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Structural requirements of phenol derivatives for direct activation of chloride currents via $GABA_A$ receptors

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Abstract

Propofol directly activates γ-aminobutyric acid ($GABA_A$) receptors in the absence of the natural agonist. This mechanism is supposed to contribute to its sedative–hypnotic actions. We studied the effects of seven structurally related phenol derivatives on chloride inward currents via rat $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, heterologously expressed in HEK 293 cells in order to find structural determinants for this direct agonistic action. Only compounds with the phenolic hydroxyl attached directly to the benzene ring and with aliphatic substituents in *ortho* position to the phenolic hydroxyl activated chloride currents in the absence of GABA. Concentrations required for half-maximum effect were 980 μM for 2-methylphenol, 230 μM for 2,6-dimethylphenol, 200 μM for thymol, and 23 μM for propofol. Drug-induced chloride currents showed no desensitisation during the 2-s application. These results show that the position of the aliphatic substituents with respect to the phenolic hydroxyl group is the crucial structural feature for direct $GABA_A$ activation by phenol derivatives. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: $GABA_A$ receptor; Propofol; Phenol derivative

1. Introduction

A substituted benzene ring is the common structural characteristic of local anaesthetics (Ehring et al., 1988; Glowka et al., 1991) and the general anaesthetic propofol (2,6-diisopropylphenol). While propofol (Rehberg and Duch, 1999; Saint, 1998; Saint and Tang, 1998; Veintemilla et al., 1992), as well as other phenol derivatives (Haeseler et al., 1999; Haeseler et al., 2000) and local anaesthetics both block voltage-operated Na^+ channels, they differ profoundly in their effects on the major receptor for inhibitory neurotransmission in the mammalian brain, the γ-aminobutyric acid ($GABA_A$)-receptor. Local anaesthetics like lidocaine, bupivacaine, procaine, benzocaine and cocaine have been shown to inhibit GABA-induced currents, a mechanism that might underlie central nervous toxicity of local anaesthetics (Hara et al., 1995; Sugimoto et al., 2000; Ye et al., 1997). In contrast, propofol not only potentiates GABA-evoked Cl^- currents, but also activates Cl^- currents through GABA receptors in the absence of GABA (Belelli et al., 1996; Hales and Lambert, 1991; Sanna et al., 1995a,b, 1999). Some experimental evidence suggests that this direct receptor activation in the absence of the agonist might determine the sedative–hypnotic as opposed to the anticonvulsant actions of propofol (Sanna et al., 1999). The focus of this in vitro investigation was to determine the structural features of a phenolic compound required for this latter effect.

2. Methods

2.1. Cell culture, transfection

The receptor complex consisting of $\alpha_1\beta_2\gamma_2$ subunits is considered a dominant human receptor combination in vivo (Lambert et al., 1997; McKernan and Whiting, 1996). Rat $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subunits were transiently transfected into transformed human embryonic kidney cells

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(HEK 293). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), 100 U ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin at 37°C in a 5% CO_2/air incubator. For transfection, cells were suspended in a buffer containing 50 mM K_2HPO_4 and 20 mM K-acetate, pH 7.35. For co-transfection of rat α_1, β_2, and γ_2 $GABA_A$ receptor subunits, the corresponding cDNA, each subcloned in the pcDM8 expression vector (Invitrogen, San Diego, USA) was added to the suspension. Plasmid DNA for the three $GABA_A$ receptor subunits was used in a ratio of 1:2:2 for α_1:β_2:γ_2. To visualize transfected cells, they were co-transfected with cDNA of green fluorescent protein. For transfection, we used an electroporation device by EquiBio (Kent, UK). Transfected cells were replated on glass-coverslips and incubated 15–24 h before recording.

2.2. Solutions

Purified 2,6-diisopropylphenol (propofol) was a gift from ZENECA (Plankstadt, Germany). 2-Methylphenol, 2,6-dimethylphenol, 3,5-dimethyl-4-chlorophenol, 3-methylphenol and thymol were from Sigma (Deisenhofen, Germany). Benzyl alcohol was from FLUKA (Deisenhofen, Germany). 2,6-Diisopropylphenol, 2,6-dimethylphenol, 3,5-dimethyl-4-chlorophenol and thymol were prepared as 1 M stock solution in ethanol, light-protected and stored in glass vessels at −20°C. 2-Methylphenol, 3-methylphenol and benzyl alcohol were dissolved directly in bath solution immediately before the experiments. Concentrations were calculated from the amount injected into the glass vials. Drug-containing vials were vigorously vortexed for 60 min. γ-Aminobutyric acid (GABA) was obtained from Sigma (St. Louis, MO, USA) and was dissolved directly in bath solution. Control experiments were performed with GABA 1 mM in bath solution, the test solution contained different concentrations of the drugs with or without GABA 1 mM. The amount of the diluent ethanol corresponding to the highest drug concentration used was tested separately. Patch electrodes contained [mM] KCl 140, $MgCl_2$ 2, EGTA 11, HEPES 10, glucose 10; the bath solution contained [mM] NaCl 162, KCl 5.3, $NaHPO_4$ 0.6, KH_2PO_4 0.22, HEPES 15, glucose 5.6.

2.3. Experimental set-up

Standard whole-cell experiments (Hamill et al., 1981) were performed at −60 mV membrane potential. A tight electrical seal of several GΩ formed between the cell membrane and a patch-clamp electrode allows inward currents due to agonist-induced channel activation to resolve in the pA range. In order to match the rapid kinetics of ligand-activated ion channels and to imitate the physiologic transmitter release, an ultra fast liquid filament switch technique (Franke et al., 1987) was used to apply the agonist in pulses of 2-s duration. The agonist and/or the drug under investigation was applied to the cells via a smooth liquid filament achieved with a single outflow (glass tubing 0.15 mm i.d.) connected to a piezo crystal. The cells were placed at the interface between this filament and the continuously flowing background solution. When a voltage pulse was applied to the piezo, the tube was moved up and down onto or away from the cell under investigation. Time for solution exchange with this technique was < 100 μs measured with an open pipette and high electrolyte gradient (Bufler et al., 1996). Correct positioning of the cell with respect to the liquid filament was ensured by applying a 2-s saturating (1 mM) GABA pulse before and after each test experiment. Care was taken to ensure that amplitude and shape of the GABA-activated current had stabilized before proceeding with the experiment. Test solution and GABA (1 mM) were applied via the same glass-polytetrafluoroethylen perfusion system, but from separate reservoirs. The 2nd reservoir containing the test solution was filled immediately before the test experiment, starting with the lowest drug concentration tested. The contents of the reservoirs were mixed at a junction immediately before entering the superfusion chamber. The solution in the tube was switched from control solution (1 mM GABA) to the respective test solution using a manual valve. Methylene-blue was added to the control solution in order to make the exchange by the colour-free test solution clearly visible. About 10 s are required for an exchange of solutions within the tube (Scheller et al., 1997). Between pulses, patches were bathed in a continuously flowing background solution. A 2-min interval between the pulses guaranteed full recovery of the channels from desensitisation. Drugs were applied either alone, for determining their direct agonistic effects, or together with a saturating (1 mM) concentration of GABA to test possible modulatory effects on channel desensitisation. All drugs were applied in pulses of 2-s duration. A new cell was used for each drug and each protocol; at least three experiments were performed for each drug.

2.4. Current recordings and analysis

For data acquisition and further analysis, we used the EPC9 digitally controlled amplifier in combination with Pulse and Pulse Fit software (HEKA Electronics, Lambrecht, Germany). Input resistance of the patch pipettes was at 6–10 MΩ. Only small cells were used for the experiments, capacitances ranged from 8 to 12 pF. Currents were filtered at 2 kHz. Fitting procedures were performed with a non-linear least-squares Marquardt–Levenberg algorithm, details are provided in the appropriate figure legends or in the Results section. The maximum current response induced by a compound acting directly as an agonist was expressed as percentage of the maximum response to 1 mM GABA in the absence of drug immediately following the respective test experiment. Concentration–response data obtained for agonists were fitted ac-

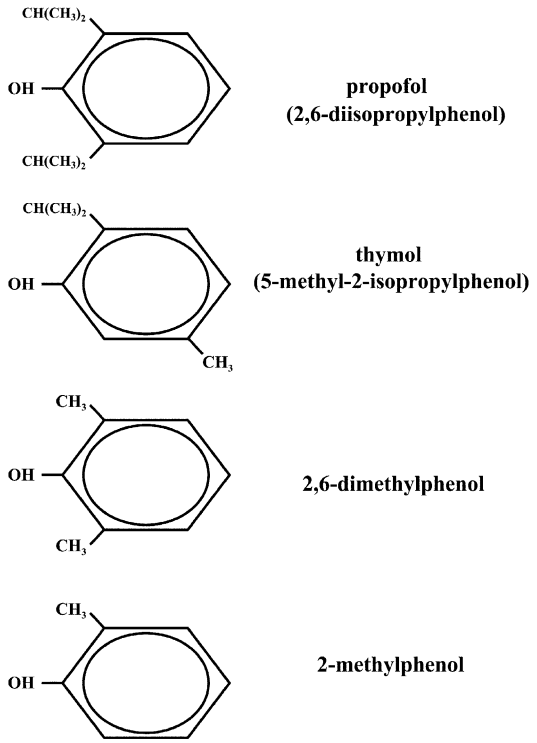

Fig. 1. Structures of the four *ortho*-alkylated phenolic compounds achieving direct receptor activation in this study.

cording to ($I_{Cl} = [1 + (EC_{50}/[C])^{n_H}]^{-1}$), where I_{Cl} is the current induced by the respective concentration [C] of the agonist, normalized to the maximum inward current induced by this agonist, EC_{50} is the concentration required to evoke a response amounting to 50% of their own maximal response and n_H is the Hill coefficient.

2.5. Statistics

All data are presented descriptively as mean ± S.D. Statistical analysis of the concentration–response plots was performed in order to reveal differences in potency between the compounds. Curve fitting and parameter estimation of the Hill curves was performed using the program MS Excel 2000. The differences between the parameter values (EC_{50} and n_H) of all independent data sets were tested using Student's *t*-test. The null hypothesis of no parameter difference was rejected when p was < 0.05.

3. Results

Expression of rat $\alpha_1\beta_2\gamma_2$ mRNA in HEK 293 cells generated $GABA_A$ receptors that showed a GABA-activated inward current with amplitudes ranging between 100 and 500 pA following saturating (1 mM) concentrations of the agonist when the cells were voltage-clamped at −60 mV. A total of 17 cells were included in the study. The current transient showed a fast increase, followed by a triphasic decay. Triexponential fits ($I(t) = a_0 + a_1\exp(-t/\tau_{fast}) + a_2\exp(-t/\tau_{interm}) + a_3\exp(-t/\tau_{slow})$) to the current decay yielded time constants τ of 25.3 ± 4.9 (τ_{fast}), 200.6 ± 37.1 ($\tau_{intermediate}$) and 2500 ± 700 ms (τ_{slow}), respectively. Steady-state current that did not desensitize in

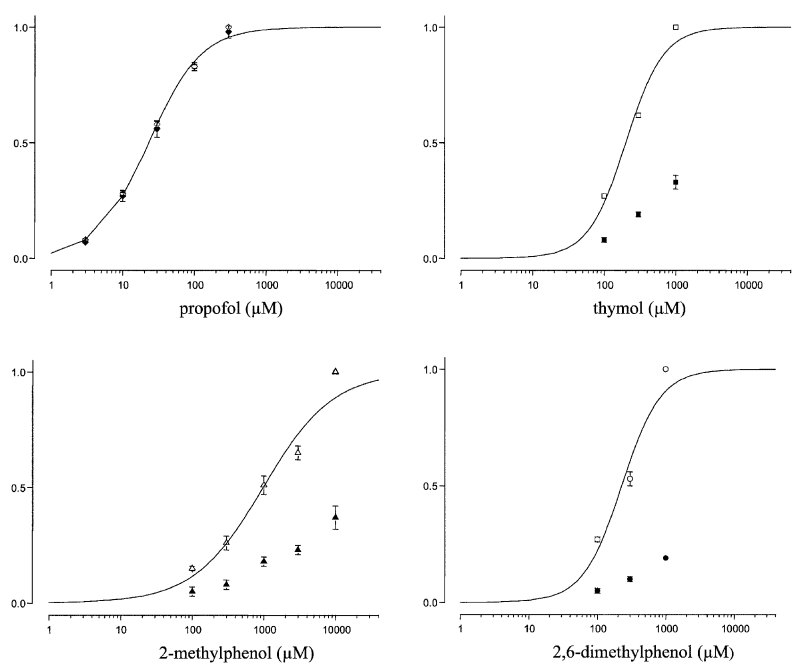

Fig. 2. Normalized Cl^- currents activated in the absence of GABA (mean ± S.D.; $n \geq 4$), plotted against the concentration of the agonist applied on a logarithmic scale. Currents were normalized either to maximum value in the presence of 1 mM GABA (filled symbols) or to maximum value achieved by saturating concentrations of the compound (empty symbols). Solid lines are Hill fits ($I_{Cl} = (1 + (EC_{50}/[C])^{n_H})$) to the data.

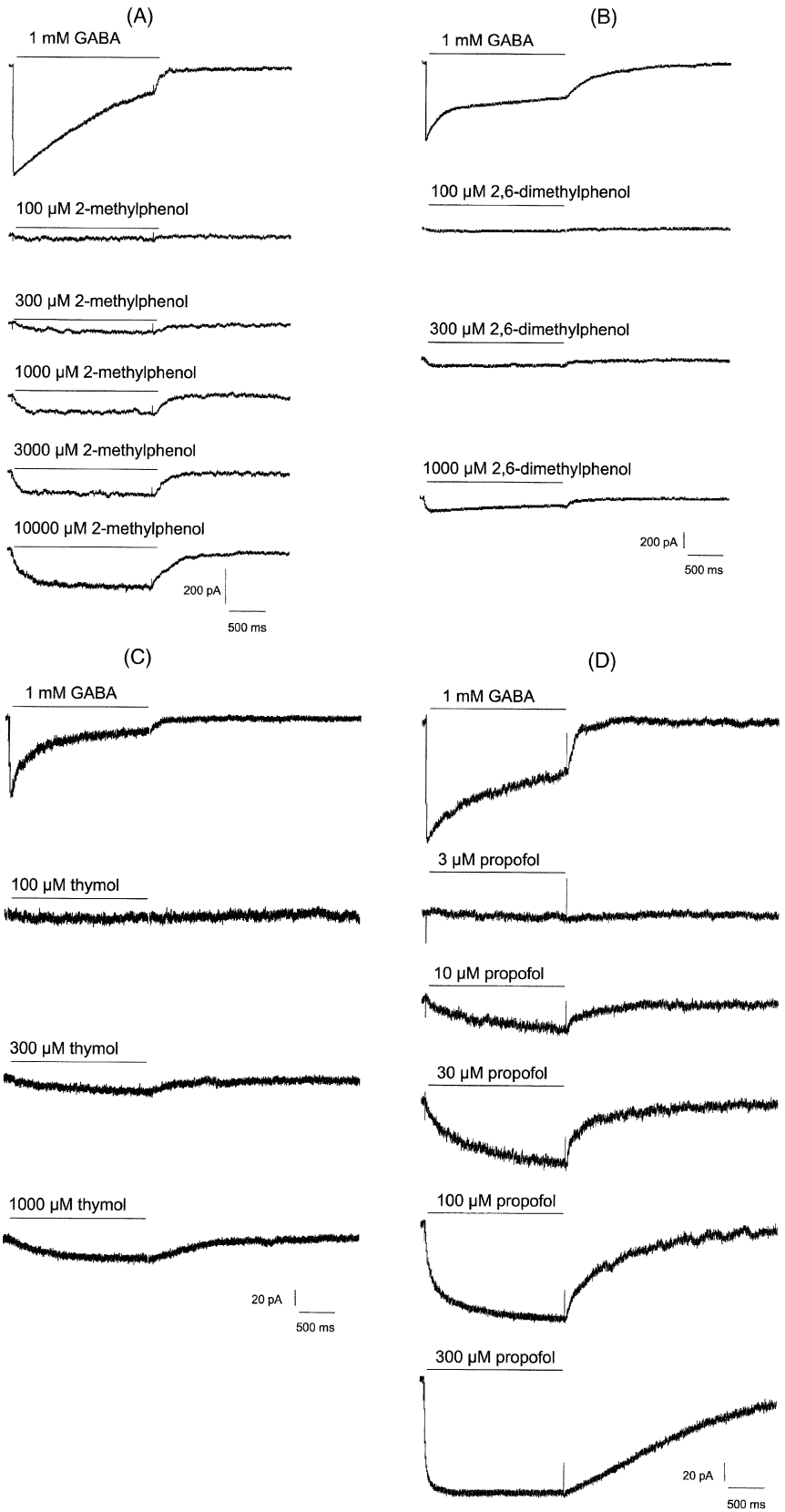

Fig. 3. (A–D) Representative current traces elicited by 2-s application of either 2-methylphenol (100–10,000 µM), 2,6-dimethylphenol (100–1000 µM), thymol (100–1000 µM) and propofol (3–300 µM), with respect to the current elicited by 1 mM GABA in the same experiment (upper trace). Tracings were obtained from one HEK 293 cell expressing $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors for each compound. GABA-induced currents show rapid desensitisation during the 2-s application, while drug-induced currents did not desensitize.

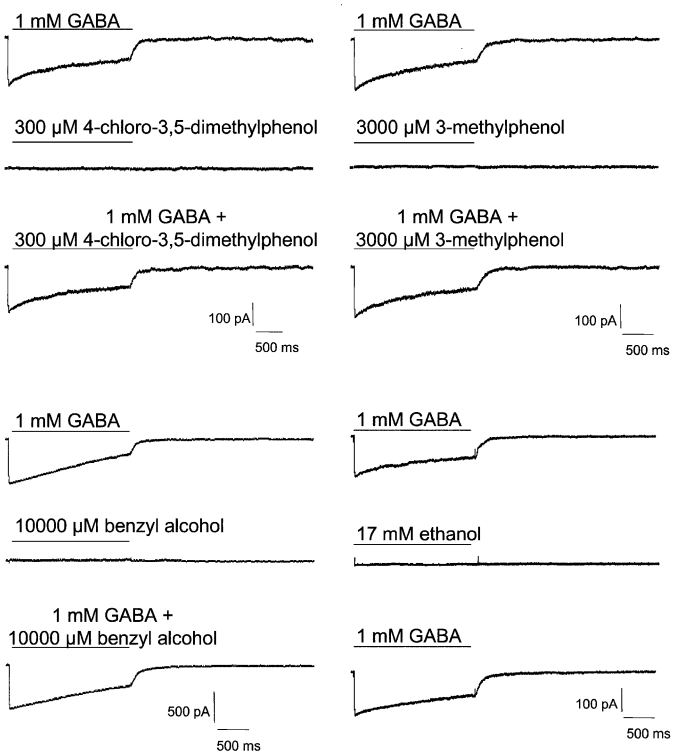

Fig. 4. Representative tracings in the presence of 3,5-dimethyl-4-chlorophenol, 3-methylphenol and benzyl alcohol, applied with or without saturating concentrations (1 mM) of GABA. Neither 3,5-dimethyl-4-chlorophenol nor 3-methylphenol or benzyl alcohol were able to activate Cl^- currents in the absence of GABA. Neither of the drugs modulated the response to saturating concentrations of GABA when coapplied with the agonist. The diluent ethanol in a concentration corresponding to the highest drug concentration applied did not activate Cl^- currents.

the presence of 1 mM GABA was at $32.6 \pm 8.9\%$ of the peak current amplitude.

When applied without GABA, only drugs alkylated in *ortho* position to the phenolic hydroxyl group (2-methylphenol, 2,6-dimethylphenol, thymol and propofol) directly activated receptor-mediated inward current in a concentration-dependent manner (Fig. 1).

Currents reached 37.3 ± 5.4 ($n = 5$), 19.2 ± 0.01 ($n = 4$), 32.8 ± 3.1 ($n = 4$) and 98.1 ± 2.6 ($n = 6$) % of the maximum GABA (1 mM) response in the presence of saturating concentrations of 2-methylphenol, 2,6-dimethylphenol, thymol and propofol, respectively.

Hill fits to the dose–response curves depicted in Fig. 2 gave EC_{50} values of 983.3 ± 88.8, 227.3 ± 10.5, 200.5 ± 4, and 23.2 ± 0.5 µM for receptor activation induced by 2-methylphenol, 2,6-dimethylphenol, thymol and propofol, respectively (see Fig. 2). The Hill coefficients (n_H) were 0.9, 1.5, 1.6 and 1.2. Potency to activate Cl^- currents increased significantly from 2-methylphenol to 2,6-dimethylphenol ($p < 0.001$), and slightly from 2,6-dimethylphenol to thymol ($p = 0.01$). All agonists, however, were significantly less potent than propofol ($p < 0.01$).

As illustrated by the tracings in Fig. 3, desensitisation and deactivation of Cl^- currents elicited by the three compounds uniformly showed a pattern that was different from GABA-activated currents. While GABA-induced currents showed rapid desensitization during the 2-s application, drug-induced currents did not desensitize. This lack of desensitization was equally observed in saturating concentrations of all compounds and was independent from the peak current amplitude achieved.

Drugs with methyl groups attached in *meso* position to the phenolic hydroxyl (3-methylphenol and 3,5 dimethyl-4-chlorophenol) or inserted between the benzene ring and

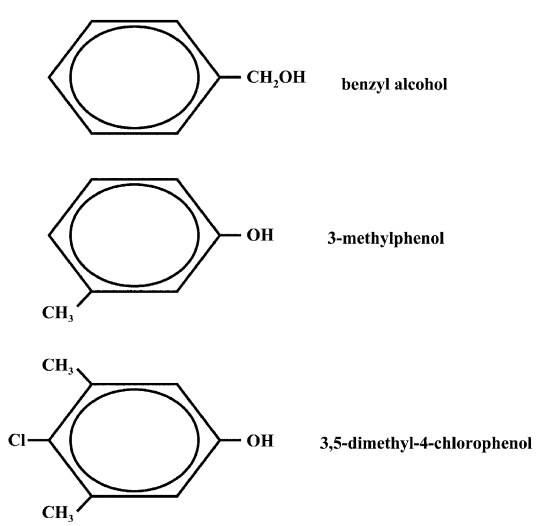

Fig. 5. Structures of the phenol derivatives that did not show direct agonist activity at $GABA_A$ receptors.

the phenolic hydroxyl (benzyl alcohol) did neither activate the receptor when applied without GABA, nor modulate the GABA response when applied together with the agonist (see Figs. 4 and 5).

4. Discussion

$GABA_A$ receptors are the main receptors for inhibitory neurotransmission in the mammalian brain. It is generally acknowledged that the efficacy of structurally diverse anxiolytic, anticonvulsant or sedative–anaesthetic drugs is related to their ability to enhance the function of the $GABA_A$/ionophore receptor complex (Banks and Pearce, 1999; Belelli et al., 1996; Cordato et al., 1999; Gage and Robertson, 1985; Hales and Lambert, 1991; Jones et al., 1992; Krampfl et al., 1998). Comparative studies of allosteric modulators of mammalian $GABA_A$ receptors revealed that the intravenous anaesthetics propofol and, with 10-fold lower potency, pentobarbitone, do not only modulate GABA effects, but are capable of directly activating $GABA_A$ receptors in the absence of GABA (Belelli et al., 1996; Hales and Lambert, 1991; Sanna et al., 1995a,b). There is some experimental evidence stressing the role of direct activation of inhibitory chloride currents in the absence of GABA in propofol-induced hypnosis. The ability of a propofol analogue to induce sedation and sleep (with respect to merely anticonvulsant and anticonflict actions) was paralleled by its ability to produce direct receptor activation in the absence of GABA (Sanna et al., 1999).

Our findings show that only compounds with (a) the unsubstituted phenolic hydroxyl attached directly to the benzene ring and (b) a methyl or isopropyl group inserted in *ortho* position to the phenolic hydroxyl were able to induce chloride inward currents via $GABA_A$ receptors in the absence of GABA. Potency was increased more than fourfold when a second methyl group was inserted in *ortho* position, indicating that the ability of a phenolic compound to activate $GABA_A$ receptors is associated with 2,6-di-alkyl-substitution. The compound with only one isopropyl group in *ortho* position (thymol) was slightly more potent than 2,6-dimethylphenol; propofol (2,6-diisopropylphenol) possessed the highest potency. The concentration-dependence for agonist effects in case of propofol confirms previous results (Belelli et al., 1996).

Our findings might provide a molecular basis for the anaesthetic activity of *ortho*-alkylated phenol derivatives revealed by animal experiments, where the highest potency to induce sedation and sleep was associated with 2,6-di-alkyl-substitution (James and Glen, 1975). However, the threefold difference in anaesthetic potency between 2,6-dimethylphenol and propofol seen in mice (James and Glen, 1975), compared to an eightfold difference in agonist activity at the GABA receptor level indicates that other factors might be involved in the anaesthetic effects of phenol derivatives seen in vivo.

Activation of chloride currents by the four agonistic drugs showed a pattern that was different from the activation of these currents by the natural agonist GABA. While the currents induced by 1 mM GABA showed triphasic desensitization (Krampfl et al., 2000; Wetzel et al., 1999), drug-induced currents uniformly showed no desensitization, even at saturating concentrations. Thus, a continuous presence of a phenolic compound might produce long-lasting hyperpolarization of the postsynaptic membrane.

Knowledge of differences in the interaction of drugs with channels and receptors at the molecular level has contributed to some understanding of differences in the pharmacological profile seen clinically. Consequently, structure–function analysis of drug effects at different channels and receptors may provide a basis for the design of drugs targeting different receptors with different potencies, translating into a desirable pattern of inhibitory or excitatory effects. Interestingly, some phenol derivatives have previously shown to be potent blockers of voltage-operated K^+ (Elliott and Elliott, 1997) and Na^+ (Haeseler et al., 1999, 2000) channels, with the halogenated compound 4-chloro-*m*-cresol being more potent than lidocaine (Haeseler et al., 1999). While the potency to block voltage-operated Na^+ channels seems to be higher in halogenated compounds (Haeseler et al., 1999), halogenation of the propofol molecule did not further increase its ability to activate $GABA_A$ receptors (Trapani et al., 1998).

The use of virtually all local anaesthetics is limited due to central nervous toxicity occurring with higher brain concentrations. Especially local anaesthetic-induced convulsions are attributed to the depression of inhibitory circuits in the central nervous system, among others the inhibition of GABA-induced chloride currents (Hara et al., 1995; Sugimoto et al., 2000; Ye et al., 1997). Results from our study suggest that *ortho*-alkylated phenol derivatives might represent an interesting alternative, as some compounds can be expected to combine sedative–hypnotic effects due to activation of inhibitory circuits in the central nervous system, with lidocaine-like actions due to inhibition of voltage-operated Na^+ channels.

In conclusion, our work provides a molecular basis for structure–function considerations with respect to direct agonist effects of phenol derivatives on $GABA_A$ receptors.

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