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GABA released from cultured cortical neurons influences the modulation of *t*-[^{35}S]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}S]butylbicyclophosphorothionate ([^{35}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}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}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}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.

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 [^{3}H]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 [^{3}H]muscimol binding (it even showed a slight increase) and increased [^{3}H]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 [^{3}H]muscimol and [^{3}H]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 et al., 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

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binding assay in living cultured cells and its correlation with GABA released from intact cells and chloride influx through the $GABA_A$ receptor.

2. Materials and 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). [^{35}S]TBPS (specific activity ranged from 3.4–6.7 TBq/mmol during the entire experimental period) and $^{36}Cl^-$ (>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 (1.6×10^6 cells/ml) was seeded in 24×-multi-well 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. [^{35}S]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 $CaCl_2$, 1.4 mM MgCl, 1 mM NaH_2PO_4, 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 [^{35}S]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 [^{35}S]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 $^{36}Cl^-$ 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 $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 15.2 mM $NaHCO_3$ and 5.5 mM glucose, adjusted at pH 7.4) and cell cultures were incubated in a humidified 5% CO_2/95% 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 μL buffer solution containing $^{36}Cl^-$ (14,800 Bq/ml). Picrotoxinin was added when indicated. After removing the $^{36}Cl^-$ 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

2.6.1. 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).

2.6.2. 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 μg/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±S.E.M., of 3 experiments performed in different culture preparations, except when otherwise stated. Each experiment