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Characterization of the electrophysiological and pharmacological effects of 4-iodo-2,6-diisopropylphenol, a propofol analogue devoid of sedative-anaesthetic properties

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1 Several derivatives and analogues of the general anaesthetic 2,6-diisopropylphenol (propofol) have been recently synthesised with the aim of exploring the structure-activity relationships.

2 In the present study, the effects of one such compound, 4-iodo-2,6-diisopropylphenol (4-I-Pro), on γ-aminobutyric acid type A ($GABA_A$) receptors *in vitro* were compared with its *in vivo* effects in rodents. Human $GABA_A$ receptors were expressed in *Xenopus* oocytes, and the actions of 4-I-Pro on receptor function were compared with those of propofol by two-electrode voltage-clamp recording.

3 Similar to propofol, 4-I-Pro directly activated Cl^- currents in the absence of GABA at all combinations of receptor subunits tested. However, the efficacy of 4-I-Pro in inducing direct activation of $\alpha1\beta2\gamma2S$ receptors was markedly less than that of propofol.

4 Similarly to propofol, 4-I-Pro potentiated in a concentration-dependent manner GABA-evoked Cl^- currents measured at different $GABA_A$ receptor constructs.

5 As expected, intraperitoneal injection of propofol induced sedation, ataxia, and loss of the righting reflex in rats. In contrast, administration of 4-I-Pro failed to produce any of these behavioural effects.

6 Administration of 4-I-Pro to rats reduced in a dose-dependent manner the incidence of tonic-clonic seizures induced by pentylenetetrazol and induced an anticonflict effect as measured in the Vogel test.

7 Microdialysis revealed that, like propofol, administration of 4-I-Pro reduced acetylcholine release in the hippocampus of freely moving rats.

8 These results demonstrate that para-substitution of the phenol ring of propofol with iodine yields a compound that exhibits anticonvulsant and anticonflict effects, but is devoid of sedative-hypnotic and anaesthetic properties. Thus, 4-I-Pro possesses pharmacological characteristics more similar to anxiolytic and anticonvulsant drugs than to general anaesthetics.

Keywords: Propofol; 4-iodo-propofol; general anaesthesia; $GABA_A$ receptors; Cl^-- currents; *Xenopus* oocytes; voltage-clamp; exploratory activity; anticonflict effect; anticonvulsant activity

Abbreviations: ANOVA, analysis of variance; GABA, γ-aminobutyric acid; 4-I-Pro, 4-iodo-propofol; LRR, loss of righting reflex; propofol, 2,6-diisopropylphenol

Introduction

Propofol (2,6-diisopropylphenol) is a widely used intravenous anaesthetic agent that is chemically unrelated to other anaesthetics (Langley & Heel, 1988; Sebel & Lowdon, 1989). Since its introduction into clinical practice about a decade ago, propofol has proven efficacious for the induction and maintenance of general anaesthesia and sedation (McCollum *et al*., 1987; Gunawardene & White; 1988; Langley & Heel, 1988; Green & Jonsson, 1993). Thus, propofol-induced anaesthesia is rapid in onset and readily controlled, and is associated with rapid recovery and a reduced incidence of nausea and vomiting (Gunawardene & White, 1988; Langley & Heel, 1988). In addition, propofol exhibits antiseizure activity both in laboratory animals and in humans with status epilepticus (Simpson *et al*., 1988; Lowson *et al*., 1990; Al-Hader *et al*., 1992; De Riu *et al*., 1992).

Although a unified theory capable of explaining the precise molecular mechanisms of action of propofol and most other general anaesthetics is still lacking, substantial experimental evidence indicates that γ-aminobutyric acid type A ($GABA_A$) receptors are major targets of propofol, being implicated in both the behavioural and pharmacological actions of this agent (Tanelian *et al*., 1993; Franks & Lieb, 1994). Thus, neurochemical and electrophysiological studies have revealed that propofol, similar to other general anaesthetics such as alphaxalone, pentobarbital, and etomidate, enhances the function of $GABA_A$ receptors in various regions of rat brain (Collins, 1988; Concas *et al*., 1990, 1991; Hales & Lambert, 1991; Peduto *et al*., 1991; Lin *et al*., 1992; Hara *et al*., 1993).

The actions of propofol at $GABA_A$ receptors are complex and include potentiation of GABA-induced responses, direct receptor activation in the absence of GABA, and alteration of receptor desensitization (Concas *et al*., 1991; Hales & Lambert, 1991; Hara *et al*., 1993; Orser *et al*., 1994; Sanna *et al*., 1995a,b). However, the importance of each of these actions in the induction of general anaesthesia is not clear.

Studies on the effects of propofol at recombinant $GABA_A$ receptors have revealed that its modulation of GABA-evoked

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Cl^- currents does not appear to be strictly dependent on the presence of any one receptor subunit, being manifest with all subunit combinations tested (Jones *et al*., 1995; Sanna *et al*., 1995b; Davies *et al*., 1997). In contrast, the direct activation of $GABA_A$ receptors by propofol, an action shared by pentobarbital and etomidate, requires the presence of a β subunit (Sanna *et al*., 1995a,b; 1997; Cestari *et al*., 1996); the β subunit, however, does not appear to be important for the direct effect of the steroid anaesthetic alphaxalone (Sanna *et al*., 1995a,b; 1997). Despite the high degree of amino acid sequence homology among $GABA_A$ receptor subunits, homomeric receptors composed of recombinant ρ_1 subunits are insensitive to propofol as well as to other general anaesthetics (Mihic & Harris, 1996). Together, these results have led to the notion that propofol interacts directly with specific domains of $GABA_A$ receptors. Further characterization of the sites of interaction of propofol as well as that of other general anaesthetics with $GABA_A$ receptors may be achieved by the construction of subunit chimaeras and site-directed mutagenesis (Mihic *et al*., 1997).

It may be possible to modify the molecular structure of propofol in order to optimize all its various (anaesthetic, sedative, anticonvulsant) activities or to yield drugs with more selective actions. With this goal in mind, we have recently synthesized several propofol derivatives and congeners, and analysed the structure-activity relations of such compounds with neurochemical and electrophysiological methods. Introduction of halogen or benzoyl substituents in the para position of the phenyl group of propofol yielded a series of molecules that inhibit the binding of *t*-[^{35}S]-butylbicyclophosphorothionate to $GABA_A$ receptors and potentiate GABA-evoked currents at these receptors with an efficacy similar to or higher than that of propofol (Trapani *et al*., 1998). Among such derivatives, 4-iodo-propofol (4-I-Pro) (Figure 1) exhibits molecular and pharmacological actions that differ markedly from those of propofol. We now describe the neurochemical, electrophysiological, pharmacological, and behavioural actions of 4-I-Pro and compare them with those of the parent compound.

Methods

Synthesis of 4-I-Pro

A solution of iodine monochloride (0.81 g, 5 mmol) in 30 ml of acetic acid was added dropwise to a stirred solution of propofol (1.78 g, 10 mmol) (Aldrich, Gillingham, U.K.) in 30 ml of acetic acid at room temperature. The resulting mixture was stirred for 3 h at room temperature, and then allowed to evaporate under reduced pressure. The residue was subjected to chromatography on a column of silica gel with petroleum ether:ethyl acetate (95:5, v v^{-1}) as eluent to provide 1.2 g (40% yield) of 4-I-Pro as a yellow oil (boiling point, 90–92°C at 1 mmHg). IR (Infrared spectroscopy) 3575 cm^{-1}; [^{1}H]-NMR (Proton magnetic resonance) ($CDCl_3$ as solvent), chemical shifts from tetramethylsilane δ: 1.20 (doublet, $J=6.5$ Hz, 12H, CH_3), 3.05 (septet, $J=6.5$ Hz, 2H, C-$H(CH_3)_2$), 4.70 (singlet, 1H, OH), 7.20 (s, 2H, Ar-H); MS (Mass spectroscopy), m z^{-1} 304 (83, M^+), 289 (base). Elemental analysis $(C_{12}H_{17}IO)$C,H.

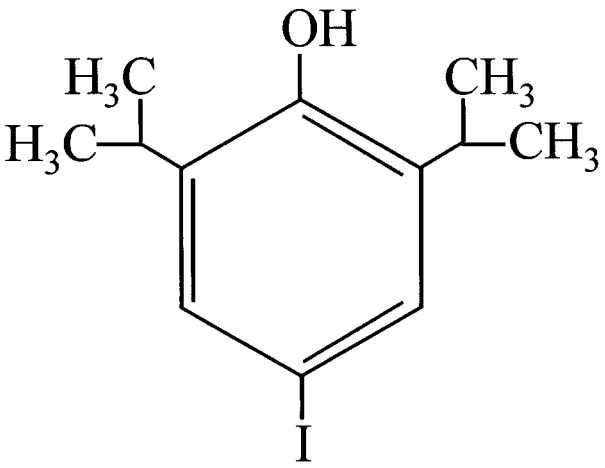

Figure 1 Chemical structure of 4-iodo-2,6-diisopropylphenol (4-I-Pro).

In vitro *studies*

Preparation of cDNAs Complementary DNAs encoding the human α1, β1, β2, β3, and γ2S $GABA_A$ receptor subunits were subcloned into the pCDM8 expression vector (Invitrogen, San Diego, CA, U.S.A.) (Hadingham *et al*., 1993) and purified with Wizard Plus Miniprep kits (Promega, Madison, WI, U.S.A.). After resuspension in sterile distilled water, the cDNAs were stored at −20°C until use.

Isolation of Xenopus *oocytes and microinjection of cDNAs* Adult *Xenopus laevis* females were obtained from Dipl. Biol.-Dipl. Ing. Horst Kähler (Hamburg, Germany). Stage V and VI oocytes were isolated with the use of fine surgical forceps after manual dissection of the ovary and exposed to collagenase type IA as described previously (Sanna *et al*., 1995a). Various combinations of cDNAs encoding different $GABA_A$ receptor subunits (total of 1.5 ng of DNA in 30 nl) were injected into oocyte nuclei with the use of a 10-μl glass micropipette (tip diameter, 10–15 μm). The injected oocytes were then transferred to modified Barth's saline [(in mM) NaCl 88, KCl 1, $NaHCO_3$ 2.4, HEPES-NaOH (pH 7.5) 10, $MgSO_4$ 0.82, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.91] supplemented with sodium pyruvate (2 mM), penicillin (10,000 U l^{-1}), streptomycin (10 U l^{-1}), gentamicin (50 U l^{-1}), and theophylline (0.5 mM), and were stored at 20°C until use (usually for up to 4 days, during which time they were transferred to fresh medium each day).

Electrophysiological recording Electrophysiological recording was initiated 18–24 h after cDNA injection and was performed as previously described (Sanna *et al*., 1995a). In brief, oocytes were placed in a rectangular recording chamber (volume, 100 μl) and continuously perfused with modified Barth's saline at a flow rate of 2 ml min^{-1} at room temperature. Oocytes were impaled at the animal pole with two glass microelectrodes (0.5–5 MΩ) filled with filtered 3 M KCl, and were voltage-clamped at −70 mV with an Axoclamp 2-B amplifier (Axon Instruments, Burlingame, CA, U.S.A.). The resting membrane potential usually ranged between −30 and −50 mV. Drugs were applied for 20 s; in particular, propofol and 4-I-Pro were perfused alone, for determining their direct effects, or together with GABA, for determining their modulatory effects. Intervals of 5 min were allowed between applications of low concentrations of GABA (Sigma, St Louis, MO, U.S.A.) alone and of $\geqslant$10 min between applications of GABA at higher concentrations or of propofol and 4-I-Pro. Propofol and 4-I-Pro were prepared in dimethyl sulphoxide as stock solutions (10 mM), aliquoted in small fractions, and frozen at −20°C until use. Before each experiment, propofol and 4-I-Pro were diluted to the

appropriate concentration (1–250 μM) with modified Barth's solution. The concentration of dimethyl sulphoxide ranged from 0.01–2.5% and did not modify GABA-induced currents. Data from electrophysiological experiments were expressed as a percentage of control current responses obtained with GABA alone. The GABA control response was measured before and after each drug application to take into account possible shifts in the control current. Potentiation of GABA-induced currents by propofol or 4-I-Pro was defined as the per cent increase of the control GABA response; the GABA control response was GABA EC_{20}, defined as the concentration of GABA producing 20% of the maximum GABA response; this concentration was determined experimentally for each cell at the beginning of every experiments. Concentration-response curves were fitted (Jandel Sigmaplot 4.01) with the logistic equation:

$$I = I_{max}/(1 + C/EC_{50})^{n_H}$$

where I_{max} is the maximum current observed, C is the drug concentration, EC_{50} the concentration of drug producing 50% of the maximum response, and n_H the Hill coefficient.

Each oocyte represents a single *n* value, and oocytes from different frogs were used for each experiment.

In vivo *studies*

Animals Male CD-1 mice (Charles River, Como, Italy) with body masses of 25–30 g and male Sprague-Dawley CD rats (Charles River) with body masses of 200–225 g were maintained under a 12 h light/dark cycle at a temperature of 23±2°C and 65% humidity. After arrival at the animal facility, the animals were acclimatized for a minimum of 7 days, during which they had free access to food and water.

Drug administration Propofol and 4-I-Pro were dissolved by adding a drop of Tween 80 per 5 ml of saline and were administered intraperitoneally in a volume of 0.3 ml per 100 g of body mass.

Acute sedative-hypnotic effects in rats Sedative and hypnotic actions of propofol and 4-I-Pro were evaluated by monitoring signs of intoxication for 60 min after the administration of a single dose of drug using an adaptation of the scale by Majchrowicz (1975). This scale, originally designed for assessing the degree of intoxication in rats by acute administration of ethanol, was used in this study because of

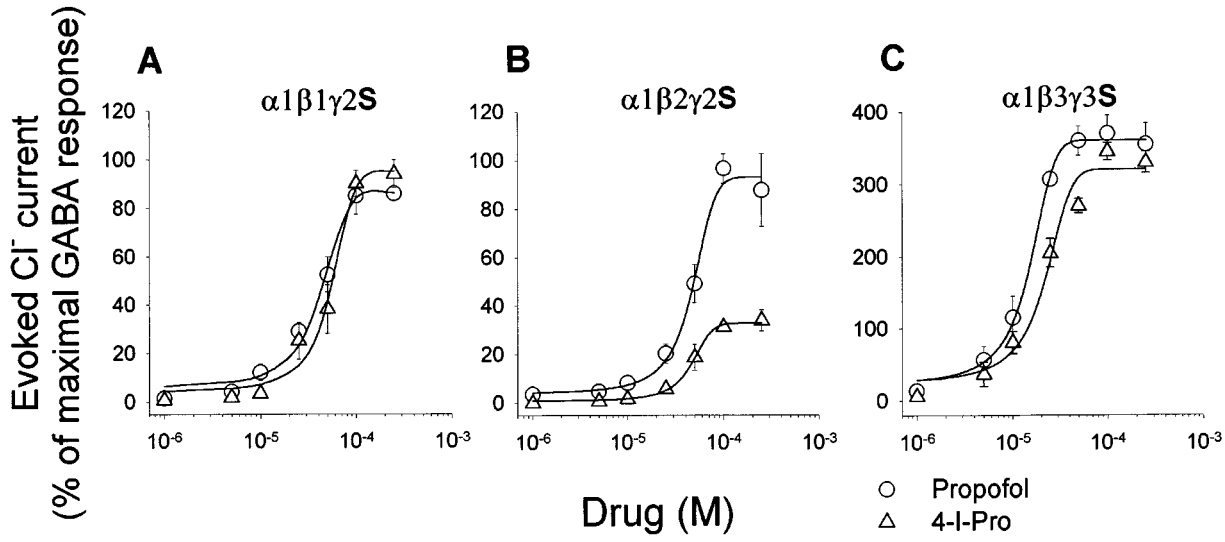

Figure 2 Direct effects of propofol and 4-I-Pro on Cl^- currents in *Xenopus* oocytes expressing $GABA_A$ receptors containing different β subunit isoforms. Chloride currents evoked by various concentrations (1–250 μM) of propofol or 4-I-Pro were measured in *Xenopus* oocytes expressing $\alpha1\beta1\gamma2S$ (A), $\alpha1\beta2\gamma2S$ (B), or $\alpha1\beta3\gamma2S$ (C) receptor subunit assemblies. Data are means±s.e.mean (n=8–10) and are expressed as a percentage of the control response obtained with 10 mM GABA.

Table 1 Direct activation of Cl^- currents by propofol and 4-I-Pro in *Xenopus* oocytes expressing recombinant human $GABA_A$ receptors

Receptor construct	n	*Maximal activation (%)*	*EC_{50} (μM)*	*Hill coefficient*
Propofol				
$\alpha1\beta1\gamma2S$	8	86±3	38±2.1	1.9±0.2
$\alpha1\beta2\gamma2S$	10	97±6	47±5.8	2.2±0.4
$\alpha1\beta3\gamma2S$	10	371±25*	13±1.6*	2.3±0.4
4-I-Pro				
$\alpha1\beta1\gamma2S$	10	94±6	58±4.4	2.1±0.3
$\alpha1\beta2\gamma2S$	9	34±4**	45±2.8	2.3±0.4
$\alpha1\beta3\gamma2S$	9	345±12*	20±6.9*	1.8±0.3

Maximal direct activation of Cl^- currents is expressed as a percentage of the control response obtained with 10 mM GABA; values are means±s.e.mean for the indicated number (n) of oocytes. *$P<0.05$ vs $\alpha1\beta1\gamma2S$ or $\alpha1\beta2\gamma2S$ receptors (Student's t-test). **$P<0.05$ vs effect of propofol at $\alpha1\beta2\gamma2S$ receptors (Student's t-test).

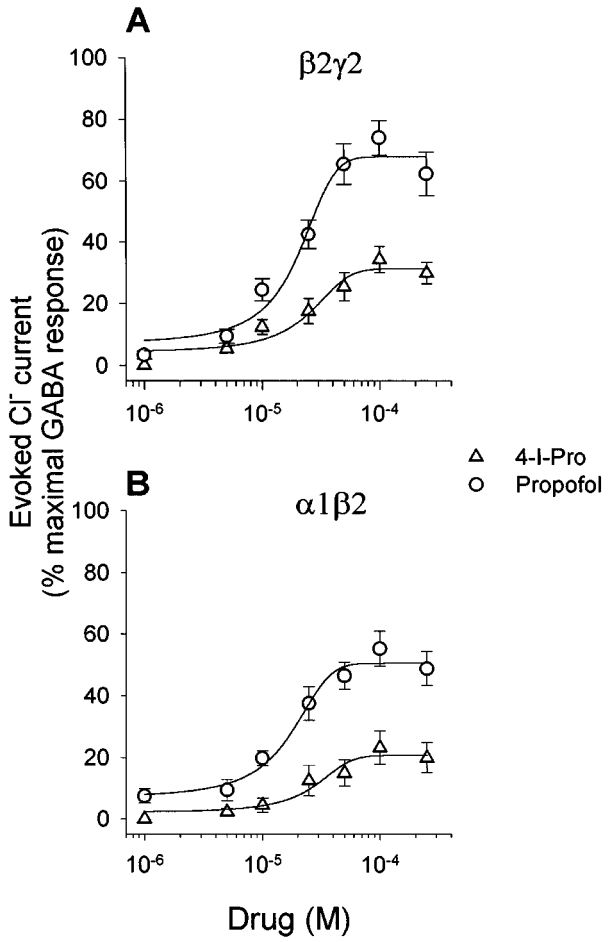

Figure 3 Direct effects of propofol and 4-I-Pro on Cl^- currents in *Xenopus* oocytes expressing $\beta2\gamma2S$ (A) or $\alpha1\beta2$ (B) $GABA_A$ receptors. Chloride currents evoked by various concentrations (1–250 μM) of propofol or 4-I-Pro were measured and expressed as a percentage of the control response obtained with 10 mM GABA. Data are means ± s.e.mean ($n = 6–8$).

the many similarities between the intoxicant effect of this drug and that of the general anaesthetic propofol. The occurrence of a set of different behavioural signs, ranging from 'sedation' to 'loss of righting reflex' was recorded and expressed as a percentage of animals presenting a given behavioural sign. The loss of the righting reflex was used as an index of the hypnotic (anaesthetic) action of these drugs, and the duration of this action was recorded.

Exploratory behaviour in mice To study the influence of propofol and 4-I-Pro on exploratory behaviour, we placed groups of five mice in a new cage (39 × 39 cm, with criss-crossing infrared beams) immediately after the injection of drug or vehicle. Exploratory behaviour was then measured for 20 min with an Omnitech behaviour meter (Omnitech Electronics, Columbus, Ohio, U.S.A.), which monitors horizontal and vertical movements as the total number of interruptions of the vertical and horizontal light beams, respectively.

Anticonvulsant activity against pentylenetetrazol-induced seizures in rats Groups of ten rats were injected intraperitoneally with various doses of 4-I-Pro or vehicle 12 min before the administration of pentylenetetrazol (55 mg kg^{-1}, i.p.) dissolved in physiological saline. Rats were then observed for 1 h to determine the incidence and time to onset of convulsions. All of the animals in the control group exhibited generalized tonic-clonic convulsions within 1–3 min.

Vogel's anticonflict test Rats were deprived of water for 24 h before the anticonflict test, which was performed as previously described (Corda *et al*., 1983). Briefly, rats were placed in a clear Plexiglas box (20 × 28 × 20 cm) with a stainless steel grid floor, and the box was enclosed in a sound-attenuated ventilated chamber (Lafayette Instruments, Lafayette, IN, U.S.A.). Water was provided through a stainless steel drinking tube that extended 1 cm into the box, 3 cm above the floor. The drinking tube and the grid floor were connected to a

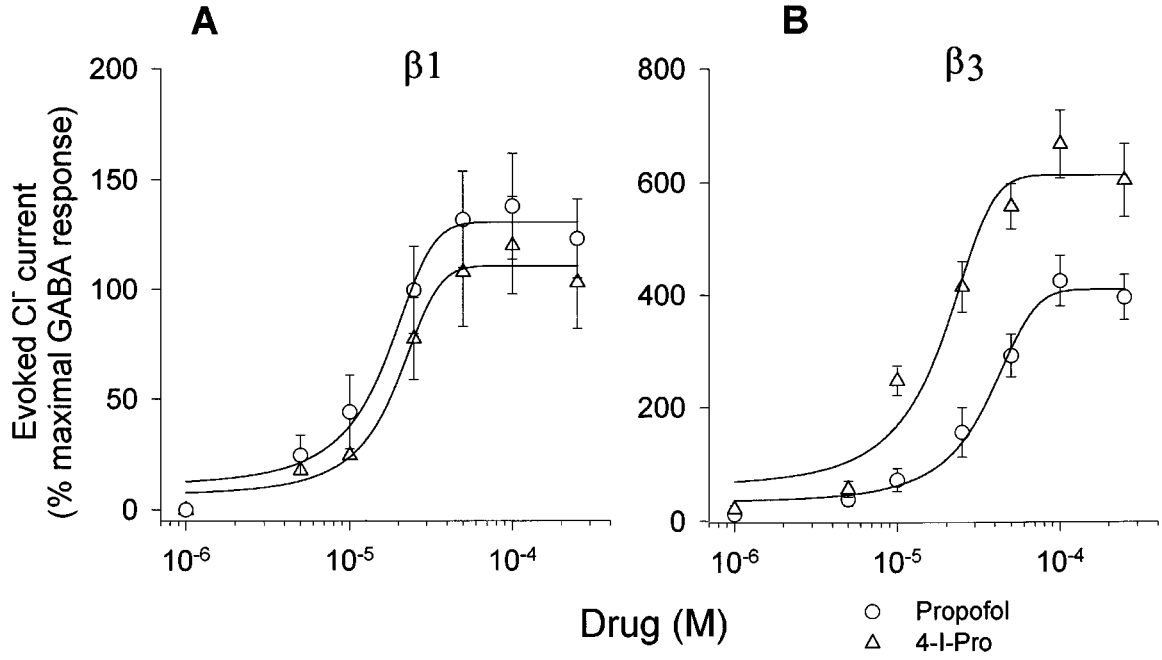

Figure 4 Direct effect of propofol and 4-I-Pro on Cl^- currents in *Xenopus* oocytes expressing $\beta1$ (A) or $\beta3$ (B) homomeric $GABA_A$ receptors. Chloride currents evoked by various concentrations (1–250 μM) of propofol or 4-I-Pro are expressed as a percentage of the control response obtained with 10 mM GABA. Data are means ± s.e.mean ($n = 6–12$).

constant-current shock generator and to a 'drinkometer'. The shock generator delivered one shock (0.6 mA), lasting 0.5 s, after each cumulative drinking period of 15 licks, termed a 'licking period'. For measurement of unpunished drinking, the electric shock was omitted. A counter recorded the total number of licking periods. Experiments were performed between 14:00 and 19:00 h, and the animals were injected with vehicle or 4-I-Pro 5 min before the test.

Measurement of acetylcholine release by microdialysis Rats were anaesthetized with chloral hydrate (0.4 g kg^{-1}, i.p.), and a dialysis tube with a wet outer diameter of 320 μm

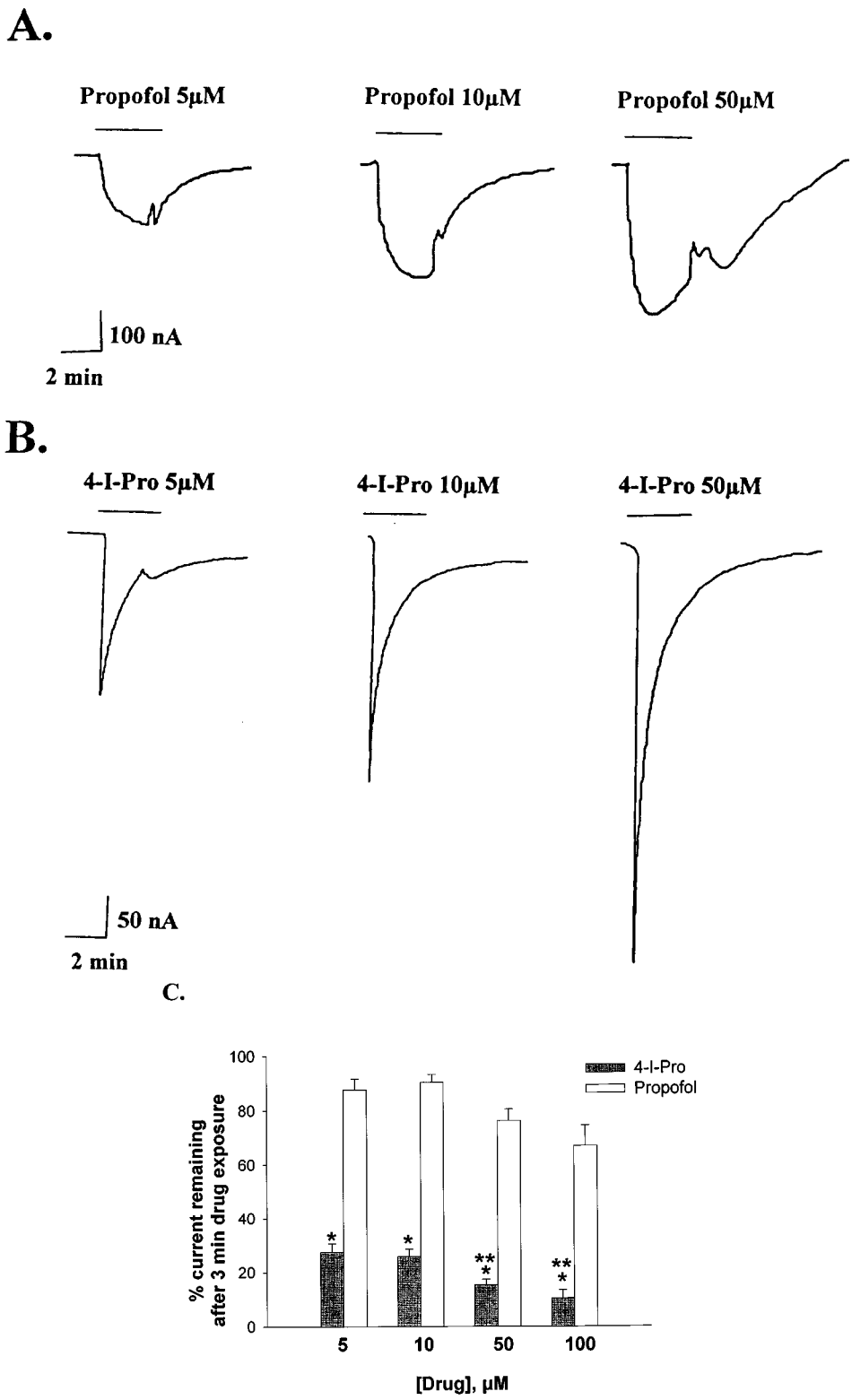

Figure 5 Different kinetic properties of propofol- or 4-I-Pro induced Cl^- currents. In (A) and (B), recordings were obtained from single oocytes expressing $\alpha 1\beta 2\gamma 2S$ $GABA_A$ receptors. Oocytes were exposed to each drug for 3 min in the absence of GABA. In (C), data are expressed as the percentage of current amplitude remaining after 3 min of drug exposure, assuming as 100% the initial current amplitude measured at the beginning of drug exposure. Values are means $\pm$ s.e.mean ($n = 25-42$ different oocytes). $^*P < 0.05$ vs propofol-induced currents; $^{**}P < 0.05$ vs 5 μM 4-I-Pro induced currents (Student's t-test).

(AN 69-HF; Hospal-Dasco, Bologna, Italy) was implanted at the level of the hippocampus according to the Paxinos atlas (A−3 from the bregma, V−3 from the dura). Surgery was performed according to the transversal microdialysis technique as described previously (Imperato *et al.*, 1992). The dialysis tube, containing a tungsten wire inside as a rigid support and not connected to the stainless steel cannula, was held directly in the micromanipulator of the stereotaxic instrument for insertion into the hippocampus. This technique minimizes tissue damage and reduces the glial reaction around the dialysis tube. Ringer solution (in mM) [KCl 3, NaCl 125, $CaCl_2$ 1.3, $MgCl_2$ 1.0, $NaHCO_3$ 23, and potassium phosphate buffer (pH 7.3) 1.5] was pumped through the dialysis probe at a constant rate of 2 μl min^{-1}. Samples (40 μl) were collected every 20 min.

To achieve detectable amounts of acetylcholine in the dialysate, we added 0.1 μM neostigmine to the Ringer's solution. Acetylcholine was measured by high-performance liquid chromatography with electrochemical detection as described (Damsma & Westerink, 1991); the detection limit was 0.05 pmol per injection. Experiments were initiated 24 h after implantation of the dialysis tube. The average concentration of neurotransmitter in the last three samples before treatment was taken as 100%, and all subsequent, posttreatment values were expressed as a percentage of basal values.

Statistical Analysis

Data are presented as means ± s.e.mean. Electrophysiological data were analysed by Student's *t*-test. Data from behavioural experiments were analysed by Student's *t*-test and Fisher's exact probability test. For microdialysis experiments, between-groups comparisons were performed by two-way ANOVA for repeated measures; *post hoc* comparisons were performed by Newman-Keuls test.

Results

Electrophysiological effects of 4-I-Pro and propofol in vitro

Direct activation of $GABA_A$ receptors by propofol and 4-I-Pro Propofol directly activates Cl^- currents in the absence of GABA in *Xenopus* oocytes expressing recombinant $GABA_A$ receptors (Sanna *et al.*, 1995a,b) as well as in neurons or transfected mammalian cells (Hales & Lambert, 1991; Hara *et al.*, 1993; Jones *et al.*, 1995). We therefore compared the GABA-mimetic actions of propofol and 4-I-Pro in *Xenopus* oocytes expressing recombinant human $GABA_A$ receptors composed of $\alpha1$ and $\gamma2S$ subunits together with $\beta1$, $\beta2$, or $\beta3$. Propofol (1–250 μM) and 4-I-Pro (1–250 μM) each activated Cl^- currents in a concentration-dependent manner with all three combinations of receptor subunits tested (Figure 2); the maximal effects were apparent at drug concentrations of 100–250 μM. These currents were reversibly inhibited by the $GABA_A$ receptor blocker picrotoxin (1–50 μM) (data not shown).

The maximal current activation, expressed relative to the effect of a saturating concentration (10 mM) of GABA, induced by 4-I-Pro at $\alpha1\beta1\gamma2S$ or $\alpha1\beta3\gamma2S$ receptors was similar to that induced by propofol at the respective subunit assemblies (Figure 2 and Table 1). In contrast, the maximal effect of 4-I-Pro at receptors containing the $\beta2$ subunit was only ~35% of that of propofol. As previously shown (Sanna *et al.*, 1995b), the maximal effect of propofol was similar at $GABA_A$ receptors containing the $\beta1$ or $\beta2$ subunit, but it was approximately four times greater at receptors containing the $\beta3$ subunit; this difference was accompanied by a corresponding decrease in the median effective concentration (EC_{50}) for propofol at $\beta3$-containing receptors (Table 1). The maximal direct effect of 4-I-Pro, although lower at $\beta2$-containing receptors than at receptors containing $\beta1$, was also greatest at receptors containing the $\beta3$ subunit.

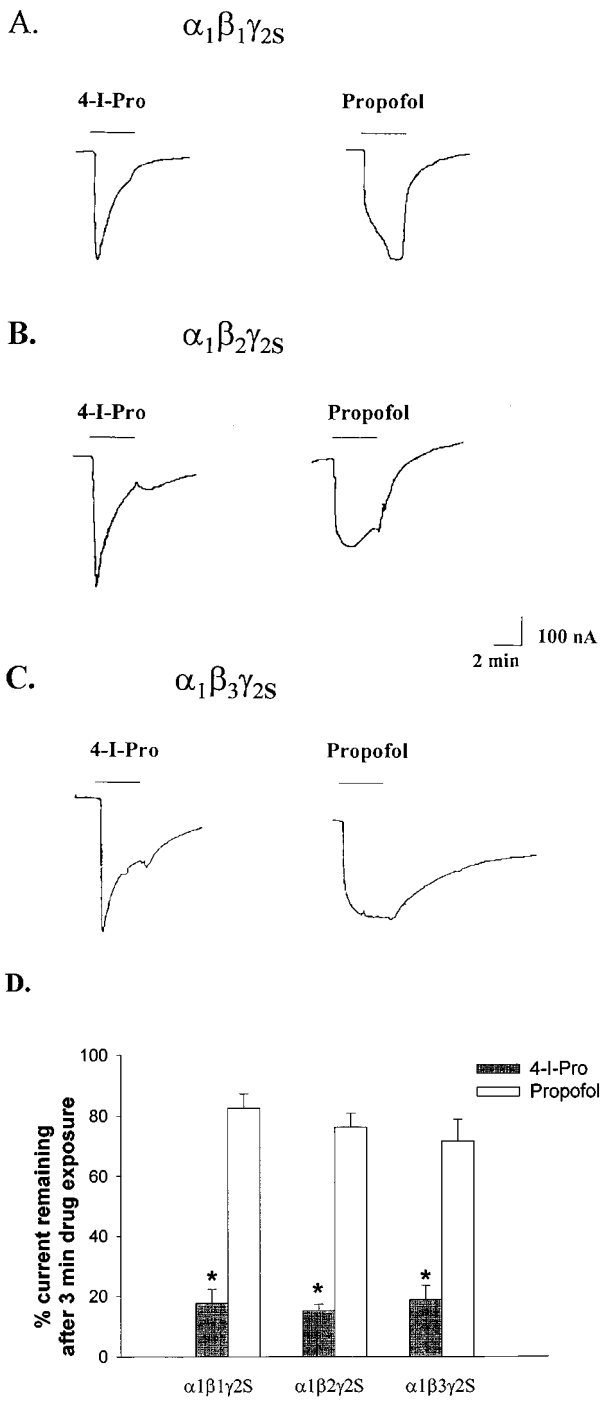

Figure 6 Distinct kinetic profile of $GABA_A$ receptor-mediated Cl^- currents induced by 4-I-Pro or propofol. Recordings were obtained from single oocytes expressing $\alpha1\beta1\gamma2S$ (A), $\alpha1\beta2\gamma2S$ (B), or $\alpha1\beta3\gamma2S$ (C) receptors. Oocytes were exposed to each drug (50 μM) for 3 min (horizontal bars) in the absence of GABA. In (D), data are expressed as the percentage of current amplitude remaining after 3 min of drug exposure, assuming as 100% the initial current amplitude measured at the beginning of drug exposure. Values are means ± s.e.mean (n = 12–42 different oocytes). *P < 0.05 vs propofol-induced currents (Student's *t*-test).

Consistent with the difference in efficacies between 4-I-Pro and propofol at $\alpha1\beta2\gamma2S$ $GABA_A$ receptors, the direct effects of 4-I-Pro at $\beta2\gamma2S$ and $\alpha1\beta2$ receptors were markedly smaller than those of propofol at these receptor constructs (Figure 3). In contrast, the direct effects of 4-I-Pro were similar to those of propofol at $\alpha1\beta1$, $\beta1\gamma2S$, $\alpha1\beta3$, and $\beta3\gamma2S$ receptors (data not shown). The high sensitivity of $\beta3$-containing receptors to the direct effects of 4-I-Pro and propofol was also apparent when the actions of these drugs were examined with homomeric receptors composed of only $\beta1$ or $\beta3$ (Figure 4). The maximal activation of Cl^- currents induced by the two compounds at $\beta3$ receptors was approximately three to five times that apparent at $\beta1$ receptors. In addition, whereas the efficacy of 4-I-Pro was similar to that of propofol at $\beta1$ receptors, it was greater than that of propofol at $\beta3$ receptors.

We next compared the Cl^- currents induced by continuous (3 min) application of 4-I-Pro and propofol. As shown previously (Sanna *et al*., 1995b), prolonged exposure of oocytes expressing $\alpha1\beta2\gamma2S$ receptors to propofol (5–100 μM) in the absence of GABA resulted in a sustained Cl^- current that did not decrease in the presence of the drug and that showed a slow decay after drug washout (Figure 5). In contrast, prolonged perfusion of 4-I-Pro produced a current that decreased in amplitude very rapidly in the presence of the drug and showed a faster recovery after drug washout (Figure 5). The different kinetic pattern of 4-I-Pro appeared to be dependent on drug concentration (Figure 5) but was not influenced by the receptor subunit composition (Figure 6), and was detected in five of nine oocytes expressing $\alpha1\beta1\gamma2S$ receptors, in 34 of 42 oocytes expressing $\alpha1\beta2\gamma2S$ receptors, and in eight of eight oocytes expressing $\alpha1\beta3\gamma2S$ receptors.

Modulation of GABA-evoked Cl^- currents by propofol and 4-I-Pro Similar to propofol, 4-I-Pro potentiated Cl^- currents induced by GABA (EC_{20}) at $\alpha1\beta1\gamma2S$, $\alpha1\beta2\gamma2S$, and $\alpha1\beta3\gamma2S$ receptors. However, while this effect was clearly evident at low concentrations (1–25 μM) of modulator (Figure 7), due to the high degree of direct activation it was not possible to separate and evaluate the modulatory action at higher drug concentrations.

Sedative-hypnotic effects of 4-I-Pro and propofol

Intraperitoneal administration of propofol (20 or 40 mg kg^{-1}) to rats induced a range of pharmacological effects in a dose-dependent manner. These effects included an initial sedation and ataxia as well as a rapid (within 1–3 min after injection) induction of general anaesthesia, indicated by loss of the righting reflex (Table 2). In contrast, administration of 4-I-Pro

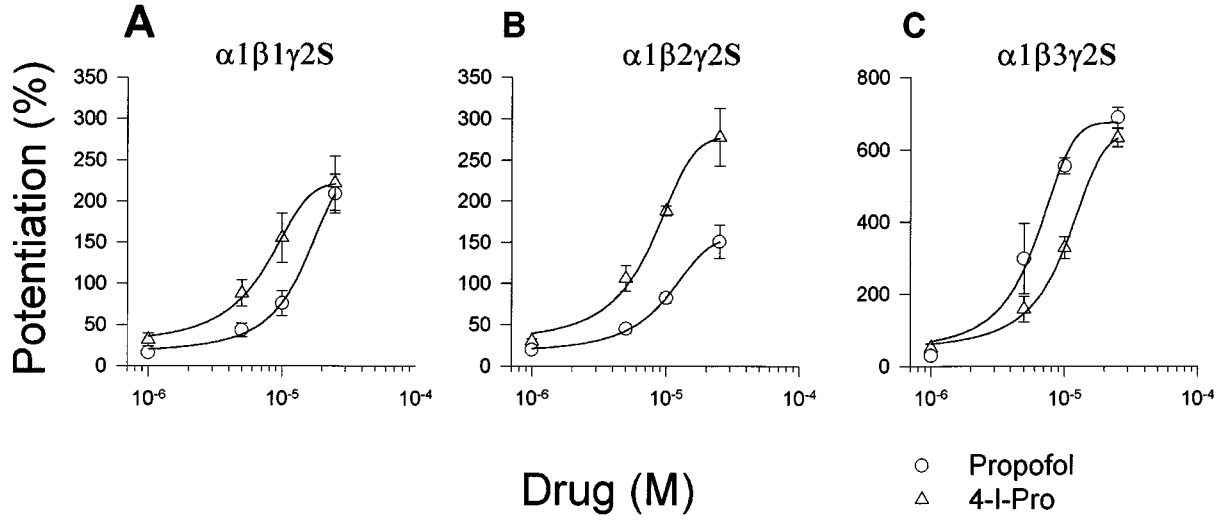

Figure 7 Modulatory effects of propofol and 4-I-Pro on GABA-induced Cl^- currents in *Xenopus* oocytes expressing $GABA_A$ receptors containing different β subunits. The effects of various concentrations (1–25 μM) of propofol or 4-I-Pro on Cl^- currents induced by GABA (EC_{20}) were measured in oocytes expressing $\alpha1\beta1\gamma2S$ (A), $\alpha1\beta2\gamma2S$ (B), or $\alpha1\beta3\gamma2S$ (C) receptors. Data are means ± s.e.mean (n = 8–12) and are expressed as a per cent potentiation of the control response to GABA (EC_{20}) alone. Actual EC_{20} values for GABA at the different receptors were determined experimentally for each oocyte: $\alpha1\beta1\gamma2S$, 5–15 μM (mean ± s.e.mean, 9.3 ± 0.9 μM); $\alpha\beta2\gamma2S$, 2–10 μM (5.4 ± 0.9 μM); $\alpha1\beta3\gamma2S$, 1–10 μM (3.5 ± 0.6 μM).

Table 2 Sedative-hypnotic effects of propofol and 4-I-Pro in rats

			Treatment		
Behavioural sign	*Propofol* (20 mg kg^{-1})	*Propofol* (40 mg kg^{-1})	*4-I-Pro* (20 mg kg^{-1})	*4-I-Pro* (40 mg kg^{-1})	*4-I-Pro* (60 mg kg^{-1})
Neutrality	0	0	100	100	100
Sedation	100	100	0	0	0
Ataxia 1	100	100	0	0	0
Ataxia 2	90	100	0	0	0
Ataxia 3	80	100	0	0	0
LRR	70*	100**	0	0	0

Groups of ten rats were injected intraperitoneally with propofol or 4-I-Pro, observed for the next 60 min. Values are expressed as percentage of animals displaying a given behavioural sign. The loss of righting reflex (LRR) was used as an index of the hypnotic (anaesthetic) action of each drug. *The means ± s.e.mean duration was 9 ± 3 min. **The means ± s.e.mean duration was 22 ± 5 min.

(20–60 mg kg^{-1}) did not induce any of these depressant effects (Table 2).

Effects of 4-I-Pro and propofol on exploratory behaviour

Consistent with the lack of ataxic-anaesthetic effects of 4-I-Pro in rats, this drug (60 mg kg^{-1}) had no significant effect on exploratory behaviour in mice, as assessed by interruption of horizontal and vertical beams of infrared light during the 20-min test period (Table 3). In contrast, propofol (20 and 40 mg kg^{-1}) markedly suppressed exploratory behaviour in a dose-dependent manner; thus, at 40 mg kg^{-1}, propofol reduced horizontal and vertical activity by 70 and 85%, respectively (Table 3).

Antiseizure activity of 4-I-Pro

4-I-Pro reduced in a dose-dependent manner the incidence of tonic-clonic seizures produced by pentylenetetrazol (55 mg kg^{-1}, i.p.) (Table 4). Whereas a dose of 20 mg kg^{-1} was ineffective, 4-I-Pro at 30, 45, and 60 mg kg^{-1} reduced the number of animals that developed seizures by 30, 50, and 100%, respectively; the drug did not have a significant effect on the time to onset of seizure activity in animals that exhibited convulsions. Complete inhibition of seizure activity was also obtained with sedative-hypnotic doses (20–40 mg kg^{-1}, i.p.) of propofol (data not shown).

Table 3 Effects of propofol and 4-I-Pro on exploratory behaviour in mice

	Exploratory behaviour	
Treatment	*Horizontal beam interruptions* 20 min^{-1}	*Vertical beam interruptions* 20 min^{-1}
Vehicle	2368±138	163±30
Propofol (20 mg kg^{-1})	414±146*	110±24*
Propofol (40 mg kg^{-1})	700±168	24±6*
4-I-Pro (60 mg kg^{-1})	2008±138	130±16

Data are means±s.e.mean of three separate experiments each with five mice per experimental group. Vehicle or drug administered immediately before recording of exploratory behaviour. *$P<$ vs vehicle-treated animals (Student's *t*-test).

Table 4 Effect of 4-I-Pro on pentylenetetrazol-induced seizures in rats

Treatment	*Latency of seizures* (min)	*No. of animals with seizures*
Vehicle	1.33±0.1	10/10 (100%)
Propofol (20 mg kg^{-1})	1.56±0.5	5/10 (50%)*
Propofol (30 mg kg^{-1})	1.51±0.6	2/10 (20%)*
Propofol (45 mg kg^{-1})		0/10 (0%)*
Propofol (60 mg kg^{-1})		0/10 (0%)*
4-I-Pro (20 mg kg^{-1})	1.40±0.3	10/10 (100%)
4-I-Pro (30 mg kg^{-1})	1.46±0.1	7/10 (70%)
4-I-Pro (45 mg kg^{-1})	1.20±0.4	5/10 (50%)*
4-I-Pro (60 mg kg^{-1})		0/10 (0%)*

Rats were injected intraperitoneally with propofol, 4-I-Pro or vehicle 12 min before administration of pentylenetetrazol (55 mg kg^{-1}, i.p.). Animals were observed for the next hour and the latency of tonic-clonic seizures and percentage of animals with seizures were recorded. *$P<0.05$ vs vehicle-treated animals (Fischer's exact probability test).

Anticonflict action of 4-I-Pro

Administration of 4-I-Pro (60 mg kg^{-1}, i.p.) elicited an anticonflict effect in rats. This treatment markedly increased (3.6 fold) the number of licking periods during punishment (Figure 8A), without having any effect on unpunished drinking behaviour (Figure 8B). In contrast, propofol (20 mg kg^{-1}) reduced the number of licking periods under the nonpunishment condition (vehicle, 83±5 licking periods 3 min^{-1}; propofol, 26±3 licking periods 3 min^{-1}).

Effects of propofol and 4-I-Pro on hippocampal acetylcholine release

Propofol (10–40 mg kg^{-1}, i.p.) induced a dose-dependent decrease in basal acetylcholine release in the hippocampus of freely moving rats (Figure 9); at doses of 20 and 40 mg kg^{-1},

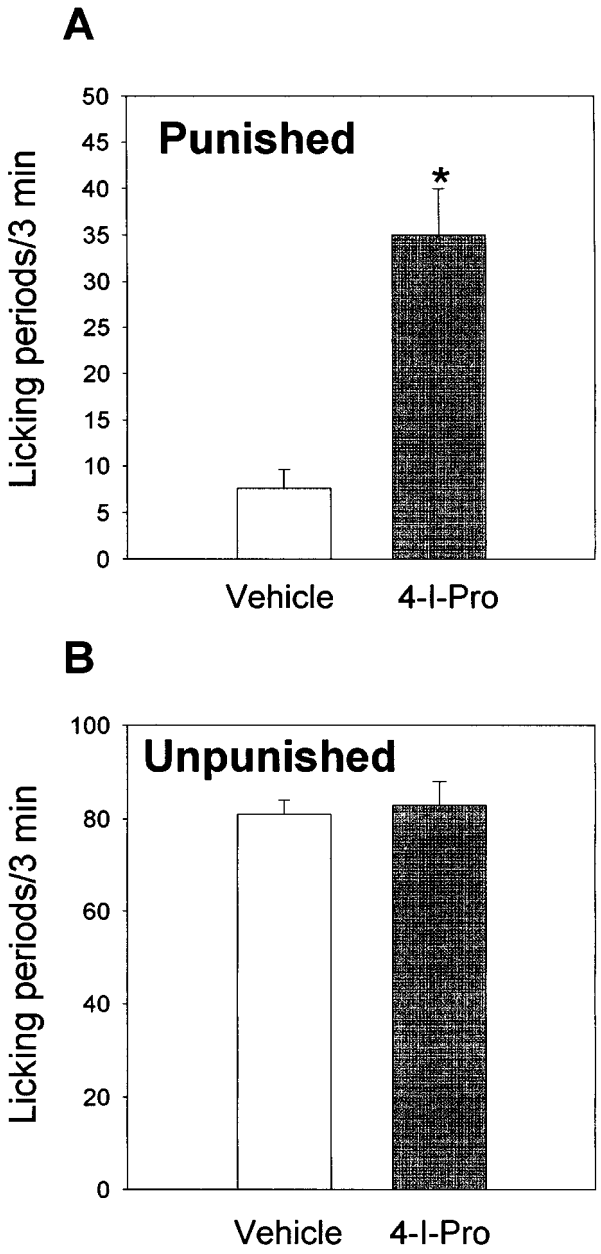

Figure 8 Anticonflict effect of 4-I-Pro in rats. Rats were injected with vehicle or 4-I-Pro (60 mg kg^1, i.p.) 5 min before administration of the Vogel anticonflict test. Data are means±s.e.mean of 7–12 rats per group and represent the number of licking periods per 3 min under conditions of punishment (A) or nonpunishment (B). *$P<0.001$ vs vehicle-treated group.

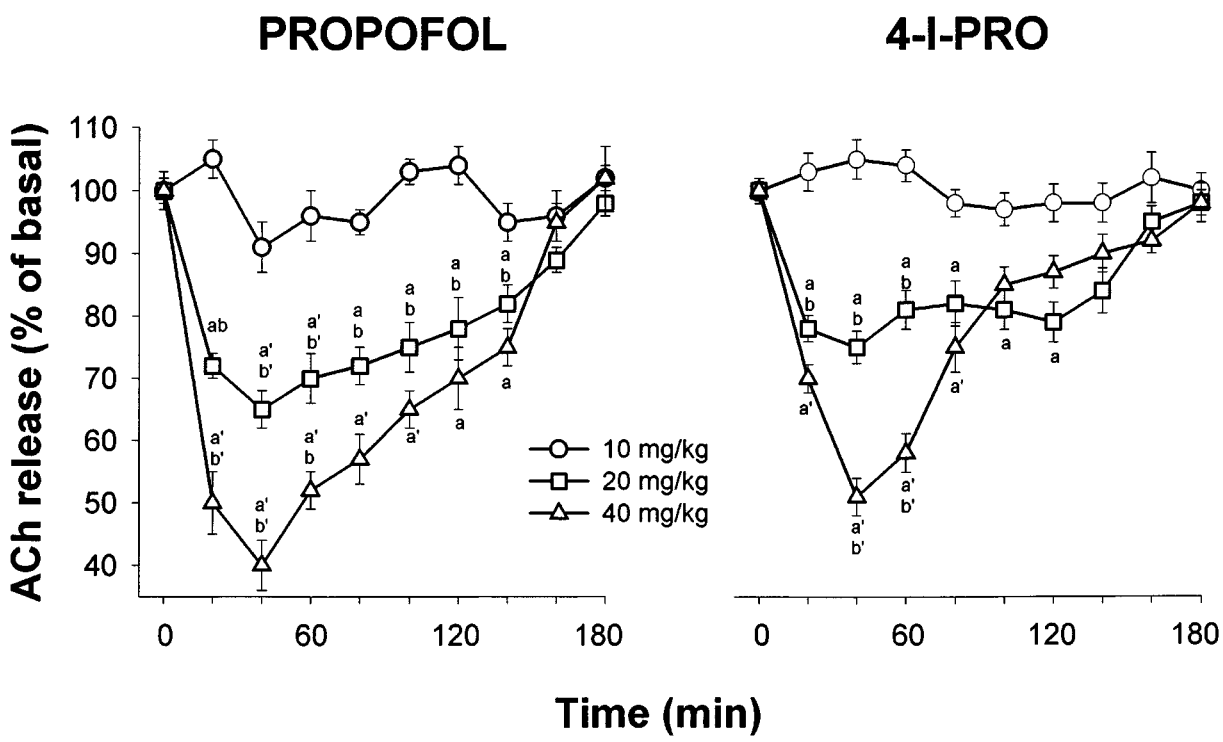

Figure 9 Effects of propofol and 4-I-Pro on basal acetylcholine (ACh) release from the hippocampus of freely moving rats. Data are means ± s.e.mean of at least five rats and are expressed as a percentage of basal values. For the effect of propofol, ANOVA revealed a significant main effect of treatment [$F(9,119) = 78.986$; $P < 0.001$], a significant main effect of repeated measures [$F(2,119) = 167.984$; $P < 0.001$], and a significant interaction between factors [$F(18,119) = 31.845$; $P < 0.001$]. For the effect of 4-I-Pro, ANOVA revealed a significant main effect of treatment [$F(9,119) = 86.745$; $P < 0.001$], a significant main effect of repeated measures [$F(2,119) = 194,746$; $P < 0.001$], and a significant interaction between factors [$F(18,119) = 27.928$; $P < 0.001$]. $^{a}P < 0.05$, $^{a'}P < 0.01$ vs basal values. $^{b}P < 0.05$, $^{b'}P < 0.01$ vs previous dose.

the drug inhibited acetylcholine output by ~35 and 60%, respectively. The effect was maximal 40 min after drug injection and was still apparent (~30% inhibition) at 120 min, with release returning to control values by 180 min. 4-I-Pro exerted a similar effect (Figure 9). The threshold dose (20 mg kg^{-1}) induced a 30% decrease in hippocampal acetylcholine release, whereas a dose of 40 mg kg^{-1} inhibited release by ~50%. Again, the maximal effect was apparent 40 min after drug administration, with release returning to basal values by 180 min.

Discussion

By exploring the structure-activity relations of several propofol derivatives and congeners, we have recently shown that substitution at the para position of the phenol ring of propofol with groups having different stereoelectronic properties yields compounds that act at $GABA_A$ receptors with efficacies and potencies similar to or distinct from those of propofol but which induce pharmacological effects that differ from those of the parent compound (Trapani *et al*., 1998). In particular, substitution with iodine yields a compound that, in contrast to propofol, is devoid of sedative-hypnotic and anaesthetic properties, but is a potent and effective modulator of $GABA_A$ receptors. In the present study, we have characterized the electrophysiological, pharmacological, and behavioural effects of 4-I-Pro.

For the *in vivo* experiments, we compared the effects of 4-I-Pro and propofol at doses of 20–60 mg kg^{-1}, administered intraperitoneally as a single bolus. At these doses, propofol induces an array of depressant effects in rodents that range from sedation and ataxia to loss of the righting reflex and sleep. In contrast, administration of 4-I-Pro did not elicit any of these behavioural signs. Although we have not evaluated directly the ability of 4-I-Pro to cross the blood-brain barrier and reach the brain, evidence suggests that this drug does enter the central nervous system. First, compared with propofol, 4-I-Pro is more liposoluble, having a calculated (CLOG P computer program, version 3.54) octanol $water^{-1}$ partition coefficient 26 times that of the parent compound as a result of the iodine substituent (Trapani *et al*., 1998). Second, 4-I-Pro induces central pharmacological effects with high potency and efficacy. Similar to propofol and at the same doses, 4-I-Pro exhibits anticonvulsant and anticonflict actions and it reduces acetylcholine release in the hippocampus of freely moving rats. However, unlike propofol, these actions of 4-I-Pro are not accompanied by sedation, a reduction in motor activity, and sleep. In this respect, 4-I-Pro may represent a prototype of a new class of compounds with a pharmacological profile more similar to that of antiepileptic and anxiolytic drugs than to that of general anaesthetics.

It is important to determine whether these differences in the pharmacology of propofol and 4-I-Pro are attributable to a difference in the interaction of the two drugs with the $GABA_A$ receptor complex. In addition, knowledge of such differences in the actions of these drugs at $GABA_A$ receptors may contribute to our understanding of the molecular events responsible for the induction of general anaesthesia as well as of those that underlie anticonvulsant and anxiolytic effects. We have thus addressed this issue by comparing the electrophysiological effects of 4-I-Pro and propofol in *Xenopus* oocytes expressing recombinant human $GABA_A$ receptors. 4-I-Pro markedly potentiated GABA-evoked Cl^- currents at all receptor subunit

combinations tested. This action was characterized by a potency and efficacy similar to those of propofol, with the exception that the apparent potency of 4-I-Pro at $\alpha1\beta2\gamma2S$ receptors was significantly higher than that of propofol. These data suggest that substitution with iodine in the para position of propofol does not abolish the ability to modulate GABA responses. Therefore, the lack of sedative-anaesthetic effects of 4-I-Pro does not appear to be attributable to a reduced modulatory efficacy at $GABA_A$ receptors. This conclusion is somewhat unexpected given that previous studies have revealed a high correlation between the anaesthetic efficacy of many compounds and their ability to potentiate GABAergic function (Tanelian *et al*., 1993; Franks & Lieb, 1994). On the other hand, the ability of 4-I-Pro to modulate GABA responses is consistent with its anticonvulsant and anticonflict effects. Many other compounds that enhance $GABA_A$ receptor function, including benzodiazepines, β-carbolines, imidazoquinolines, imidazopyridines, cyclopyrrolones, and loreclezole, also exhibit anxiolytic and anticonvulsant actions. Our data further suggest that positive modulation of $GABA_A$ receptors by propofol is not the only likely effect responsible for the induction of general anaesthesia.

Propofol, as well as other injectable general anaesthetics such as pentobarbital, etomidate, and alphaxalone, directly activates $GABA_A$ receptors in the absence of GABA (Hales & Lambert, 1991; Hara *et al*., 1993; Sanna *et al*., 1995a,b, 1997; Davies *et al*., 1997). This effect is apparent at higher concentrations than those effective in modulating the GABA response. Nevertheless, in the present study, activation of Cl^- currents by propofol and 4-I-Pro was detected at drug concentrations as low as $5-10\ \mu M$, which are in the pharmacological range, suggesting that this effect may contribute to the pharmacological actions of these drugs. The direct effects of propofol observed in the present work appear to be quantitatively higher than those reported by other workers. For example, Orser *et al*. (1994) found that the maximum activation of Cl^- currents by propofol in hippocampal neurones was 31% that of maximum of GABA response, while Wafford *et al*. (1996) using *Xenopus* oocytes expressing $\alpha1\beta1\gamma2$ $GABA_A$ receptors, detected a 25% maximum activation. This difference may likely be attributable to the fact that in our experiments maximum GABA responses were determined by applications of 10 μM GABA which may have produced a higher desensitization rate and thus underestimated the true GABA maximum.

The direct activation of $GABA_A$ receptors by propofol is influenced by receptor subunit composition; in particular, the β subunit may be especially important because it appears to contain the site of interaction with propofol (Sanna *et al*., 1995a). Such a role for β subunits has also been demonstrated for the general anaesthetics pentobarbital and etomidate (Thompson *et al*., 1995; Sanna *et al*., 1997). In the present study, the direct effect of 4-I-Pro at $\alpha1\beta2\gamma2S$ receptors was markedly smaller than that of propofol, whereas the two drugs showed similar effects at $\alpha1\beta1\gamma2S$ and $\alpha1\beta3\gamma2S$ receptors. The differential response of $\beta2$-containing receptors to the direct effects of 4-I-Pro and propofol was also apparent with $\alpha1\beta2$ and $\beta2\gamma2S$ subunit combinations. It is possible that the domain of the $\beta2$ subunit that interacts with propofol to mediate direct activation of the receptor is less accessible to 4-I-Pro because of the presence of the iodine substitute, resulting in a much weaker effect. The fact that the two drugs show similar modulatory efficacies at all subunit combinations tested further supports the hypothesis that direct activation and allosteric modulation of $GABA_A$ receptors by propofol and other general anaesthetics are mediated through different sites of the receptor complex (Sanna *et al*., 1995a,b, 1997; Thompson *et al*., 1995).

We have also confirmed and extended previous observations (Sanna *et al*., 1995b) on the effects of propofol at homomeric β receptors by showing that the direct effects of propofol and 4-I-Pro, relative to that of GABA, at receptors containing the $\beta3$ subunit are markedly greater than those at receptors containing $\beta1$. This difference was most apparent with $\beta1$ and $\beta3$ homomeric receptors; the maximal direct activation by propofol or 4-I-Pro, relative to GABA, at $\beta3$ receptors was three to five times that apparent at $\beta1$ receptors. The large direct effect of propofol observed at $\alpha1\beta3\gamma2S$ receptors are unusual if compared to other reports (Krasowski *et al*., 1997). In this respect, the possibility that co-expression of $\alpha1\beta3\gamma2S$ receptors along with homomeric $\beta3$ receptors, which may have overemphasized the effect of propofol, cannot be ruled out. It is thus unlikely that the $\beta3$ subunit plays an important role in the sedative-anaesthetic actions of propofol.

Our data also show that the direct action of 4-I-Pro at $\alpha1\beta1\gamma2S$, $\alpha1\beta2\gamma2S$, and $\alpha1\beta3\gamma2S$ receptors differs from that of propofol when examined during prolonged drug perfusion. In 55–100% of oocytes tested, the decrease of Cl^- currents activated by the continuous exposure to 4-I-Pro occurred much faster than in the case of propofol. Our data show that this effect of 4-I-Pro detectable also at low drug concentrations, is dependent on drug concentrations, and occurs in any of the subunit constructs studied. While this effect could represent an important factor in determining the different pharmacological action of 4-I-Pro with respect to propofol, the molecular mechanism has not been assessed and might involve either receptor desensitization or channel blockade.

While we have observed quite consistently the non-reducing current induced by prolonged propofol perfusion in the absence of GABA in oocytes (Sanna *et al*., 1995b), to our knowledge, a similar effect has not been reproduced in neurones. For instance, Hara *et al*. (1993) reported, that, recording in voltage-clamped rat hippocampal pyramidal neurones, perfusion of propofol ($1-200\ \mu M$) elicited currents that showed desensitization depending on the drug concentration. Thus, we have to consider the possibility that our observation may be restricted to oocytes, but may not occur in neurones. Thus, it becomes very difficult and too speculative to correlate this oocyte finding with the pharmacological effects of 4-I-Pro observed in rats.

Together, our data indicate that the most important differences between the actions of 4-I-Pro and propofol at $GABA_A$ receptors relate to the direct activation of the receptor; this effect of 4-I-Pro was weaker at $\beta2$-containing receptors and of shorter duration at all subunit combinations tested than that of propofol. Whether these differences are responsible for the lack of sedative-anaesthetic actions of 4-I-Pro cannot be readily determined. In addition, the correlation of the *in vitro* and *in vivo* data may be further complicated on the basis of the species differences, as rats and mice were used for behavioural experiments, while human clones were used in electrophysiological studies. However, in view of the prominence of $\alpha1\beta2\gamma2S$ receptor subpopulations in the mammalian central nervous system (McKernan & Whiting, 1996), the reduced sensitivity of these receptors to direct activation by 4-I-Pro may contribute to the unusual pharmacological profile of this anticonvulsant and anxiolytic drug.

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