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Cyclohexanol analogues are positive modulators of $GABA_A$ receptor currents and act as general anaesthetics in vivo

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ABSTRACT

$GABA_A$ receptors meet all the pharmacological criteria required to be considered important general anaesthetic targets. In the following study, the modulatory effects of various commercially available and novel cyclohexanols were investigated on recombinant human γ-aminobutyric acid ($GABA_A$, $\alpha_1\beta_2\gamma_{2s}$) receptors expressed in *Xenopus* oocytes, and compared to the modulatory effects on GABA currents observed with exposures to the intravenous anaesthetic agent, propofol. Submaximal EC_{20} GABA currents were typically enhanced by co-applications of 3–300 μM cyclohexanols. For instance, at 30 μM 2,6-diisopropylcyclohexanol (a novel compound) GABA responses were increased ~3-fold (although similar enhancements were achieved at 3 μM propofol). As regards rank order for modulation by the cyclohexanol analogues at 30 μM, the % enhancements for 2,6-dimethylcyclohexanol ~ 2,6-diethylcyclohexanol ~ 2,6-diisopropylcyclohexanol ~ 2,6-di-sec-butylcyclohexanol ≫ 2,6-di-tert-butylcyclohexanol ~ 4-tert-butylcyclohexanol > cyclohexanol ~ cyclopentanol ~ 2-methylcyclohexanol.

We further tested the potencies of the cyclohexanol analogues as general anaesthetics using a tadpole in vivo assay. Both 2,6-diisopropylcyclohexanol and 2,6-dimethylcyclohexanol were effective as anaesthetics with EC_{50}s of 14.0 μM and 13.1 μM respectively, while other cyclohexanols with bulkier side chains were less potent. In conclusion, our data indicate that cyclohexanols are both positive modulators of $GABA_A$ receptors currents and anaesthetics. The positioning and size of the alkyl groups at the 2 and 6 positions on the cyclohexanol ring were critical determinants of activity.

1. Introduction

Intravenous general anaesthetics are widely used in surgical settings and typically have the advantage of rapid onset and offset of action. For instance, propofol is a commonly used intravenous anaesthetic agent (Langley and Heel, 1988) that is postulated to render patients unconscious through positive modulation of $GABA_A$ receptor currents in the central nervous system (Franks and Lieb, 1994; Krasowski and Harrison, 1999; Trapani et al., 2000).

$GABA_A$ receptors are the predominant ionotropic receptors for fast inhibitory neurotransmission in the mammalian central nervous system (CNS). Their pentameric structure is composed of different subunits (α_{1-6}, β_{1-4}, γ_{1-3}, δ, ε, π, and θ) forming membrane-spanning chloride-selective ion channel complexes that are activated through the binding of GABA (Barnard et al., 1998). In the mammalian central nervous system, the predominant $GABA_A$ receptor combination appears to be $\alpha_1\beta_2\gamma_2$ (McKernan and Whiting, 1996). Propofol at clinically relevant concentrations is a potent enhancer of neuronal GABAergic currents and directly activates GABA currents at supraclinical concentrations (Hales and Lambert, 1991). Given the importance of propofol as a general anaesthetic, several studies have used structure-activity relationship (SAR) analyses to assess the anaesthetic properties of propofol analogues and reported that the aliphatic groups adjacent to the hydroxyl group are critical for activity (Krasowski et al., 2001; Lingamaneni et al., 2001; Sanna et al., 1999; Trapani et al., 1998).

Recent studies demonstrated the potential for the monoterpenoid menthol to act as a positive modulator of $GABA_A$ receptor currents (Hall et al., 2004a; Zhang et al., 2008) and as a general anaesthetic (Hall et al., 2004a). Further studies described related monoterpenoid alcohols and ketones (e.g. borneol, camphor, menthone and carvone; Hall et al., 2004a; Granger et al., 2005) as positive modulators of $GABA_A$ receptors and were supported by reports of receptor modulation by monoterpenes including citronellol and pinene (Aoshima and Hamamoto, 1999), thujone (Hold et al., 2000) and

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thymol (Priestley et al., 2003). Interestingly, menthol and propofol share some structural similarities including adjacency of an isopropyl group to the hydroxyl group on their respective rings (a phenol ring for propofol, and a cyclohexanol ring for menthol) and may also share common sites of action on $GABA_A$ receptors (Watt et al., 2008). Binding assays in chick forebrain highlighted stereoselectivity of menthol's activity at $GABA_A$ receptors and the importance of the positioning of a freely-rotating non-polar isopropyl group in proximity to a polar hydroxyl group for activity (Corvalan et al., 2009). Given the potential for cyclohexanol analogues to act as receptor modulators and anaesthetics, and the interest in developing novel anaesthetic agents, there have been few studies to date that have systematically explored the structure-activity relationship for cyclohexanol-based compounds (see Hall et al., 2004a; Granger et al., 2005).

In the following study, we used the *Xenopus* oocyte expression system to investigate modulation of recombinant human wild-type $GABA_A$ receptors ($\alpha_1\beta_2\gamma_{2s}$) by commercially available and novel cyclohexanol analogues. We further explored whether the cyclohexanol analogues act as general anaesthetics in an established loss of tadpole righting reflex assay (Downes and Couragen, 1996).

2. Materials and methods

2.1. Xenopus oocyte expression

cDNAs encoding for the α_1, β_2, γ_{2s} subunits of human $GABA_A$ receptors were kindly provided by Dr. Paul J. Whiting (Merck, Sharp & Dohme Research Laboratories, UK). The $GABA_A$ receptor subunit cDNAs were prepared in pcDNA3.1+ vector and stored at −20 °C prior to injection as described previously (Hall et al., 2004b).

Wild-type $\alpha_1\beta_2\gamma_{2s}$ $GABA_A$ receptor subunits were routinely co-expressed in *Xenopus* oocytes. Briefly, *Xenopus laevis* (Xenopus Express, Plant City, FL, USA) were anaesthetised with 1.25% tricaine/1.75% sodium bicarbonate and oocytes were harvested through laparotomy. Batches of eggs were treated with 1 mg/ml collagenase D (Roche Diagnostics Corporation, Indianapolis, IN, USA) in (mM) 82 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES (pH: 7.6) for 80 min at room temperature on a shaking platform. Oocytes were transferred to a solution (ND-96) containing (mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES with 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin and 5 μg/ml tetracycline, pH: 7.6 (antibiotics from Invitrogen, Rockville, MD, USA). Eggs were defolliculated manually by repetitive rolling on plastic Petri dishes. Plasmids were introduced by nuclear injection using a Nanoject II (Drummond Scientific Co., Broomall, PA, USA). For all eggs, the injection volume was 32 nl with concentrations of the $GABA_A$ receptor α_1 and β_2 subunit cDNAs at 12 ng/μl and the γ_{2s} subunit at 6 ng/μl. Injected oocytes were maintained in ND-96 with antibiotics at 16 °C. All animal maintenance and oocyte harvest procedures were approved by Smith College's Institutional Animal Care and Use Committee (IACUC).

2.2. Electrophysiology

Between 1 and 3 days after cDNA injection, injected oocytes were screened for GABA-evoked currents in a 100 μl oocyte chamber (Warner Instruments Corp., Hamden, CT, USA). All experiments were performed at room temperature (20–23 °C) unless stated otherwise. Eggs were placed in a small depression, animal-pole face up, and continually superfused at 5 ml/min with ND-96 (less antibiotics). Recordings were made using standard two-electrode voltage-clamp technique with an OC-75 C clamp (Warner Instruments Corp.). Glass micropipettes (World Precision Instruments, Sarasota, FL, USA) were fabricated using a two-stage pull (Narishige, Tokyo, Japan) and filled with 3 M KCl giving resistances of 1–3 MΩ. For all experiments impaled oocytes were voltage-clamped at −50 mV. All drug stock solutions were dissolved in ND-96 immediately prior to use, and solutions applied via gravity feed (5 ml/min) using an automated switching device (ALA Scientific Instr., Westbury, NY, USA). Currents were digitised at 200 Hz, recorded and analysed using pClamp 6.0 software (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA). Solution switches to GABA (in the absence or presence of drugs) were applied until currents were determined to have achieved peak amplitudes. There was at least 2 min exposure to control recording solution between drug switches to allow adequate washout and recovery from receptor desensitisation.

Currents evoked by 30 μM concentration of GABA were routinely measured, a concentration that was determined to represent ~ EC_{20} (see Hall et al., 2004b). GABA currents were completely blocked by bicuculline and picrotoxin and incorporation of the γ_{2s}-subunit was confirmed by insensitivity of the evoked currents to block by Zn^{2+}, by a rightward shift in the GABA dose-response plots relative to recordings from wild-type $\alpha_1\beta_2$ receptors and through positive modulation by the benzodiazepine, flunitrazepam (data not shown, see also Hall et al., 2004b).

Dilutions of drugs (1–300 μM) 2,6-diisopropylphenol (propofol), cyclohexanol, cyclopentanol, 2-methylcyclohexanol, 2-tert-butylcyclohexanol, 4-tert-butylcyclohexanol, 2,6-dimethylcyclohexanol, 2,6-diethylcyclohexanol, 2,6-diisopropylcyclohexanol, 2,6-di-tert-butylcyclohexanol and 2,6-di-sec-butylcyclohexanol were prepared by adding quantities of 1 M stock solutions in dimethyl sulphoxide (DMSO) to the recording solution. Testing higher doses (≥1 mM) was considered unreliable given the insolubility of the agents at these concentrations. Reservoirs for control and drug applications contained equivalent DMSO concentrations up to 0.1% that were determined to have no effect on GABA currents alone (data not shown). 2,6-Dimethylcylohexanol was obtained as a mixture of three diastereomers from Acros Organics, USA. Other chemicals (except 2,6-diethylcyclohexanol, 2,6-diisopropylcyclohexanol, 2,6-di-tert-butylcyclohexanol and 2,6-di-sec-butylcyclohexanol) were purchased from Sigma-Aldrich, St. Louis, MO, USA (unless stated otherwise).

2,6-Diethylcyclohexanol, 2,6-diisopropylcyclohexanol, 2,6-di-tert-butylcyclohexanol and 2,6-di-sec-butylcyclohexanol were prepared either by catalytic hydrogenation of the corresponding phenol or reduction of the corresponding 2,6-disubstituted cyclohexanone (see Synthesis). It should be noted that, with the 2,6 substituents as methyl, ethyl, isopropyl or tert-butyl, the cyclohexanols were synthesised as mixtures of three diastereomers (either cis/cis, cis/trans or trans/trans) in varying proportions depending on the alkyl group (Table 1). With the chiral sec-butyl group, eight isomers (four meso forms and four pairs of enantiomers) were detected in the mixture by ^1H and ^{13}C nmr. In each case the GABA response and anaesthetic activity were recorded using the isomer mixtures.

Currents were initially measured using pClamp 6.0 software (Axon Instruments Inc.) and then further analyses were carried out with Origin software (OriginLab Corp., Northampton, MA). All collated data are expressed as mean ± standard error of the mean (S.E.M.), and were calculated from at least n = 5 individual oocytes for every data point.

Table 1
Relative percentages of diastereomers in synthesised cyclohexanol analogues.

R	^1H nmr, δ CHOH			^{13}C nmr, δ CHOH[a]		
	cis,cis (%)	cis,trans(%)	trans,trans(%)	cis,cis	cis,trans	trans,trans
Me	3.52 (57)	3.32 (17)	2.67 (26)	75.0	77.6	82.1
Et	3.77 (44)	3.56 (14)	2.85 (42)	66.9	71.0	78.2
Pr^i	4.03 (56)	3.47 (27)	3.12 (17)	66.2	68.8	72.8
Bu^t	4.42 (>90)	4.26 (*ca.* 5)	3.48 (*ca.* 5)	69.6	n.o.	n.o

n.o.: not observed.
[a] Assignments based on intensity.