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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.

2.3. Synthesis

2,6-Diethylcyclohexanol: A solution of 2,6-diethylaniline (9 g, 60 mmol) in water (180 ml) and concentrated H_2SO_4 (120 ml) was stirred and cooled to 5 °C. Ice (30 g) was added followed by a solution of sodium nitrite (4.5 g) in water (120 ml) added over 50 s with vigorous stirring. The light orange solution was transferred to a flask containing 50% aqueous H_2SO_4 (150 ml) already heated to *ca.* 50 °C. The mixture was then heated at 90 °C for 15 mins, cooled to room temperature, evaporated and the residue sublimed at 40–50 °C and 0.25 mmHg to give 2,6-diethylphenol as a white crystalline solid (5 g, 55%), m.p. 37–38 °C, (see Noble et al., 1971) m.p. 36.0–36.5 °C. ^{1}H nmr ($CDCl_3$) δ, 1.15 (t, 6H), 2.53 (q, 4H), 4.62 (1H, OH), 6.75 (t, 1H), and 6.91 (d, 2H); ^{13}C δ 13.9, 23.0, 120.5, 126.5, 151.2. Reduction of 2,6-diethylphenol was effected using 4 g of the phenol with 0.5 g Raney nickel catalyst at 200 °C and a hydrogen pressure of 50 atms. The product was dissolved in CH_2Cl_2 (25 ml), filtered free of catalyst and the solvent evaporated to give 2,6-diethylcyclohexanol (3.8 g, 97%) as a mixture of three diastereomers (see Table 1). ^{1}H nmr, $CDCl_3$, δ, 0.86–0.94, four triplets centred at 0.876, 0.886, 0.894 and 0.919 (total 6H, J = 7.5 Hz for each, 4 × CH_3); 1.10–1.88 (multiplets, 12H); 2.85 (dt, 0.4H), 3.56 (dt, 0.14H) and 3.77 (d, 0.44H); ^{13}C, δ, 11.0, 11.1, 11.8, 14.2, 23.1, 24.0, 25.1, 25.6, 26.0 (2), 26.2, 29.1 (2), 30.1, 30.6 ($\underline{C}H_2 + \underline{C}H_3$); 38.9, 42.8, 44.4, 46.5 (all-$\underline{C}$H-); 66.9, 71.0, 78.2 (all $\underline{C}$HO).

2,6-Diisopropylcyclohexanol: 2,6-Diisopropylphenol (propofol, 10 g, 56 mmol) was catalytically reduced under the conditions described above to give a mixture of three diastereomers (7.2 g, 40 mmol, 70%), b.p. 130 to 135 °C at 30 mm (Kugelrohr, see Coffield et al., 1957) b.p. 130.5 to 132 °C at 30 mm. ^{1}H nmr ($CDCl_3$) δ, 0.7–1.5 (m, 22H), 3.12 (t, 0.06H), 3.46 (br d, 0.25H), and 4.03 (br m, 0.69H); ^{13}C δ, 20.6, 20.7, 20.9, 21.1, 21.5, 21.6, 24.1, 25.9, 26.1, 29.1, 29.2, (all unassigned), 20.7, 21.1, 23.6, 29.4, {−$\underline{C}$H(CH_3)$_2$}, 43.0, 47.9, 49.3, 50.6, {−$\underline{C}$H-CH(OH)-} and 68.2, 72.8, {−$\underline{C}$H-(OH)-} (See Table 1).

2,6-Di-tert-butylcyclohexanol: The catalytic hydrogenation of 2,6-di-tert-butylphenol (10 g, 50 mmol) over Ra-Ni at 200 °C and 50 atm H_2 pressure yielded 2,6-di-tert-butyl cyclohexanone (9 g, 91%) as a mixture of *cis* and *trans* isomers, b.p. 135 to 145 °C at 35 mm^2^{1}H nmr ($CDCl_3$) δ 0.97 (s,~17H, *cis* isomer, $CHCl_3$) 0.86 (s,0.5H), 1.025 (0.5H) - *trans* isomer plus multiplets at 1.43–1.90 m (3H), 1.94 m (1H), and 2.14 m (4H); ^{13}C nmr ($CDCl_3$) δ, 26.5, 27.6,, 31.8, 32.0, 62.1, and 214.8 (*cis* isomer); 21.4, 26.5, 27.9, 32.6, 58.6, 221 (*trans* isomer). A solution of 2,6-di-tert-butylcyclohexanone (1.07 g, 4.8 mmol) in tertahyrofuran (THF, 10 ml) was added to a solution of $LiAlH_4$ (0.48 g 2.3 mmol) in THF (5 ml) and the mixture was heated under reflux for 24 h. After cooling, the mixture was treated with water (5 ml) and 5% eq HCl (5 ml); the organic layer was then separated, dried and evaporated. Recrystallisation of the residue from hexanes gave white crystals of 2,6-di-tert-butylcyclohexanol (0.82 g, 81%). Found: C, 78.79; H, 13.71; $C_{14}H_{28}O$ requires, C, 79.18; H, 13.29%. ^{1}H nmr ($CDCl_3$) δ 0.95–1.90 m (26H), 3.46 (q, 0.05H), 4.18 (br 0.05H), 4.42 (br d, 0.9H); ^{13}C nmr ($CDCl_3$) cis, cis isomer, δ 21.2, 26.8, 28.8, 32.8, 52.7, 69.6 (CHOH); ^{13}C peaks for the cis, trans and trans, trans isomers were not assignable.

2.4. In vivo Xenopus laevis tadpole anaesthetic assay

General anaesthetic potencies of selected cyclohexanols were determined as previously described (Downes and Couragen, 1996; Krasowski et al., 2001) for *Xenopus laevis* tadpoles (Xenopus One; Ann Arbor, MI) in the prelimb-bud stage of development, corresponding to stages 43 to 50 (Nieuwkoop and Faber, 1956). Tadpoles were maintained in dechlorinated tap water in an aerated aquarium at 16–18 °C and brought to room temperature prior to experimentation. The anaesthetic endpoint, referred to as loss of righting reflex (LORR, a measure of immobility), is defined as a lack of purposeful and sustained swimming response after a gentle inversion with a smooth glass rod (Downes and Couragen, 1996). In randomised blind experiments, 10 tadpoles were placed in beakers containing 300 ml of tap water, with or without the addition of a test compound. All drugs (2,6-diisopropylphenol (propofol), 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 added from stock dimethyl sulfoxide (DMSO) solutions to water to give final concentrations of 0.3, 1, 3, 10, 30, 100 and 300 μM drug, and all beakers were standardised to contain 0.033% (v/v) DMSO. No anaesthetic actions or lethality were observed at this concentration of DMSO alone.

The number of anaesthetised tadpoles was recorded every 10 min for up to 120 min, after which the tadpoles were returned to fresh tap water, and recovery monitored. To lessen the risk of lethality, tadpoles were removed from beakers in which the drug completely ablated all tadpole movement before 120 min. Instances in which a tadpole failed to recover from a particular drug concentration were scored as a lethal event rather than anaesthesia. We used an Excel macro to analyse the tadpole concentration-response data according to the method of Waud, using the quantal analysis equation:

$$p = (100XI^n)\left[I^n + (EC_{50})^n\right]^{-1}$$

where p is the percentage of the population anaesthetised, I is the anaesthetic concentration, n is the slope factor, and EC_{50} is the concentration for a half-maximal anaesthetic effect (Waud, 1972). Data were plotted using Origin 7.0 software (OriginLab Corp., Northampton, MA). All tadpole maintenance and experimental procedures were approved by Smith College's Institutional Animal Care and Use Committee (IACUC).

3. Results

3.1. Cyclohexanols are positive modulators of $GABA_A$ receptor currents

We investigated the modulation of sub-maximal GABA currents by a variety of cyclohexanol analogues including cyclohexanol itself, cyclohexanols with a single aliphatic chain in the *ortho* and *para* positions, and di-substituted cyclohexanols with increasingly bulky aliphatic groups (methyl to butyl, see Fig. 1) in both *ortho* positions. We focused our studies on 2,6-di-substituted cyclohexanols given the structural similarities to propofol and relatively straightforward syntheses from the phenols for the analogues that were not commercially available (see Materials and methods).

Oocytes were routinely screened for wild-type $\alpha_1\beta_2\gamma_{2s}$ receptor expression by applications of 30 μM GABA that evoked ~ EC_{20} currents (effective concentration that evoked 20% of maximal current, see Hall et al., 2004b). As widely reported (Hales and Lambert, 1991;

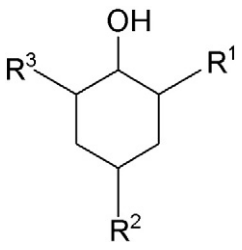

Fig. 1. Structures of the selection of the cyclohexanols used in this study. (a) cyclohexanol: R^1, R^2, R^3 = H (b) 2-methylcyclohexanol: R^1 = methyl; R^2, R^3 = H (c) 2-tert-butylcyclohexanol: R^1 = tert-butyl; R^2, R^3 = H (d) 4-tert-butylcyclohexanol: R^2 = tert-butyl; R^1, R^3 = H (e) 2,6-dimethylcyclohexanol: R^1, R^3 = methyl; R^2 = H (f) 2,6-diethylcyclohexanol: R^1, R^3 = ethyl; R^2 = H (g) 2,6-diisopropylcyclohexanol: R^1, R^3 = isopropyl; R^2 = H (h) 2,6-di-sec-butylcyclohexanol: R^1, R^3 = sec-butyl; R^2 = H (i) 2,6-di-tert-butylcyclohexanol: R^1, R^3 = tert-butyl; R^2 = H.

Krasowski et al., 2001; Krasowski and Harrison, 1999; Watt et al., 2008), co-applications of propofol produced dose-dependent potentiations of the GABA responses (Figs. 2A, 3A) e.g. with 3 μM propofol resulting in ~3-fold enhancements of the EC_{20} GABA current. Pre-exposure to propofol did not affect the extent of current modulation upon subsequent co-application (not shown) with directly-activated currents evident at higher concentrations of propofol > 10 μM (Fig. 2H).

Co-applications of 30 μM cyclohexanols produced a variety of effects on control GABA and responses (Figs. 2, 3) ranging from strong (up to 5-fold control current) to weak positive modulation or minimal effect. The rank order for positive modulation of GABA EC_{20} currents was: propofol ≫ 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 (Figs. 2, 3) while 2-tert-butylcyclohexanol acted only as an inhibitor (Fig. 3B). All the cyclohexanol effects were fully reversible upon washout (see Fig. 2B). Pre-exposure to the cyclohexanols did not affect the extent of current modulation upon subsequent co-application with GABA (e.g. for 2,6-diethylcyclohexanol, Fig. 2G). Also, none of the cyclohexanols tested (up to 300 μM) evoked any directly-activated current in expressing or uninjected oocytes (e.g. Fig. 2G). The current enhancements by cyclohexanols with aliphatic chains in both *ortho* positions were typically pronounced and were consistently larger than for equivalent concentrations of mono-substituted cyclohexanols (Figs. 2E, 3); for example, for 30 μM 2,6-dimethylcyclohexanol the potentiation was 446 ± 47% while for 30 μM 2-methylcyclohexanol the potentiation was only 5.6 ± 2.2% (Figs. 2B,E and 3A,B).

Positive modulations by cyclohexanol and cyclopentanol were negligible; for instance, at 100 μM cyclopentanol and cyclopentanol produced only 10–20% current enhancements of the GABA currents (Figs. 2F, 3B).

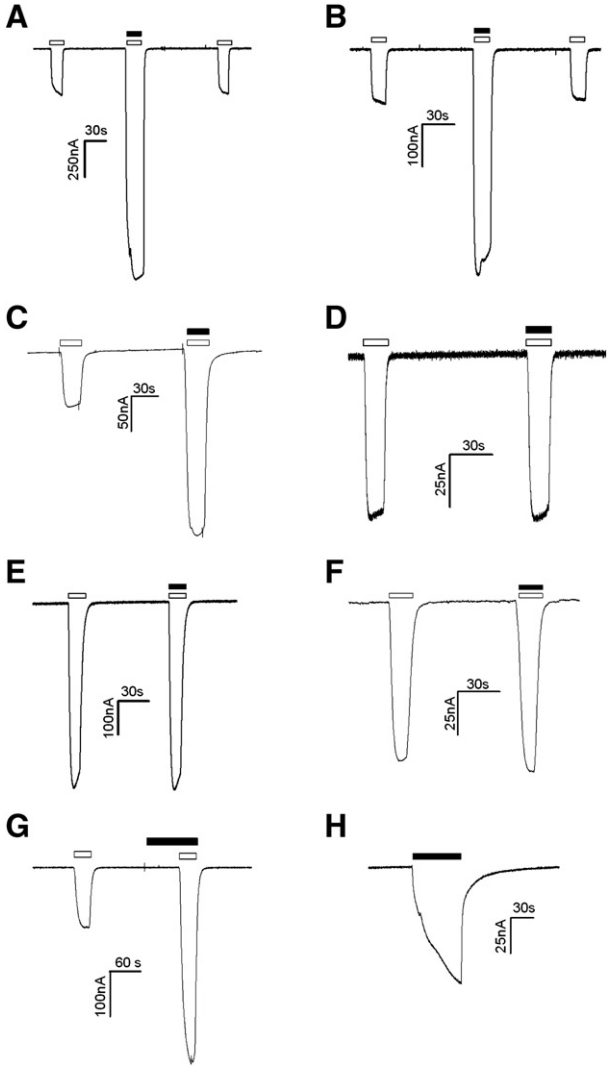

Fig. 2. Current recordings illustrating modulation by cyclohexanols of $GABA_A$ receptor activity. Oocytes were held at −50 mV and 30 μM GABA was applied for the duration of the open boxes above each trace. Co-application of 30 μM drug (except propofol) is indicated by the filled boxes with A) 3 μM propofol, B) 2,6-dimethylcyclohexanol, C) 2,6-diisopropylcyclohexanol, D) 2,6-di-tert-butylcyclohexanol, E) 2-methylcyclohexanol, and F) cyclohexanol. G) an oocyte was pre-exposed to 30 μM of 2,6-diethylcyclohexanol (filled box) prior to co-exposure with GABA (open box). Pre-exposure did not affect the extent of the current enhancements nor did the cyclohexanol evoke any current when applied in the absence of GABA. H) By contrast 30 μM propofol (filled box) evoked a directly-activated current. Note: the modulatory effects were fully reversible upon washout for all compounds tested, as shown for (A) and (B).

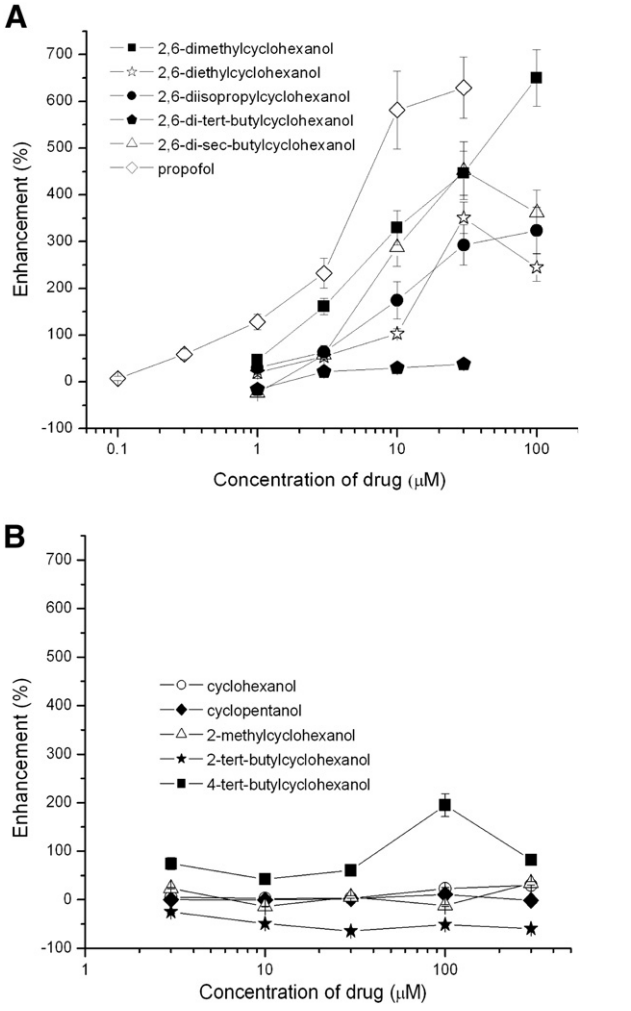

Fig. 3. Cyclohexanols with aliphatic chains in both *ortho* positions are more potent positive modulators of $GABA_A$ receptors than mono-substituted cyclohexanols. Relative modulations of EC_{20} GABA (30 μM) responses were compared by co-applying increasing concentrations of cyclohexanols (or propofol). A) Dose-dependency for effects of cyclohexanols with aliphatic chains in the 2,6-positions and propofol on $GABA_A$ receptors. With the exception of 2,6-tert-butyl cyclohexanol, the 2,6-substituted cyclohexanols were effective positive modulators of GABA currents with propofol demonstrating the most potent modulation. Error bars are S.E.M.s for n ≥ 5 oocytes. B) Concentration-response graph for effects of cyclopentanol, cyclohexanol and mono-substituted cyclohexanols on $GABA_A$ receptor currents. These compounds were considerably less potent modulators of GABA currents. Error bars are S.E.Ms. for n ≥ 5 oocytes.

3.2. Cyclohexanols act as general anaesthetics, possibly via action at $GABA_A$ receptors

Since the cyclohexanol series were observed to share positive modulation of $GABA_A$ receptor currents with the intravenous anaesthetic propofol, we explored the potential for cyclohexanols to act as anaesthetics by inducing a loss of righting reflex in *Xenopus* tadpoles. Typically the anaesthetic action of the cyclohexanols reached equilibrium within 60 min when minimum EC_{50} values calculated from the Waud equation (see 'Methods') were recorded (Fig. 4A, B). The rank order of anaesthetic potencies (n>30 tadpoles) was: propofol (1.7 ± 0.4 μM) ≫ 2,6-dimethylcyclohexanol (13.1 ± 3.0 μM) ~ 2,6-diisopropyl-cyclohexanol (14.0 ± 3.0 μM) > 2,6-di-tert-butyl-cyclohexanol (23.1 ± 4.8 μM) ~ 2,6-di-sec-butyl-cyclohexanol (23.6 ± 5.9 μM) > 2,6-diethylcyclohexanol (32.2 ± 9.4 μM) > 2-tert-butyl-cyclohexanol (54.3 ± 4.0 μM) ~ 4-tert-butyl-cyclohexanol (61.1 ± 12.7 μM). It was noted that the errors associated with the determinations of the anaesthetic EC_{50}s were typically large (~20–30% of the EC_{50} values) which may reflect individual variability in sensitivity of the tadpoles to the tested agents. 2,6-dimethylcyclohexanol was considered a moderately potent anaesthetic producing anaesthesia at ~7-fold the EC_{50} value reported for propofol using a similar assay (Krasowski et al., 2001, and this study). Cyclohexanol, as reported previously (Watt et al., 2008), did not anaesthetise any tadpoles (n = 30) up to 300 μM and was therefore considered inactive.

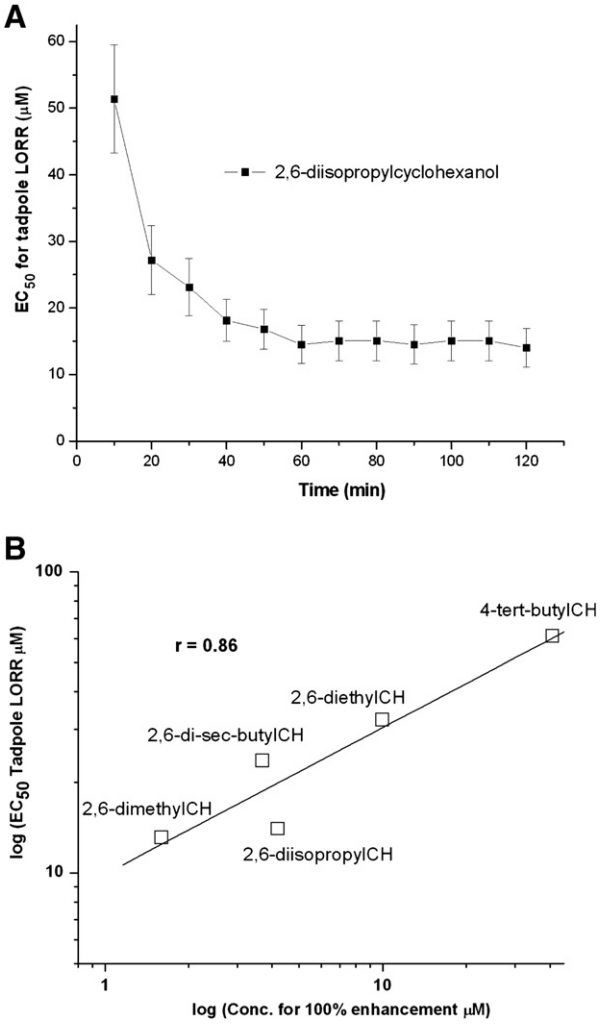

Fig. 4. Cyclohexanols act as general anaesthetics in a tadpole loss of righting reflex assay, possibly via action at $GABA_A$ receptors. A) 2,6-diisopropylcyclohexanol EC_{50} values for loss of righting reflex in tadpoles were calculated over time (10–120 min) using the Waud equation (see Materials and methods). 2,6-diisopropylcyclohexanol gave a minimal EC_{50} of 14.0 + 3.0 μM (n = 30 tadpoles) after 60 min. Error bars indicate S.E.Ms. B) Cross-correlation analysis of the potencies of the cyclohexanols for producing loss of righting reflex in tadpoles and for potentiation of $GABA_A$ receptor currents (I_{GABA}). From tadpole loss of righting reflex assays, minimal EC_{50} values were calculated for 2,6-dimethylcyclohexanol, 2,6-diethylcyclohexanol 2,6-diisopropylcyclohexanol, 2,6-di-sec-butylcyclohexanol and 4-tert-butylcyclohexanol (n>30 tadpoles). The tadpole anaesthesia EC_{50} values were plotted against the concentration of the compound that produced an estimated 100% enhancement of 30 μM GABA responses in oocyte recordings. Concentrations that produced 2-fold (100%) enhancement were estimated through a linear fit between points in Fig. 3A and B immediately below and above the 100% level of enhancement level. Potencies are expressed as log (concentration) with concentrations in μM units. Note: 2,6 di-tert-butylcyclohexanol data were not included in this analysis since this agent never produced 100% enhancement of the GABA current at any concentration tested (see Fig. 3A).

Finally, for cyclohexanols in Fig. 3 the correlation coefficient between the potencies of cyclohexanol analogues for producing loss of righting reflex in tadpoles and for the estimated concentration of the agent that produced a 100% enhancement of GABA current was calculated (Fig. 4B). There was a linear relationship between these values with a correlation coefficient r = 0.86. This level of correlation is suggestive of the involvement of $GABA_A$ receptor modulation in producing anaesthesia. Furthermore, agents that were ineffective at enhancing GABA currents (e.g. all the cyclohexanols in Fig. 3B except 4-tert-butylcyclohexanol) also lacked potency as anaesthetics (e.g. 2-tert-butyl-cyclohexanol and 4-tert-butyl-cyclohexanol gave tadpole anaesthesia EC_{50} values of 54.3 ± 4.0 μM and 61.1 ± 12.7 μM, respectively). Nevertheless, given the variability in the anaesthetic EC_{50} values, and the deviations of the points from the linear fit and limited number of points in Fig. 4B, other molecular mechanisms for producing anaesthesia certainly cannot be excluded.

4. Discussion

Propofol, 2,6-diisopropylphenol, and its analogues have been shown to be potent positive modulators of $GABA_A$ receptor currents, and to act as sedatives and anaesthetics in vivo (Krasowski et al., 2001). Given our previous findings that menthol (2-isopropyl-5-methylcyclohexanol) can evoke similar (albeit less pronounced) responses (Hall et al., 2004a) possibly via equivalent sites on $GABA_A$ receptors (Watt et al., 2008), we investigated a series of cyclohexanol analogues for receptor modulation and for their actions as anaesthetics. The major findings of this study were that (1) some cyclohexanol analogues were potent positive modulators of recombinant $GABA_A$ receptor currents (2) aliphatic groups in both *ortho* positions adjacent to the hydroxyl group were important for effective modulation of GABA currents (3) addition of a single aliphatic group on the cyclohexanol ring was not sufficient to confer activity (4) substitutions with bulkier aliphatic groups (e.g. tert-butyl) reduced activity (5) in a tadpole loss of righting reflex assay, cyclohexanol analogues that were potent $GABA_A$ receptor modulators were also effective general anaesthetics.

Propofol is evidently a far more potent positive modulator of GABA receptor currents than any of the cyclohexanols tested (e.g. EC_{50} for propofol ~ 3-fold less than for 2,6-dimethylcyclohexanol, see Fig. 3A). It is intriguing that the most potent of the cyclohexanols (2,6-dimethylcyclohexanol, 2,6-diethylcyclohexanol, 2,6-diisopropylcyclohexanol, 2,6-di-sec-butylcyclohexanol) possess some structural similarities to the intravenous general anaesthetic. Although propofol is a phenol with a planar ring structure and the cyclohexanols adopt chair structures, they share equivalent 2,6 positioning of the aliphatic chains adjacent to their respective hydroxyl groups (Fig. 1). The *ortho* positioning of an aliphatic chain has been shown to be an important requirement for activity of propofol analogues for modulation (Krasowski et al., 2001; 2002) and for direct activation of $GABA_A$ receptor currents (Mohammadi et al., 2001). Since propofol is a phenol, the positioning of the hydroxyl and isopropyl groups would be planar in relation to the ring. For cyclohexanol-based structures the stereochemistry of these molecules is such that the most stable chair

conformations place the hydroxyl and aliphatic groups equatorially. Thus, the arrangement in space for these substituents on the cyclohexanols is similar to a planar molecule like propofol. It is noted that in this study there was no attempt to isolate the individual stereoisomers of the cyclohexanol analogues tested. Stereo-selective action for positive modulation of GABA currents has been widely reported for different enantiomers of general anaesthetics (Hall et al., 1994; Tomlin et al., 1998), and evidently cyclohexanol-based compounds are no exception (Corvalan et al., 2009). Therefore, it is possible that certain isolated diastereomers or even enantiomers of the cyclohexanol analogues may be more potent as regards both receptor modulation and anaesthetic action.

A surprising result of the current study was that potencies for $GABA_A$ receptor modulation by 2,6-dimethylcyclohexanol, 2,6-diethylcyclohexanol, 2,6-diisopropylcyclohexanol and 2,6-di-sec-butylcyclohexanol were approximately equal (see Fig. 3A). By contrast, for the 2,6-substituted phenols, Krasowski et al. (2001) demonstrated that the increasing size of the aliphatic chains (up to sec-butyl) resulted in increased potency for GABA modulation and of anaesthetic action. This may be because the phenols are rigid and therefore the size and shape of the alkyl group is crucial for activity. On the other hand the cyclohexanol chair conformation is fluxional and therefore the size and shape of the alkyl groups is less important to complexation with the active site. In the same study (Krasowski et al., 2001) it was also shown that the addition of di-tert-butyl groups in both ortho positions rendered the phenols inactive. Likewise, in our study 2,6-di-tert-butylcyclohexanol was minimally effective as both a receptor modulator and as an anaesthetic, presumably due to the excessively bulky nature of these groups. Finally, when the cyclohexanol analogues were mono-substituted with aliphatic chains in the ortho- and para-positions, the compounds had little activity as either receptor modulators or anaesthetics. As an extension of this principle, cyclohexanol itself produced minimal positive enhancement of GABA currents ($30.1 \pm 10.3\%$ at 300 μM) and no anaesthesia.

We explored the potential for the cyclohexanol analogues to act as general anaesthetics. Assessing the loss of righting reflex in tadpoles using an established procedure (Downes and Couragen, 1996; Krasowski et al., 2001) enabled us to derive EC_{50}s for most of the drugs tested. For instance, the EC_{50} for 2,6-diisopropylcyclohexanol to induce a loss of the righting reflex in tadpoles reached a minimum (14.0 ± 3.0 μM) after 60 min exposure (Fig. 4A). It is recognised that many factors may contribute to the reported anaesthetic potency of a given agent including relative lipophilicity, drug uptake, and subsequent metabolism. Indeed, it might be expected that the compounds with larger aliphatic chains (e.g. 2,6-di-sec-butylcyclohexanol) would be more lipophilic and thus more readily absorbed into tadpole tissues. Nevertheless, it was 2,6-dimethylcyclohexanol and 2,6-diisopropylcyclohexanol that were shown to be moderately potent general anaesthetics with $EC_{50} = 13.1 \pm 3.0$ μM and 14.0 ± 3.0 μM, respectively while the EC_{50} value for 2,6-di-sec-butylcyclohexanol was determined to be 23.6 ± 5.9 μM. By comparison, we recorded an EC_{50} value of 1.7 ± 0.4 μM for propofol-induced loss of righting reflex in agreement with previous studies (Krasowski et al., 2001; Tonner et al., 1997). The most potent cyclohexanols were therefore at least 8-fold less potent than propofol in producing anaesthesia (see Fig. 4B). However, although no toxicity data are currently available for the novel cyclohexanol analogues, such compounds are typically well tolerated (e.g. see Thorup et al., 1983 for menthol) and may present interesting leads as novel anaesthetics with therapeutic indices within respectable ranges. Furthermore, the activity of the cyclohexanols might be enhanced by use of a single diastereomer (note the relative percentages of cis/cis, cis/trans and trans/trans diastereomers in Table 1).

Finally, a GABAergic mechanism for the general anaesthetic action of the cyclohexanol analogues was somewhat supported by correlation analyses of EC_{50} for loss of righting reflex vs. the level of current enhancement (Fig. 4B, albeit with a correlation coefficient of $r = 0.86$ based on only 5 data points). There is a possibility that, as well as having different pharmacokinetic and pharmacodynamic profiles, the cyclohexanol analogues mediate their anaesthetic actions through other receptor interactions. For instance, 2-isopropyl,4-methylcyclohexanol (i.e. menthol) is a potent activator ($EC_{50} \sim 80$ μM) of the cold- and menthol-sensitive receptor-1 (CMR1), also known as the transient receptor potential channel, TRPM8 which is known to be expressed in sensory neurones (McKemy et al., 2002). Furthermore, alcohols (e.g. octanol) have also been shown to be effective inhibitors of gap junction permeability (Chanson et al., 1989). Thus, the cyclohexanols could potentially impact motor output by modulating transmission at electrical synapses between motoneurones in *Xenopus* tadpoles (see Perrins and Roberts, 1995).

5. Conclusion

We have demonstrated that cyclohexanols with short aliphatic chains in both *ortho* positions act as potent positive allosteric modulators of $GABA_A$ receptors and may have the potential to act as general anaesthetics. Although we suggest that the cyclohexanols may be producing their hypnotic effect via modulation of GABA currents, they may not be acting *via* GABAergic mechanisms alone. For future studies, the design of novel cyclohexanol-based anaesthetics may depend on isolating individual diastereomers (or possible enantiomers) and exploring the potential for their stereoselective action both in vitro and in vivo.

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