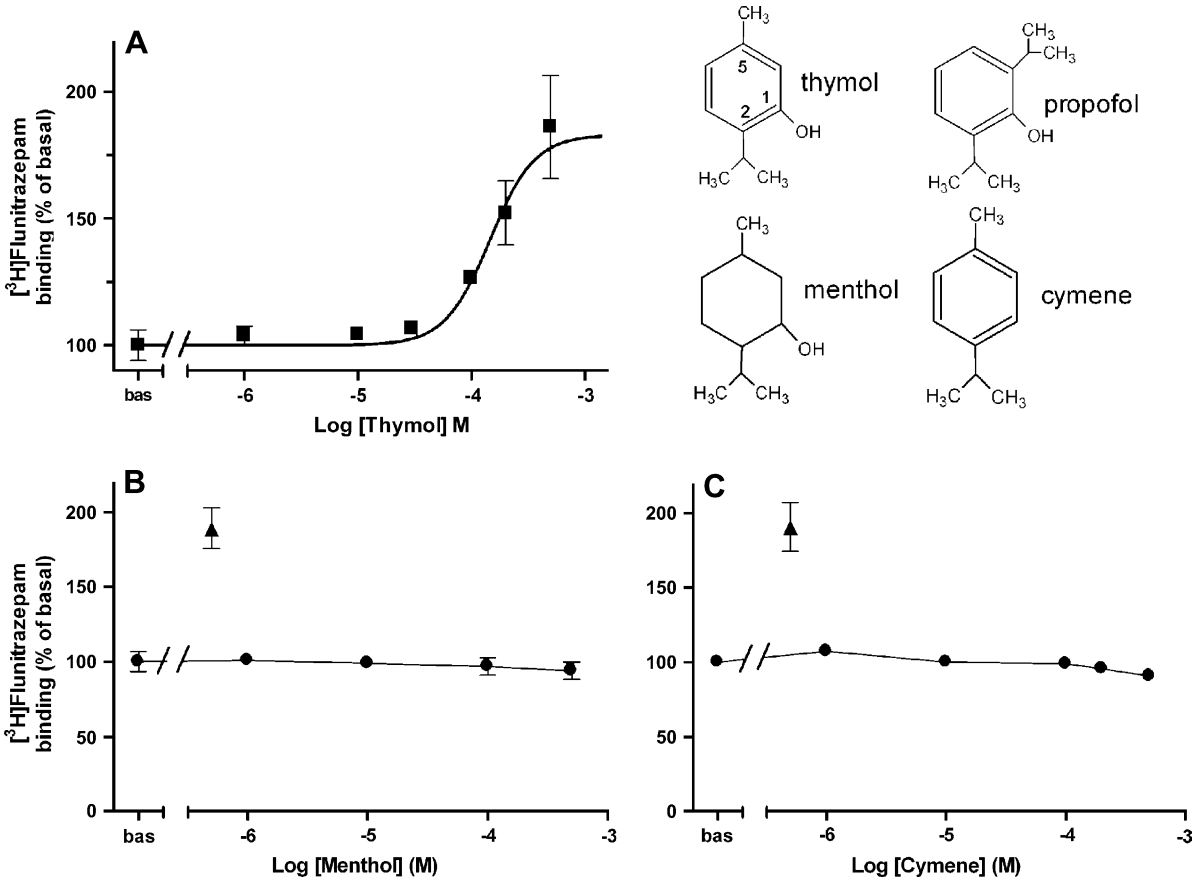

Fig. 2. Concentration–response curves for [^{3}H]flunitrazepam (2.5–4 nM) binding in the presence of (A) thymol, (B) menthol, and (C) cymene, and their chemical structures. [^{3}H]Flunitrazepam binding was performed in intact cultured cortical neurons at 25 °C for 30 min. Results are expressed as the percentage of basal binding (bas) in the absence of the test agent. Each point represents the mean ± S.E.M. (n = 2–3) of independent experiments, each performed in triplicate. Basal [^{3}H]flunitrazepam specific binding was 244 ± 14 fmol/mg protein (n = 7). The triangles in B and C correspond to the effects of 100 μM GABA used as internal control. The chemical structure of propofol is included for comparison.

uptake of GABA by these neurons could result in a significant reduction of the initial GABA concentration in the exposure medium. Exposure of the cells to 100 μM GABA for 30 min or to 5 μM GABA for 7 s (like in the [^{3}H]flunitrazepam or $^{36}Cl^-$ uptake assays) did not significantly reduce the initial concentration of GABA in the incubation medium (final concentrations were 89 ± 6% and 97 ± 3% with respect to the initial GABA concentration, respectively). Furthermore, the release of endogenous GABA by cells exposed to basal conditions for both assays resulted in a medium concentration of 34 ± 4 nM and 18 ± 1 nM, respectively. These concentrations do not elicit $GABA_A$ receptor-mediated responses (Vale et al., 1997, 1999; Bali and Akabas, 2004).

3.2. Effects of thymol on [^{3}H]flunitrazepam binding at the $GABA_A$ receptor

Thymol induced a concentration-dependent increase in [^{3}H]flunitrazepam binding in primary cultures of cortical neurons with an EC_{50} value of 130.9 μM and a maximum response (E_{max}) of 183% with respect to basal binding (Table 1, Fig. 2A). Under the same experimental conditions, increments induced by 100 μM GABA and 200 μM thymol were not additive: 196.9 ± 9.7% and 149.8 ± 8.7% for GABA and thymol, respectively, and 208.9 ± 2.7% for GABA plus thymol (n = 2–5). Increases in [^{3}H]flunitrazepam binding induced by thymol or GABA were completely inhibited by the competitive $GABA_A$ receptor antagonists bicuculline and SR95531, while the non-competitive antagonist picrotoxinin significantly reduced any increases (Fig. 3A,B).

3.3. Effects of thymol on chloride flux through the $GABA_A$ receptor

Thymol induced a concentration-dependent increase of $^{36}Cl^-$ uptake in primary cultures of cortical neurons when applied in the absence of GABA (Fig. 4, Table 1). Bicuculline, picrotoxinin, and SR95531 blocked the increase of $^{36}Cl^-$ uptake induced by thymol, like that induced by GABA (Fig. 5A and B, respectively). The inhibition of $^{36}Cl^-$ uptake and [^{3}H]flunitrazepam

Table 1
Effects of thymol on [^{3}H]flunitrazepam binding and Cl^- flux in primary cultures of cortical neurons

	Log EC_{50}	EC_{50} (μM)	E_{max} (%)
[^{3}H]Flunitrazepam binding	−3.885 ± 0.026 (3)	130.9	183.0 ± 12.3
$^{36}Cl^-$ uptake			
Thymol	−3.871 ± 0.128 (3)	134.6	168.8 ± 8.2
Thymol + 5 μM GABA	−4.928 ± 0.039 (3)**	11.8	202.7 ± 23.1

The values correspond to the mean ± S.E.M. of 3 independent experiments. A minimum of 6 concentrations, all determined in triplicate or quadruplicate, were used for each curve. E_{max} corresponds to the percentage of increment with respect to the basal binding. **$p < 0.01$ with respect to thymol in the absence of GABA (Student's t-test).

binding exerted by 100 μM of bicuculline was also demonstrated at 500 μM thymol (results not shown).

When thymol was applied in the presence of 5 μM GABA (the concentration that produces about 30% of the maximal effect of GABA in cortical neurons, see Fig. 1B), the potency of thymol significantly increased (EC_{50} values were 11.8 μM for thymol in the presence of GABA vs. 134.6 μM in the absence of GABA, $p < 0.01$) (Fig. 4, Table 1). The maximum net increase of $^{36}Cl^-$ uptake induced by thymol plus 5 μM GABA (102.7%) did not differ from the sum of that induced by thymol

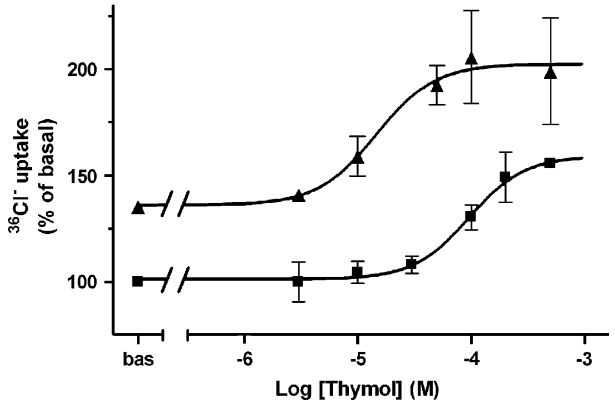

Fig. 4. Concentration–response curves for the increase in chloride influx induced by Thymol. $^{36}Cl^-$ uptake was performed in intact cultured cortical neurons at room temperature for 7 s. The values are expressed as the percentage of basal uptake (bas) in the absence of thymol. The symbols correspond to samples in the absence (■) or in the presence (▲) of 5 μM GABA. Each point represents the mean ± S.E.M. of 3–4 independent experiments, each performed in quadruplicate.

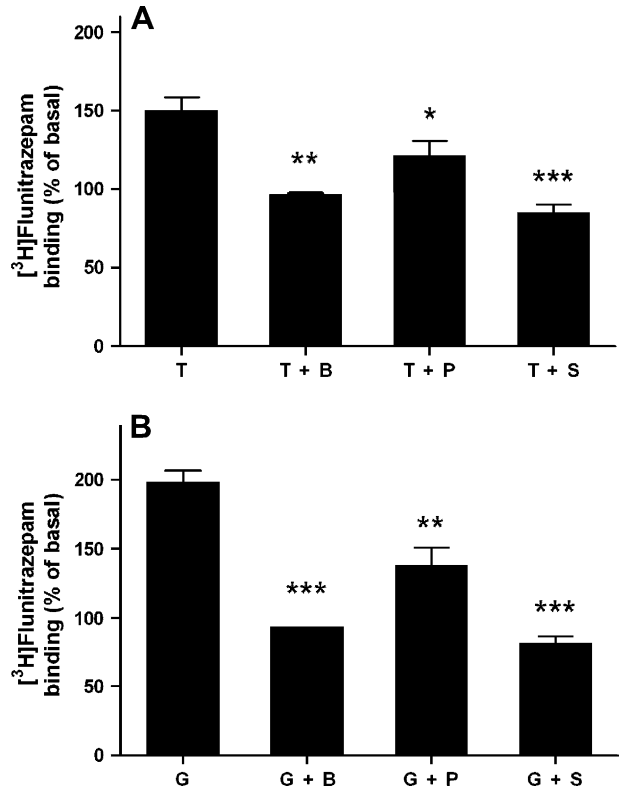

Fig. 3. Effects of bicuculline, picrotoxinin or SR95531 on the increase of [^{3}H]flunitrazepam binding induced by (A) thymol or (B) GABA. Each value represents the mean ± S.E.M. of 3–5 independent experiments, each performed in triplicate. T, 200 μM thymol; G, 100 μM GABA; B, 100 μM bicuculline; P, 100 μM picrotoxinin; S, 100 μM SR95531. *, ** and *** indicate significant differences from G or T according to the graph ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, one-way ANOVA).

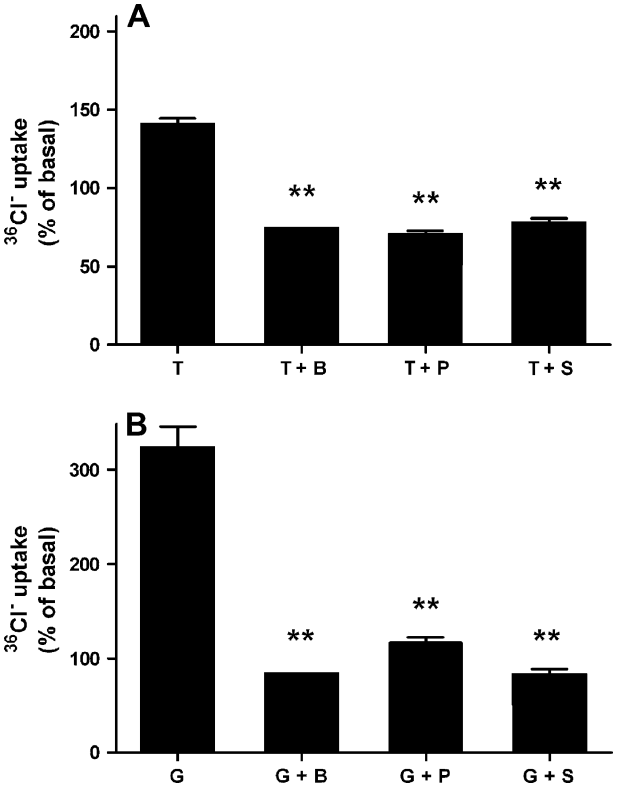

Fig. 5. Effects of bicuculline, picrotoxinin or SR95531 on the chloride influx induced by (A) thymol or (B) GABA. Each value represents the mean ± S.E.M. of 2–4 independent experiments, each performed in triplicate. T, 200 μM thymol; G, 100 μM GABA; B, 100 μM bicuculline; P, 100 μM picrotoxinin; S, 100 μM SR95531. **Significantly different from G or T ($p < 0.01$; one-way ANOVA).

(68.8%) and by 5 μM GABA alone (35.5%) when applied separately (Fig. 4, Table 1).

3.4. Effects of thymol on [^{3}H]muscimol binding at the $GABA_A$ receptor

Both [^{3}H]flunitrazepam binding and $^{36}Cl^-$ influx are increased by $GABA_A$ receptor agonists, as well as by positive allosteric $GABA_A$ receptor modulators (Vale et al., 1997, 1999). To ascertain whether thymol was a true GABA agonist or a GABA modulator, we tested the effects of thymol on [^{3}H]muscimol binding at the GABA recognition site. Thymol did not inhibit, but rather generated a concentration-dependent increase in [^{3}H]muscimol binding up to 100 μM thymol (Fig. 6). For comparison, Fig. 6 also shows the concentration-dependent inhibition of [^{3}H]muscimol binding by GABA.

3.5. Effects of thymol structural analogues on $GABA_A$ receptor

Thymol and its structural analogue, menthol, have been reported to block the neuronal sodium channel in a way that is compatible with anaesthetic activity (Haeseler et al., 2002). Both compounds have a hydroxyl, an isopropyl and a methyl group at positions 1, 2 and 5, respectively, on a six-member ring, aromatic for thymol and alycyclic for menthol (see Fig. 2). We wondered whether menthol could also be active at the $GABA_A$ receptor. Menthol did not increase [^{3}H]flunitrazepam binding (93.8 ± 5.6% with respect to control binding, in the presence of 500 μM menthol) (Fig. 2B). Cymene, a different structural thymol analogue containing an aromatic ring and the corresponding alkyl groups but not the hydroxyl group (see Fig. 2), was also inactive when tested against [^{3}H]flunitrazepam binding (90.7 ± 4.7% with respect to control binding, in the presence of 500 μM cymene) (Fig. 2C).

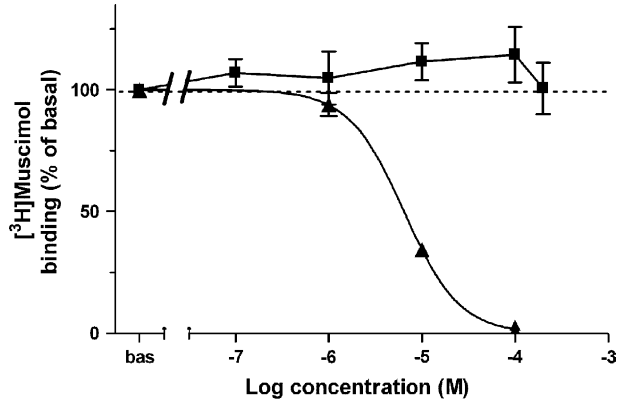

Fig. 6. Concentration–response curves for [^{3}H]muscimol (20–25 nM) binding in the presence of thymol (■) or GABA (▲). [^{3}H]Muscimol binding was performed in cultured cortical neurons at 25 °C for 20 min. Results are expressed as the percentage of basal binding (bas) in the absence of any compound. Each point represents the mean ± S.E.M. of three independent experiments (thymol) or a typical experiment (GABA), each done in triplicate. Basal [^{3}H]muscimol specific binding was 649 ± 37 fmol/mg protein (n = 3).

3.6. Pharmacophoric model

Based on the models for the potentiation and the direct activation of the $GABA_A$ receptor proposed by Krasowski et al. (2002), a pharmacophoric hypothesis was generated that represents the structural requirements of the binding site for propofol and its phenolic analogues in the $GABA_A$ receptor (Fig. 7, Table 2). This hypothesis includes three hydrophobic aliphatic features (HAL1–3), one hydrogen bond donor group (HBD), an exclusion volume (Excl), and an aromatic ring (AR), the last not included in Krasowski's model. Fig. 7 shows the best possible alignments of propofol and thymol using our hypothesis. These alignments make clear that both compounds can accommodate most of the features present in the proposed hypothesis, with the sole exception of the hydrophobic interaction in the HAL3 region. According to the software algorithm, thymol, which contains a 5-methyl substituent that in principle could align to HAL3, fits better with our hypothesis by shifting the position of the molecule, thereby placing this

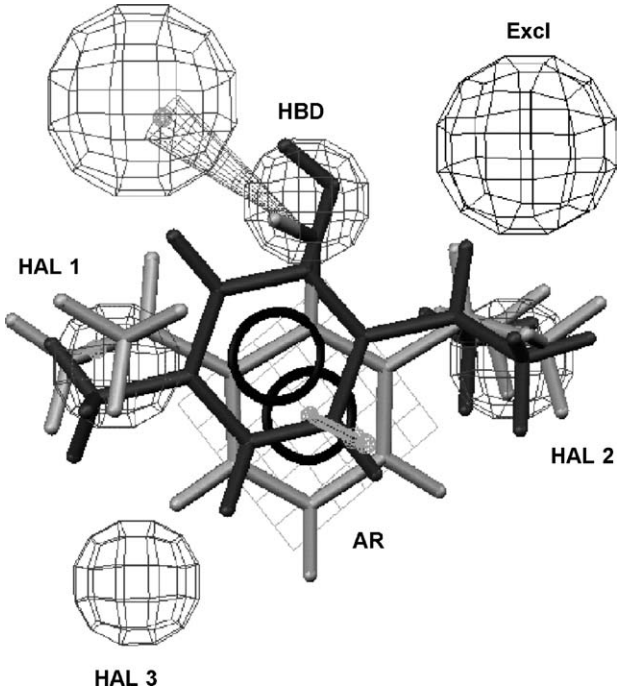

Fig. 7. Pharmacophoric hypothesis for the propofol/thymol-binding site in the $GABA_A$ receptor. The pharmacophoric features included are labelled as: HAL, hydrophobic aliphatic; HBD, hydrogen bond donor; AR, aromatic ring; and Excl, excluded volume. Thymol (dark grey) and propofol (light grey) are shown in their optimum fitted conformations.

Table 2
Distance matrix (in Å) of the pharmacophoric hypothesis shown in Fig. 6

	HAL2	HAL3	HBDc	HBDp	ARc	ARp	Excl
HAL1	3.3	6.4	3.9	4.0	3.0	4.3	7.9
HAL2		7.0	4.2	7.2	3.4	4.4	3.6
HAL3			6.4	7.3	4.1	5.3	9.6
HBDc				3.0	2.8	4.1	4.3
HBDp					5.0	5.9	6.8
ARc						3.0	5.5
ARp							5.6

Suffixes c and p stand for centroid and projection points, respectively.

methyl group in the HAL1 region. Menthol and cymene are relatively similar to the active compounds thymol and propofol in steric and hydrophobic terms (molecular volume: 179, 152, 160 and 193 $Å^3$, respectively; logP: 2.78, 3.51, 3.27 and 3.98, respectively). Nevertheless, menthol and cymene could only partially meet some of the requirements inherent to this hypothesis. Cymene cannot accommodate the presence of the essential hydrogen bond donor group, although it can satisfy the interactions with two of the hydrophobic regions (HAL1 and 2) as well as the aromatic region (AR). On the other hand, replacement of the aromatic ring present in thymol by an aliphatic one in menthol precludes its fit to our model and introduces substantial changes in the electronic properties of the molecule, which can result in a poor interaction with the $GABA_A$ receptor and, consequently, an inability to increase [^{3}H]flunitrazepam binding.

3.7. Effects of thymol exposure on cell viability in primary cultures of cortical neurons

Since thymol has been described as an effective bactericide through disruption of cell membrane or ATP generation (Shapiro and Guggenheim, 1995), we analysed its effects on cell viability and membrane integrity in neuronal cells. Fig. 8A shows that the presence of thymol up to 1 mM for 30 min and 24 h did not result in decreased neuron mitochondrial activity, as demonstrated by the capability of the cells to reduce the MTT salt, thereby assuring cell viability. Likewise, exposure to 1 mM thymol for 24 h did not result in significant differences with respect to control cells in PI incorporation, nor in the release of intracellular LDH (Fig. 8B,C). Consequently, concerns regarding any potentially harmful effects by thymol on cellular membrane integrity can be excluded.

4. Discussion

In the present work we provide clear evidence that thymol is a positive $GABA_A$ receptor modulator, as

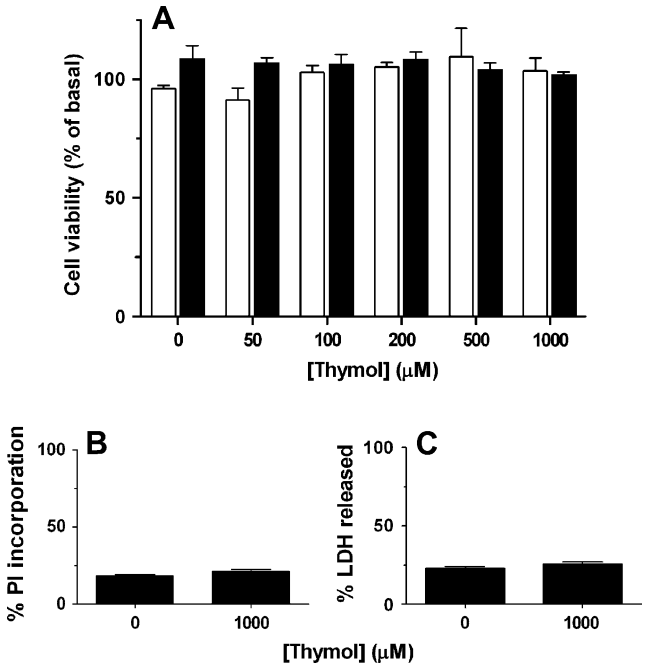

Fig. 8. Cell viability assays in the presence of thymol (0–1000 μM). The samples labelled as 0 represent control samples with DMSO 0.25%. All values are mean ± S.E.M. of 2–4 independent experiments done in triplicate. (A) MTT assay. The bars represent the percentage of cell viability with respect to basal (without thymol and DMSO) after 30 min (open bars) or 24 h (filled bars) of exposure to thymol. (B) and (C) correspond to PI and LDH assays: the bars represent the percentage of PI fluorescence or LDH released into the incubation medium with respect to 100% cell death.

demonstrated by its ability to increase both $^{36}Cl^-$ influx and [^{3}H]flunitrazepam binding, as well as by its tendency to increase [^{3}H]muscimol binding. The inhibition of these effects by $GABA_A$ receptor antagonists further support this finding. The fact that thymol, at concentrations that elicited a chloride influx, did not inhibit [^{3}H]muscimol binding precludes a direct agonist effect at the GABA recognition site. Similarly, increased [^{3}H]flunitrazepam binding by thymol precludes a benzodiazepine-like interaction at the $GABA_A$ receptor. This is consistent with the results published by Priestley et al. (2003), who found that benzodiazepine antagonists did not reduce thymol's effects on GABA-induced currents.

The concentrations of thymol that produced a direct effect on the opening of the chloride channel were similar to those that increased [^{3}H]muscimol and [^{3}H]flunitrazepam binding, suggesting that thymol may produce a conformational change of the $GABA_A$ receptor, thereby leading to direct gating of the associated chloride channel. This is consistent with data reported by Sánchez et al. (2004), who found that thymol is incorporated by membranes and allosterically modulates specific $GABA_A$ receptor recognition sites without having any effect on non-specific binding. In addition to this direct effect, thymol potentiated the chloride flux induced by GABA and this potentiation

occurred at concentrations much lower than those required for direct action. Again, this behaviour is consistent with that produced by known positive $GABA_A$ receptor modulators, including barbiturates, anaesthetics, neurosteroids, and δ-hexachlorocyclohexane (an isomer of the environmental contaminant lindane) (Pomés et al., 1994; Vale et al., 1997; Hawkinson et al., 1998; Costa, 1998; Belelli et al., 1999; Priestley et al., 2003). What all these compounds share in common is that they are central depressant agents. Accordingly, in addition to its chemical stabilising effect in halothane preparations (MacPherson, 2001), thymol might also potentiate the anaesthetic effect of these preparations.

Krasowski et al. (2002) described 4D-QSAR models for three different screens of biological activity in 27 propofol-analogue phenolic compounds. There were striking similarities between their results for the anaesthetic activity model and those relative to the direct and potentiating effects on the $GABA_A$ receptor. This led the authors to propose the $GABA_A$ receptor as the likely site of propofol's anaesthetic action. The authors then identified three key ligand–receptor interactions that determine the activity of these compounds. The most important interaction involves the hydroxyl group present in the ligands, which forms a hydrogen bond with an acceptor group on the $GABA_A$ receptor. The other important features identified were two hydrophobic interaction sites close to the two *ortho* positions of the phenolic system. These three key interactions are integral to the qualitative pharmacophoric model shown in Fig. 7, which also includes the requirement of an aromatic ring with a rather co-planar orientation relative to the HBDc, HAL1 and HAL2 features. The study by Krasowski et al. (2002) similarly suggested the existence of other potential interaction sites close to the 3, 4 and/or 5 positions of the phenolic system. We included one of these features in our pharmacophoric hypothesis, shown in Fig. 7 as a third hydrophobic aliphatic group (HAL3), to verify whether this could serve as a favourable interaction site for the thymol 5-methyl group. The best alignment for this compound, however, did not involve such an interaction. Instead, like propofol, thymol fits the 5-methyl substituent in the HAL1 region without greatly perturbing the fit of the hydroxyl group into the HBD site. This subtle difference in the way of alignment of thymol substituent in position 5 and propofol substituent in position 6 could hardly be detected by looking at the structure of the compounds. This results in an enhanced pharmacophoric model of that proposed by Krasowski et al. (2002), which might account for the better predictability of thymol activity. Menthol and cymene are sterically and hydrophobically similar to thymol and propofol, as shown by their molecular volume and logP. However, consistent with Krasowski et al. (2002), our results demonstrate that these properties do not determine their $GABA_A$-related activity. Instead, cymene's lack of that key hydrogen bond interacting group correlates with the observed lack of activity. Furthermore, while menthol contains hydroxyl and aliphatic substituents suitable for establishing certain favourable interactions with the $GABA_A$ receptor, the changes in the geometry of these substituents and, most importantly, in the electronic properties of the molecule relative to thymol due to the absence of an aromatic ring, presumably perturb, or even abolish, efficient interaction with the receptor. Accordingly, thymol, but not cymene or menthol, increased [^{3}H]flunitrazepam binding in cultured neurons, despite the fact that all of them interact with lipid membranes (Ultee et al., 2002; Turina and Perillo, 2003; Sánchez et al., 2004). It should be remembered that this $GABA_A$ receptor specificity for thymol vs. menthol was not found for voltage-dependent neuronal Na^+ channels where both compounds were found to be active, thymol proving more potent than menthol (Haeseler et al., 2002). Both effects, the increase of GABA function and the blocking of voltage-gated sodium channels by thymol, might confer to this molecule interesting sedative, anaesthetic and anti-epileptic pharmacological properties.

The pharmacophore model proposed here, to which thymol but not menthol or cymene fits, supports a similar interaction of thymol, propofol, and other alkyl phenol compounds with the $GABA_A$ receptor. This assumption is consistent with findings published by Mohammadi et al. (2001) who reported the direct activation of chloride inward currents through $GABA_A$ receptors by thymol, propofol and phenol derivatives. On the other hand, Priestley et al. (2003) suggested that thymol may interact with the $GABA_A$ receptor at a site different to that of propofol, based on a small increase of the propofol activity by thymol. However, the concentration of propofol used did not saturate the $GABA_A$ receptor in similar preparations (Mohammadi et al., 2001; Bali and Akabas, 2004). Priestley et al. (2003) also provide experimental evidence that thymol and pentobarbital do not compete for the same binding site. In agreement with this, although there are a number of possible ways in which barbiturates, such as pentobarbital or phenobarbital, can be aligned to the structures of thymol or propofol, the structural difference between those compounds precludes a good fit with the proposed pharmacophoric model. At present it remains unclear which amino acids and receptor subunits are specifically involved in the binding domains of different classes of $GABA_A$ receptor positive allosteric agonists. Recently, such amino acid residues as serine, asparagine, glycine and methionine, placed near the extracellular end of the receptor's membrane-spanning domain, were described as being at or near the binding sites of barbiturates, anaesthetics and δ-hexachlorocyclohexane (Belelli et al., 1999; Krasowski

et al., 2001; Chang et al., 2003; Pau et al., 2003; Siegwart et al., 2003; Bali and Akabas, 2004; Hall et al., 2004; Olsen et al., 2004; Schofield and Harrison, 2005). The functional groups on certain amino acid side chains (i.e. Ser and Asn) could potentially act as the hydrogen bond acceptors responsible for the essential interaction with thymol and other related phenolic compounds. On the other hand, mutagenic evidence has been reported, pointing out that substitution of Gly219 or Met286 by bulkier amino acids (i.e. tryptophan) reduce the $GABA_A$ receptor modulation exerted by barbiturates and propofol (Chang et al., 2003; Krasowski et al., 2001). The non-polar nature of these residues is compatible with the existence of three different hydrophobic regions in the proposed pharmacophoric model, therefore under this assumption it could be expected that their replacement would similarly affect the modulation capacity exhibited by thymol. Finally, the requirement of an aromatic ring suggests the presence of aromatic residues in the $GABA_A$ receptor-binding site, with which it could establish a stacking interaction; however, it could also arise from a better geometric orientation of the substituents or other electronic effects.

It should be recalled that the thymol concentrations necessary for activity at the $GABA_A$ receptor in the present study (EC_{50} 12 μM for the GABA-potentiating effect and EC_{50} 135 μM for the direct action) are lower that those found active against gram-positive and gram-negative bacteria (minimal bactericide concentration ≅ 3 mM; Shapiro and Guggenheim, 1995), as well as those described for growth inhibition of *Bacillus cereus* (0.75 mM; Ultee et al., 2002). Membrane perturbation and permeabilisation are the main modes of action for the reported antimicrobial effect of thymol, producing a rapid leakage of cytoplasmic components and a reduction of intracellular ATP content in bacteria (Shapiro and Guggenheim, 1995). In this study, thymol was not found to produce any neuronal damage, either after short (30 min) or long exposures times (up to 24 h). The lack of significant LDH leakage and PI incorporation excludes any neurotoxic effects of thymol via membrane disruption. In addition, we also provide evidence that thymol does not precipitate loss of cell viability, as demonstrated by the MTT assay. The results of this assay, based on the capability of mitochondria to reduce MTT, are also indicative of a lack of mitochondrial failure. It should be taken into account that under certain circumstances, changes in mitochondrial respiration are observed in the absence of cell death (Bolaños et al., 1994).

In summary, we have found that thymol was able to enhance GABA action at concentrations lower than those exhibiting direct activity in the absence of GABA. This effect was inhibited by competitive and non-competitive $GABA_A$ receptor antagonists, suggesting a direct interaction with the $GABA_A$ receptor. This finding, in addition to the fact that thymol increased [^{3}H]flunitrazepam binding and to a lesser extent, [^{3}H]muscimol binding, confirms that thymol is a positive allosteric modulator of the $GABA_A$ receptor. Additionally, we describe a pharmacophoric model containing three main molecular features essential for the interaction of thymol-related molecules with the $GABA_A$ receptor; namely, a hydrogen bond donor group (i.e. the phenolic hydroxyl), an aromatic ring, and two aliphatic substituents close to the hydrogen bond donor. This allosteric activity would not involve non-specific interactions between thymol and membrane components. These results reveal the exceptionally complex system of allosteric modulation involved in this ligand-gated channel representing the $GABA_A$ receptor. Moreover, this study makes clear the enormous challenges involved in assigning eventual recognition sites to many compounds known by their positive activity on the $GABA_A$ receptor.

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