GABA released from cultured cortical neurons influences the modulation of *t*-[³⁵S]butylbicyclophosphorothionate binding at the GABA_A receptor. Effects of thymol.

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Abstract

Thymol is a monoterpene that specifically interacts with synaptic neural functions in neuronal GABA-operated Cl^- channels. Here we explore the effects of thymol, and propofol as positive control, on t - $[^{35}\text{S}]$ butylbicyclophosphorothionate ($[^{35}\text{S}]$ TBPS) binding in primary cultures of cortical neurons. The study includes a meaningful analysis of the effect of various exposure buffers, and their correlation with GABA released from cells, chloride influx through the GABA_A receptor and GABA transporter activity. Cell viability was also determined. Thymol and propofol inhibited the binding of $[^{35}\text{S}]$ TBPS to cells exposed to Tris-citrate-NaCl buffer whereas a biphasic effect was observed in HEPES solution. The different effects of the two buffers analysed are due to the higher capacity of Tris-citrate-NaCl buffer to induce the release of endogenous GABA facilitating the binding of $[^{35}\text{S}]$ TBPS to its recognition site at the GABA_A receptor. Released GABA in the presence of this buffer was inhibited by the neuronal GABA transporter inhibitor SKF 100330-A. Tris-citrate-NaCl buffer also induced a chloride influx, which was reverted by picrotoxinin. TBPS binding in living cells is facilitated by GABA released from the cells, which in turn activates basal GABA_A receptor activity. The results deepen on the allosteric mechanism of thymol as positive modulator of the GABA_A receptor. Furthermore, we corroborate $[^{35}\text{S}]$ TBPS binding as an important test to verify the capacity of drugs to act on and recognize a site at the GABA_A receptor.

Keywords

Thymol; GABA_A receptor; $[^{35}\text{S}]$ TBPS binding; GABA release; Chloride uptake; Buffer effect, Neuronal GABA transporter.

1. Introduction

Thymol (2-isopropyl-5-methylphenol) is a monoterpene that is found as a component of many essential oils (Gomes-Carneiro et al., 1998). It is widely used in dental practice and in anaesthetic halothane preparations due to its anti-microbial and antioxidant properties (Shapiro and Guggenheim, 1995; MacPherson, 2001; Burt, 2004). Interest in isolated monoterpenes has been growing in recent years because of their possible pharmaceutical utility. In particular, this compound specifically interacts with synaptic neural functions on neuronal Na^+ and GABA-operated Cl^- channels (Mohammadi et al., 2001; Haeseler et al., 2002; Priestley et al., 2003; García et al., 2006) as well as with nociception-sensitive receptor channels (Lee et al., 2008). Its capacity to increase [^3H]flunitrazepam binding to its specific site in the GABA_A receptor by using synaptosomal membranes or cultured cortical neurons has been described (Sánchez et al., 2004; García et al., 2006). We also demonstrated the ability of thymol to both enhance GABA-induced chloride influx and open the chloride channel in the GABA_A receptor in the absence of GABA. The fact that this monoterpene did not inhibit [^3H]muscimol binding (it even showed a slight increase) and increased [^3H]flunitrazepam binding argues strongly for its recognition site in the receptor being different from the one for GABA and benzodiazepines. Its structural properties, which resemble those of propofol suggest a close or common recognition site in the receptor (García et al., 2006).

Positive allosteric modulators of the GABA_A receptor increase both [^3H]muscimol and [^3H]flunitrazepam binding and modify t-[^{35}S]butylbicyclophosphorothionate ([^{35}S]TBPS) binding (Vale et al., 1997; Hawkinson et al., 1998; Zeng et al., 2005; Suñol et al., 2006). [^{35}S]TBPS recognizes a site in the GABA_A receptor that binds the convulsant picrotoxin; and it is well established that positive allosteric modulators that activate the GABA_A receptor Cl^- channel also allosterically modify this site (Ghiani, 1996; Kalueff, 2007). In this work we explore the effects of thymol, with propofol as positive control, on [^{35}S]TBPS binding in primary cultures of cortical neurons in order to gain a deeper insight into its mechanism of action. TBPS binding is a powerful tool widely used to study the interaction of a drug with the GABA_A receptor. The study also includes an analysis of the effect of various exposure buffers on this binding assay in living cultured cells and its correlation with GABA released from intact cells and chloride influx through the GABA_A receptor.

2. Methods

2.1. Materials

Pregnant NMRI mice (16th day of gestation) were obtained from Charles River, Iffa Credo (St. Germain-sur-l'Arbreste, France). Plastic multi-well culture 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). [³⁵S]TBPS (specific activity ranged from 3.4 – 6.7 TBq/mmol during the entire experimental period) and ³⁶Cl⁻ (> 111 MBq/g) were procured from Perkin Elmer (Boston, MA, USA) and Amersham Life Sciences (Buckinghamshire, UK), respectively. Liquid scintillation cocktail Optiphase Hisafe 2 was obtained from Wallace Oy (Turku, Finland). Thymol, 2,6-diisopropylphenol (propofol), GABA, picrotoxinin (PTX), 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazam (MTT), trypsin, soybean trypsin inhibitor, DNase, amino acids and poly-L-lysine were obtained from Sigma Chemical Co. (St Louis, MO, USA). All the other chemicals were of analytical grade. Propidium iodide and trypan blue (0.4%) were from Molecular Probes and Gibco, respectively (Invitrogen, Spain). N-(4,4-diphenyl-3-butenyl)-guvacine (SKF-100330-A) was a gift from Smith Kline & French. Thymol and propofol 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 mice foetuses, following 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 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 modified DMEM solution (31 mM glucose and 0.2 mM glutamine), supplemented with insulin, penicillin and 10% foetal calf serum. The cell suspension (1.6x10⁶ cells/ml) was seeded in 24x-multi-well plates, pre-coated with poly-L-lysine, and incubated for 6-9 days in a humidified 5% CO₂ / 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. [³⁵S]TBPS binding

The binding assay was performed according to Pomés et al. (1993) and Vale et al. (1997). Briefly, the cells were washed 3 times with 0.5 ml of HEPES buffered saline solution (HBSS: 136 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl₂, 1.4 mM MgCl, 1 mM NaH₂PO₄, 10 mM HEPES and 9 mM glucose, adjusted at pH 7.4), pre-warmed at 37°C. The incubation system contained 1.5-3 nM [³⁵S]TBPS and compound solution in either a Tris-citrate buffered saline solution (TCBSS: 50 mM Tris-citrate and 200 mM NaCl, adjusted at pH 7.4) or HBSS in a final volume of 0.25 ml. After 30 min at 25°C the solution was removed and rinsed 3 times with 0.5 ml of cold HBSS solution. Non-specific binding was measured in the presence of 200 µM PTX. Cells were collected in 0.25 ml 0.2 M NaOH overnight and their radioactivity was determined by liquid scintillation counting (Optiphase 'Hisafe' 2). No differences were observed between protein content in the cultures exposed to TCBSS and those exposed to HBSS, measured by the Bradford method. Therefore, binding data were determined as dpm/culture. Specific [³⁵S]TBPS binding values along this study were 3.2 ± 0.9 pmol/g protein and 28.1 ± 3.0 pmol/g protein when using HBSS or TCBSS buffers, respectively.

2.4. Chloride influx

Chloride influx was determined as ³⁶Cl⁻ uptake in intact cell cultures (Vale et al., 2003; García et al., 2006). Briefly, culture medium was replaced by a pre-warmed Earle's balanced salt solution (EBSS: 116 mM NaCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄, 1 mM NaH₂PO₄, 15.2 mM NaHCO₃ and 5.5 mM glucose, adjusted at pH 7.4) and cell cultures were incubated in a humidified 5% CO₂ / 95% air atmosphere at 36.8°C. After 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 pre-incubated for 30 min at 25°C with HBSS or TCBSS buffer, according to the experiment. After this, the cells

were incubated for 30 s with 225 μ L buffer solution containing $^{36}\text{Cl}^-$ (0.4 $\mu\text{Ci/ml}$). Picrotoxinin was added when indicated. After removing the $^{36}\text{Cl}^-$ solution, each well was immediately rinsed four times with 1.5 ml cold HBSS solution. Cells were lysed by water-induced hyposmotic shock, and radioactivity was determined by liquid scintillation counting, as described above.

2.5. GABA determination

GABA concentration in the exposure medium was determined by high-performance liquid chromatography (Babot et al., 2005) after 30 min incubation at 25°C with HBSS or TCBSS (binding incubation conditions). GABA content was calculated by an external standard method with GABA dissolved in HBSS and TCBSS, since GABA fluorescence detection was different in the two buffer solutions.

2.6. Cell viability

MTT assay.- Following 7 days in vitro, the cells were exposed to HBSS or TCBSS for 30 min at 25°C, and their viability was determined. The assay was conducted essentially according to the method described by García et al. (2006). Following each exposure period, the cells were washed three times with 0.5 ml of pre-warmed HBSS solution (37°C) and then incubated for 20 min at 37°C with 0.25 ml MTT reagent solution (0.25 mg/ml). After removal of the MTT solution, 0.25 ml/well of solubilization 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 by a spectrophotometer plate reader (iEMS Reader MF; Labsystems, Helsinki, Finland).

Propidium iodide and trypan blue assay.- Cells were exposed to HBSS, TCBSS, HBSS + 0.3% Triton X-100 or TCBSS + 0.3% Triton X-100 for 30 min at 25°C in the presence of propidium iodide (PI, 7.4 $\mu\text{g/ml}$). In parallel cultures, trypan blue was added at the end of the incubation period without adding propidium iodide.

Microphotographs were taken in a fluorescence microscope equipped with phase contrast (Olympus YX70). Digital images of incorporated propidium iodide and trypan blue were captured using a ColorView camera.

2.7. Data analysis

Results are mean $\pm$ S.E.M., of 3 experiments performed in different culture preparations, except when otherwise stated. Each experiment was performed in

triplicate. Sigmoid curves were fitted to concentration-response data. A minimum of 6 concentrations, all determined in triplicate, were used for each curve. Student's *t*-test and one-way analysis of variance followed by Bonferroni's test were used for comparing different experimental conditions. Fitting and statistical analyses used GraphPad Prism (GraphPad Software Inc, San Diego, CA, USA).

3. Results

Specific [35 S]TBPS binding in basal conditions, determined as the difference between total binding and binding in the presence of PTX, was significantly higher in cultures incubated with TCBSS than in those with HBSS buffer (664 ± 47 vs. 235 ± 47 dpm/culture, respectively, $P = 0.003$, $n = 3$). This difference was because total binding was enhanced in TCBSS, whereas non-specific binding in the presence of PTX was similar in both buffers (Fig. 1A). GABA produced a biphasic effect on the specific [35 S]TBPS binding in HBSS, significantly increasing TBPS binding at $10 \mu\text{M}$ and decreasing thereafter (Fig. 1B), whereas only a decreasing effect was observed in TCBSS (Fig. 1B).

Fig. 2A shows the effect of thymol on specific [35 S]TBPS binding in intact neocortical neurons. An inhibitory effect was observed when [35 S]TBPS binding was performed in TCBSS, whereas a biphasic effect was observed in HBSS buffer. The stimulatory effect in HBSS buffer was observed up to $100 \mu\text{M}$ thymol; but $200 \mu\text{M}$ thymol completely inhibited [35 S]TBPS binding, as it did in TCBSS. Propofol inhibited [35 S]TBPS binding in TCBSS, whereas the effect in HBSS was stimulatory up to $30 \mu\text{M}$ and then inhibitory up to $100 \mu\text{M}$ (Fig. 2B). IC_{50} values for the inhibitory effects of thymol and propofol in TCBSS were $25.0 \pm 8.5 \mu\text{M}$ ($n = 3$) and $6.3 \pm 1.2 \mu\text{M}$ ($n = 2$), respectively (mean $\pm$ S.D.).

We found statistically significant differences in the extracellular GABA concentrations after incubating the cells with HBSS or TCBSS. While incubation with HBSS did not induce substantial GABA liberation, incubation with TCBSS significantly increased its free concentration (Figure 4).

A leakage of intracellular GABA due to a harmful state of the cells can be ruled out because the viability assays did not show differences between cells incubated with HBSS or TCBSS. Cells with an intact membrane are able to exclude the vital dye trypan blue, while cells with damaged membrane take up the dye. Fig. 3A shows that cells incubated with TCBSS did not take up trypan blue, whereas damaged cells exposed to TCBSS plus 0.3% Triton X100 did (Fig. 3B). Similarly, propidium iodide, which is taken up by nonviable cells and binds to DNA, only labeled Triton X100-treated cells (Fig. 3D) but not those exposed to TCBSS (Fig. 3C). Cells incubated with HBSS also excluded trypan blue and did not incorporate propidium iodide (data not shown).

Furthermore, cell viability values, measured as MTT reduction, were $98 \pm 1\%$ ($n = 2$) in TCBSS-exposed cultures, when compared with HBSS-exposed cultures.

We wondered whether the increase in extracellular GABA found in the cells incubated with TCBSS could come through reversion of the GABA transport. Fig. 4 shows that the neuronal GABA transporter inhibitor SKF-100330-A (Risso et al., 1996; Clausen et al., 2006) significantly reduced the release of GABA induced by TCBSS.

We determined whether the presence of GABA released in the extra-cellular medium in the cells incubated with TCBSS activated the GABA_A receptor and induced an influx of Cl⁻. Fig. 5 shows the uptake of ³⁶Cl⁻ measured in cells exposed to HBSS and TCBSS in the absence or presence of 200 μM PTX. Uptake of ³⁶Cl⁻ was significantly higher in TCBSS-exposed cultures than in HBSS-exposed ones. Furthermore, picrotoxinin significantly inhibited the influx of ³⁶Cl⁻ in cultures exposed to TCBSS (Fig. 5).

Bicuculline also reduced ³⁶Cl⁻ uptake (data not shown). However, exposure to HBSS did not show any such difference in the presence or absence of PTX.

4. Discussion

The GABA_A receptor is the main site of action of various GABA agonists and positive allosteric modulators, such as muscimol, benzodiazepines, barbiturates and steroids, which act as anxiolytic, sedative-hypnotic, anticonvulsant or anaesthetic drugs (MacDonald and Olsen, 1994; Whiting, 2003). This receptor also has recognition sites for GABA antagonists (for review, see Kalueff, 2007), such as bicuculline, PTX, neurotoxic pesticides or TBPS, which can be displaced by the positive drugs mentioned above (Hawkinson et al., 1998; Zeng et al., 2005). In this study, we describe thymol's inhibition of [³⁵S]TBPS binding, which together with the increase of [³H]flunitrazepam binding (García et al. 2006), confirms its activity as an allosteric positive modulator of the GABA_A receptor.

We previously demonstrated that [³⁵S]TBPS binding parameters and its inhibition by convulsant agents are equivalent in intact cultured cells and in cell membrane preparations when binding is performed in TCBSS (Pomés et al., 1993). In addition, the EC₅₀ value for propofol against [³⁵S]TBPS binding in TCBSS found in this study agrees with the value reported in EDTA-water dialyzed brain membranes (Squires and Saederup, 2000). The influence of buffer composition and washing paradigms on [³⁵S]TBPS-binding parameters in brain membrane preparation, known for a long time, results in different affinities of TBPS for the binding sites and in different profiles of binding activation/inhibition for compounds that interact with the GABA_A receptor (Squires et al., 1983; Supavilai and Karobath, 1984; Ghiani et al., 1996). In this study, we provide evidence that performing the [³⁵S]TBPS binding assay in intact cultured cells with a physiological buffer (HBSS) decreases specific binding. Furthermore, thymol and propofol had a bell-shaped effect in HBSS, similar to that described for full and allosteric agonists in recombinant GABA_A receptors (Im et al., 1994). It is noteworthy that exogenous added GABA increased HBSS-performed [³⁵S]TBPS binding up to a value similar to that obtained using TCBSS in the absence of added GABA. Some authors attribute the differences in [³⁵S]TBPS binding between different membrane preparations to their GABA concentrations (Squires et al., 1983; Ghiani et al., 1996). Ghiani et al. (1996) reported that propofol and loreclezole had a biphasic effect when [³⁵S]TBPS binding was performed on washed brain membrane preparations, whereas only the inhibitory effect was observed on non-washed membranes. Others explained the biphasic effect of the GABA agonist on [³⁵S]TBPS

binding according to its occupation of the high-affinity (enhancing effect) or low-affinity (inhibitory effect) sites (Supavilai and Karobath, 1984; Im et al., 1994), with the latter effect associated with channel openings. Thus, TBPS would need a minimal level of modulation of the GABA receptor in order to bind it. It has been demonstrated that similar GABA concentrations to the ones determined in this study after TCBSS incubation induced increments in flunitrazepam binding (~ 30% with 1 μ M GABA; Vale et al., 1997) and in chloride uptake (~ 20 % with 3 μ M GABA; García et al., 2006). The decrease of chloride influx by PTX and bicuculline in the presence of TCBSS supports the idea that the increment induced by Tris-citrate-NaCl on [35 S]TBPS binding is caused by a GABAergic modulation.

GABA_A receptors have different levels of spontaneous activity that depend on the subunits expressed (McKernan and Whiting, 1996; Wooltorton et al., 1997). This spontaneous activity is inhibited by GABA antagonists such as PTX (for review, see Mortensen et al. 2003). Such behaviour could explain the fact that PTX diminished the $^{36}\text{Cl}^-$ influx of cells incubated with TCBSS buffer. However, since this latter experimental condition induced GABA release and since there are no data about buffer effect on this spontaneous activity (HBSS did not show this performance), we assume that the PTX inhibitory effect found in this work is due to the moderate quantity of released GABA. It has also been reported that the protonation/desprotonation of amino acid residues of GABA_A receptor are responsible for pH modulation of the receptor (Huang and Narahashi, 1996; Uusi-Oukari et al., 2004). The incubation process (30 min at 25°C) did not produce changes in the pH values of the two buffers (data not shown), ruling out any pH effect. Thus, the higher specific TBPS binding obtained with TCBSS might be attributable to a higher GABA release. An important proportion of the cells in primary cultures of cortical neurons are GABAergic neurons (around 40%) and these cells express neuronal GABA transporters (Vale et al., 1999; Suñol et al., 2008). We propose that the GABA efflux process when using TCBSS is mediated by reversal of the neuronal GABA transporter, since a significant reduction of extracellular GABA was attained in the presence of the neuronal GABA transport inhibitor SKF-100330-A. As far as we know there are no reports of the interaction of Tris buffers with the GABA transport, but Li et al. (2002) have reported that Tris allosterically interact with the substrate site of the dopamine transporter, which shares with the GABA transporters the fact that both belong to the SLC6 gene family (Gether et al., 2006). Furthermore, lead inhibited the uptake of GABA and potentiated the spontaneous release of GABA from

motoneurons when Tris-HCl but not phosphate or carbonate buffers were used (Spence et al., 1985). The observed inhibition of the TCBSS-induced efflux of GABA by SKF-100330-A, found in this work, suggest a direct effect of Tris on the neuronal GABA transporter. It should be noted that the tonic conductance of the GABA_A receptor is sensitive to factors that regulate the amount of GABA surrounding the neurons, like neuronal and glial GABA transport (Glykys and Mody, 2007).

Therefore, the use of TCBSS may make TBPS binding sites more accessible in the membrane. In the physiologic buffer HBSS, opening the GABA_A receptor-operated Cl⁻ channel by GABA also made TBPS binding sites accessible to the radio-labelled agonist. In this study we show that GABA, thymol and propofol induced first an increase of [³⁵S]TBPS binding in HBSS buffer, in consonance with activation of the Cl⁻ channel (García et al., 2006). Once TBPS binding sites are fully accessible, then an inhibitory interaction of the compound/test agent with the TBPS sites occurs, producing more stepped curves.

Finally, the ability of thymol to reduce [³⁵S]TBPS binding in TCBSS, as described in this paper, confirms thymol as a positive allosteric modulator of the GABA_A receptor with a specific recognition site, which might be shared with propofol as previously suggested by the described pharmacophoric model (García et al., 2006). The pharmacophoric hypothesis includes two hydrophobic aliphatic features, one hydrogen bond donor group, an exclusion volume, and an aromatic ring, characteristics found in both molecules. The displacement potency found for thymol was 5 times lower than that determined for propofol, in agreement with their EC₅₀ values for potentiating the Cl⁻ influx induced by GABA (García et al., 2006). Table 1 summarizes the parameters of the activity of thymol and propofol on the GABA_A receptor. In this study, we show that thymol inhibits the binding of [³⁵S]TBPS in TCBSS buffer with an IC₅₀ value (25 µM; Fig. 2A), which is close to the EC₅₀ value obtained for the effect on GABA potentiation of the GABA_A receptor-operated Cl⁻ channel (12 µM; García et al., 2006). Similarly, the concentration of propofol that inhibited [³⁵S]TBPS binding in TCBSS (IC₅₀ = 6.3 µM; Fig. 2B) corroborates reported data on inhibition of [³⁵S]TBPS binding in rat brain membranes (Ghiani et al., 1996) and on potentiation of the GABA_A receptor-operated Cl⁻ channel (Bali and Akabas, 2004). The direct activation of the GABA_A receptor by thymol and propofol (EC₅₀ values ~ 130 µM and 10-23 µM, respectively; Mohammadi et al., 2001; Bali and Akabas, 2004; García et al., 2006) correlates much better with the

potentiation of [^{35}S]TBPS binding in HBSS buffer. Again, a similar relationship exists between the potentiation of [^3H]flunitrazepam binding and the potentiation of [^{35}S]TBPS binding in HBSS buffer by thymol and propofol (Ghiani et al., 1996; García et al., 2006).

In conclusion, [^{35}S]TBPS binding in living cells is an important test for verifying the capacity of drugs like thymol to act on and recognize a specific site in the GABA_A receptor but, the interpretation of the results must take into consideration the methodology employed. Depending on the buffer used in the living cell assay, thymol had only inhibitory or both inhibitory and potentiating effects on TBPS binding. Our interpretation is that thymol, by increasing GABA_A receptor function (García et al., 2006) allows the full binding of [^{35}S]TBPS binding to the receptor, and this binding site is later inhibited by thymol. If binding sites are fully accessible for TBPS, as it is the situation when TCBSS is used in the living binding assay, thymol immediately inhibits TBPS binding. Taken all together, the results of this and previous works (García et al., 2006) display a clear picture of thymol acting as positive allosteric modulator of the GABA_A receptor.

Acknowledgments

This study was supported by grants PI061212 and 2005-SGR-00826 (Ministry of Health and Generalitat de Catalunya, respectively, Spain), SECYT-UNC (Argentina) and CAEN-ISN. DAG is a member of CONICET (Argentina) and was recipient of a post-doctoral fellowship from the Fundación Carolina (Spain). IV was recipient of a CSIC Fellowship in the I3P program, co-financed with European Social Funds.

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Figure Legends

Figure 1. [^{35}S]TBPS binding in intact cultured cortical neurons. A) [^{35}S]TBPS (1.5-4 nM) was incubated with TCBSS (white bars) or HBSS buffer (diagonal bars) for 30 minutes at 25°C, alone or in the presence of 200 μM PTX. B) Effect of GABA on specific [^{35}S]TBPS binding in TCBSS buffer (squares and solid line) or HBSS buffer (triangles and dashed line). B: basal specific binding in the absence of added GABA. *: $P < 0.001$ with respect to basal binding. The data correspond to mean $\pm$ S.E.M. of 3 independent experiments determined in triplicate.

Figure 2. Concentration-response curves for the effects of thymol (A) and propofol (B) on specific [^{35}S]TBPS binding in intact cultured cortical neurons. [^{35}S]TBPS binding was performed in TCBSS (squares and solid line) or in HBSS (triangles and dashed line). The data correspond to 2-3 independent experiments done by triplicate for propofol or thymol, respectively. A minimum of 6 concentrations, all determined in triplicate, were used for each curve.

Figure 3. Microphotographs of cultured cortical neurons incubated with TCBSS (A, C) or TCBSS plus 0.3% Triton X-100 (B, D). A and B are phase contrast microphotographs of cells after trypan blue exposure. C and D are fluorescence microphotographs of cells labelled with propidium iodide. Bar size: 20 μm .

Figure 4. Extracellular GABA in cultured cortical neurons incubated with HBSS or TBSS, in the absence (white bars) or in the presence of 100 μM SKF-100330-A (black bars). * $P < 0.01$ after two way ANOVA analysis and Bonferroni post test. Results are mean $\pm$ S.D. of two experiments, each performed in triplicate.

Figure 5. Influx of $^{36}\text{Cl}^-$ is potentiated in cultures incubated with Tris-NaCl buffer. Cultures were exposed for 30 minutes at 25°C to TCBSS buffer (white bars) or HBSS buffer (diagonal bars); and then to a solution containing 0.4 $\mu\text{Ci/ml}$ $^{36}\text{Cl}^-$ in the corresponding buffers, in the absence or presence of 200 μM PTX. Values were normalized to $^{36}\text{Cl}^-$ uptake in the presence of PTX (considered as basal uptake) and correspond to mean $\pm$ S.E.M. of 3 experiments. *: $P < 0.05$ with respect to basal

binding.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Table 1. Parameters of thymol and propofol on the GABA_A receptor.

Compound	TBPS binding		Cl ⁻ influx		Flunitrazepam binding
	Inhibition (IC ₅₀ , μM)	Potentiation	Potentiation (EC ₅₀ , μM)	Activation (EC ₅₀ , μM)	Potentiation (EC ₅₀ , μM)
Thymol	<i>In TCBSS</i> 25	<i>In TCBSS</i> None	12 ^a	135 ^a	131 ^a
	<i>In HBSS</i> 100<IC ₅₀ <300	<i>In HBSS</i> Max at ~100 μM			
Propofol <i>In TCBSS</i> <i>In HBSS</i>	<i>In TCBSS</i> 5	<i>In TCBSS</i> None	2; 3 ^{b,d}	10; 23 ^{c,d}	~ 20 ^e
	<i>In HBSS</i> 10<IC ₅₀ <100	<i>In HBSS</i> Max at 10 μM			

The values correspond to the concentrations of thymol or propofol that induce different effects on the GABA_A receptor, taken either from this study or from cited references. In TBPS binding, the exposure buffer used for the parameter determination is indicated. Letters *a* to *e* correspond to values taken from references [8,40,6,41,42], respectively.