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# Effects of inhalational general anaesthetics on native glycine receptors in rat medullary neurones and recombinant glycine receptors in *Xenopus* oocytes

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**1** Glycine responses were studied under voltage clamp in *Xenopus* oocytes injected with cDNA encoding mammalian glycine receptor subunits and in rat medullary neurones. Bath application of glycine gave strychnine-sensitive currents which reversed close to the expected equilibrium potentials for chloride ions. The peak currents for the receptors expressed in oocytes fitted a Hill equation with $EC_{50} = 215 \pm 5$ $\mu$M and Hill coefficient $n_H = 1.70 \pm 0.05$ (means ± s.e.means). The peak currents from the receptors in medullary neurones fitted a Hill equation with $EC_{50} = 30 \pm 1$ $\mu$M and Hill coefficient $n_H = 1.76 \pm 0.08$. The current-voltage relationship for the receptors expressed in oocytes showed strong outward rectification (with $V_{rev} = -21 \pm 2$ mV), while that for the glycine responses from the medullary neurones in symmetrical $Cl^-$ was linear (with $V_{rev} = 3.2 \pm 0.6$ mV).

**2** Inhalational general anaesthetics, at concentrations close to their human minimum alveolar concentrations (MACs), potentiated responses to low concentrations of glycine. The potentiation observed with the recombinant receptors (between 60-220%) was approximately twice that found with the medullary neurones (between 40-80%). For both the recombinant receptors and the receptors in medullary neurones, the degree of potentiation increased in the order of methoxyflurane ≈ sevoflurane < halothane ≈ isoflurane ≈ enflurane. There was no significant difference between the potentiations observed for the two optical isomers of isoflurane.

**3** For both the recombinant and native receptors, isoflurane potentiated the currents in a dose-dependent manner at low concentrations of glycine, although at high glycine concentrations the anaesthetic had no significant effect on the glycine-activated responses. The major effect of isoflurane was to cause a parallel leftward shift in the glycine concentration-response curves. The glycine $EC_{50}$ concentration for the recombinant receptors decreased from a control value of $215 \pm 5$ $\mu$M to $84 \pm 7$ $\mu$M glycine at 610 $\mu$M isoflurane, while that for the medullary neurones decreased from a control value of $30 \pm 1$ $\mu$M to $18 \pm 2$ $\mu$M glycine at the same concentration of isoflurane. The potentiation was independent of membrane potential.

**4** Isoflurane also potentiated responses to taurine, a partial agonist at the glycine receptor. This was observed for receptors expressed in oocytes at both low and saturating concentrations of taurine. The $EC_{50}$ concentration decreased from a control value of $1.6 \pm 0.2$ to $0.9 \pm 0.1$ mM taurine in the presence of 305 $\mu$M isoflurane, while the maximum response to taurine increased from $47 \pm 2$ to $59 \pm 2$% of the maximum response to glycine.

**5** Glycine receptors, like other members of the fast ligand-gated receptor superfamily, are sensitive to clinically relevant concentrations of inhalational general anaesthetics. Effects at these receptors may, therefore, play some role in the maintenance of the anaesthetic state.

**Keywords:** General anaesthesia; glycine receptors; inhalational anaesthetics; taurine; ligand-gated ion channels

## Introduction

Glycine, the simplest of the amino acids, is one of the major inhibitory neurotransmitters in the mammalian central nervous system (for reviews, see Betz, 1992; Kuhse *et al.*, 1995) and is thought to have its predominant effects in the spinal cord and brain stem (Curtis *et al.*, 1968; Young & Snyder, 1973). However, glycine can activate chloride currents in neurones throughout the CNS. This has been shown in the hippocampus (Krishtal *et al.*, 1988; Ito & Cherubini, 1991), olfactory bulb (Trombley & Shepherd, 1994), hypothalamus (Akaike & Kaneda, 1989), medulla oblongata (Krishtal *et al.*, 1988; Lewis *et al.*, 1991), nucleus tractus solitarius (Wakamori *et al.*, 1991) and neocortex (Siebler *et al.*, 1993), as well as in *Xenopus* oocytes injected with mRNA from human cerebral cortex (Gundersen *et al.*, 1984), indicating that glycine is likely to play a significant role as a neurotransmitter in many brain regions. This is supported by *in situ* hybridization studies (Malosio *et al.*, 1991) which reveal a widespread expression of glycine receptor subunits throughout the brain.

As a neurotransmitter, glycine acts by binding to a receptor channel (GlyR) which is a member of a superfamily of fast ligand-gated ion channels. This family includes the $GABA_A$ ($\gamma$-aminobutyric acid, type A), nicotinic ACh (acetylcholine) and $5\text{-}HT_3$ (5-hydroxytryptamine, type 3) receptors (Betz, 1992; Unwin, 1993; Ortells & Lunt, 1995), all of which form ion channels with a pentameric structure. These channels can be either homomeric or heteromeric, but each of the subunits is thought to have a roughly equivalent topology consisting of a large N-terminal extracellular domain and four transmembrane segments. Several different glycine subunits have now been cloned, and these fall into two main groups, $\alpha$ (Grenningloh *et al.*, 1987; 1990b; Kuhse *et al.*, 1990) and $\beta$ (Grenningloh *et al.*, 1990a). While the $\alpha$ subunits can form

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homomeric channels on their own, the $\beta$ subunit is thought to combine with other $\alpha$ subunits to form a channel with a stoichiometry of three $\alpha$ and two $\beta$ subunits (Langosch *et al.*, 1988; Kuhse *et al.*, 1993).

The superfamily of receptors to which the GlyRs belong contain members, most notably the nicotinic ACh and $GABA_A$ receptors, that are amongst the most sensitive of all known ion channels to general anaesthetics (Franks & Lieb, 1994). There is therefore some *a priori* reason to suppose that the glycine channel may share this sensitivity, and the few anaesthetic studies on this receptor that have been published (Hales & Lambert, 1991; Wakamori *et al.*, 1991; Harrison *et al.*, 1993) support this view. Moreover, the glycine receptor appears to be unusually sensitive to the effects of high pressure (Shelton *et al.*, 1993), one of the very few antagonists of general anaesthetic action. In addition, it has recently been shown that the anaesthetic targets involved in the abolition of purposeful responses to a surgical incision may lie in the spinal cord, where glycine is known to be a key neurotransmitter. For example, decerebration (Rampil *et al.*, 1993) and spinal cord transection (Rampil, 1994) experiments in rats, and experiments (Antognini & Schwartz, 1993) with goats where anaesthetics were delivered preferentially to the brain, have shown that the sites of action of inhalational anaesthetics, as assessed by the standard determination of anaesthetic potency (lack of purposeful response to a painful stimulus), may be independent of higher brain centres. In this paper we describe the effects of a variety of inhalational general anaesthetics on recombinant glycine receptors in *Xenopus* oocytes and native glycine receptors in rat medullary neurones, as a step towards assessing their possible role in general anaesthesia.

## Methods

### *Preparation and injection of* Xenopus *oocytes*

Individual adult female *Xenopus laevis* frogs (Blades Biological, Cowden, Kent, U.K.), maintained in fresh-water holding tanks at 20–22°C, were anaesthetized by immersion in a 0.2% (w/v) solution of tricaine (3-aminobenzoic acid ethyl ester, methanesulphonate salt). Portions of the ovaries were surgically removed and teased apart with forceps. These portions were then briefly washed in 'calcium-free' oocyte incubation buffer (calcium-free OIB; composition in mM: NaCl 88, KCl 1, $NaHCO_3$ 2.4, $MgSO_4$ 0.8, HEPES 15, titrated to pH 7.5 with NaOH) before incubation in the same saline containing collagenase (Sigma, type 1A; 2 mg $ml^{-1}$) for 3 h at room temperature with constant agitation. After careful washing in calcium-free OIB to remove all traces of collagenase, the oocytes were transferred into normal OIB (composition in mM: NaCl 88, KCl 1, $NaHCO_3$ 2.4, $MgSO_4$ 0.8, $CaCl_2$ 0.4, $Ca(NO_3)_2$ 0.3, HEPES 15, titrated to pH 7.5 with NaOH). Oocytes at stages 5–6 of development were then chosen for injection by visual inspection. Selected oocytes were injected with 10 nl of Tris-EDTA buffer (1 mM Tris-HCl, 0.1 mM EDTA, titrated to pH 8.0 with NaOH) containing 0.2–20 pg of cDNA directly into the nucleus of the oocyte, with a calibrated micropipette (10–16 $\mu$m tip diameter) and a Picospritzer II valve (General Valve Corp., Fairfield, New Jersey, U.S.A.) which provided short pressure pulses of nitrogen gas. Equal amounts of the human $\alpha$1 and rat $\beta$1 clones in the pCIS eukaryotic expression vector were used. Although it has been shown (Bloomenthal *et al.*, 1994) that an excess of the $\beta$1 cDNA needs to be used for the expression of heteromeric receptors in HEK 293 cells, we were not successful in obtaining reliable expression in oocytes when the $\beta$1 subunit was injected in excess. In our experiments, therefore, it seems likely that the expressed receptors were predominantly $\alpha$1 homomers. Indeed, in preliminary experiments we found very similar anaesthetic responses from oocytes injected only with the $\alpha$ subunit. These GlyR clones were a gift from H. Betz (Neurochemistry Department, Max Planck Institut für Hirnforschung, Frankfurt, Germany). Injected oocytes were maintained in a cooled incubator (BDH, Poole, Dorset, U.K.) at 19–20°C in normal OIB containing antibiotics (penicillin 50 iu $ml^{-1}$, streptomycin 50 $\mu$g $ml^{-1}$; Life Technologies, Paisley, U.K.) in individual wells (200 $\mu$l per well) of 96-well microtitre plates (Life Technologies, Paisley) for 2 to 10 days prior to use. All chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

### *Recording technique for* Xenopus *oocytes*

Ionic currents evoked by bath application of glycine (10 $\mu$M–10 mM) were recorded using the two-electrode voltage-clamp technique with an Axoclamp 2A amplifier (Axon Instruments, Foster City, California, U.S.A.). Pipettes were fabricated from thin-walled filamented borosilicate glass capillary tubes (GC150TF, Clark Electromedical Instruments, Reading, Berkshire) using a two-stage pull (David Kopf Instruments vertical pipette puller, Model 720, Tujunga, California, U.S.A.). Electrodes were filled with 3 M KCl and had typical resistances of 0.4–0.8 M$\Omega$. Currents were filtered at 5 Hz (−3 dB, 8-pole Bessel filter; Frequency Devices, Model 902, Haverhill, Massachusetts, U.S.A.) before being digitised and stored on a computer. The saline used for electrophysiological recordings had the following composition (mM): NaCl 110, KCl 2, $MgCl_2$ 1, $CaCl_2$ 2 and HEPES 10 (titrated to pH 7.5 with NaOH). Experiments were performed at room temperature (21–23°C).

### *Preparation of rat medullary neurones*

The medulla oblongata was dissected from decapitated rats (7–14 days old, Sprague-Dawley) and cut into chunks ($\sim$1 $mm^3$). Cells were dissociated with the solutions and enzymes described by Mintz *et al.* (1992), with minor modifications. Briefly, the chunks of tissue were incubated for 5–6 min under an $O_2$ atmosphere at 37°C in (mM): $Na_2SO_4$ 82, $K_2SO_4$ 30, $MgCl_2$ 5, HEPES 10, glucose 10 (titrated to pH 7.4 with NaOH) containing 3 mg $ml^{-1}$ protease (Sigma type XXIII) and 0.001% phenyl red indicator. The protease solution was then decanted and the tissue resuspended in the same solution but with the protease replaced by 1 mg $ml^{-1}$ trypsin inhibitor (Sigma type II-O, chicken egg white) and 1 mg $ml^{-1}$ bovine serum albumin (Sigma fraction V) at 37°C, and then left to cool to room temperature (21–23°C) under an $O_2$ atmosphere. A few chunks were then withdrawn, gently triturated by 10–15 passages through the tip of a fire-polished Pasteur pipette, and the cell suspension deposited uniformly in a glass-bottomed recording bath (bath volume $\sim$300 $\mu$l) containing extracellular recording solution (mM): NaCl 150, KCl 4, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 10 (titrated to pH 7.4 with NaOH). Most of the cells adhered to the glass within 5 min and neurones were easily identified by their size (10–15 $\mu$m diameter) and distinctive shape. After the cells had adhered, they were continually superfused ($\sim$1 ml $min^{-1}$) with extracellular recording solution. After each recording, the bath was cleaned and a fresh aliquot of cells added. Cells were used within 3 h of dissection.

### *Recording technique for rat medullary neurones*

Ionic currents evoked by the application of glycine (3–1000 $\mu$M) were recorded using the standard whole-cell voltage-clamp technique (Hamill *et al.*, 1981) with an Axopatch 200 amplifier (Axon Instruments, Foster City, California, U.S.A.). A rapid perfusion system was used whereby solutions could be applied to the cells through a pair of fused glass tubes (750 $\mu$m diameter) positioned $\sim$600 $\mu$m from a chosen neurone. The tubes could be translated hydraulically so that the solution bathing the neurone could be rapidly switched from one composition to another. Recording pipettes were fabricated from thin-walled filamented borosilicate glass capillary tubes (GC150TF, Clark Electromedical Instruments, Reading, Berkshire) using a two-stage pull (Narishige PB-7 micropipette puller, Tokyo, Japan). After light fire-polishing, they were fil-

led with the internal recording solution (composition, mM): CsCl 140, NaCl 3, HEPES 10, EGTA 11, Mg-ATP 2 (titrated to pH 7.2 with CsOH). These pipettes, with typical resistances of 3–6 MΩ, readily formed 'giga-ohm' seals with selected neurones which, upon establishing the whole-cell configuration, were voltage-clamped at −60 mV. A few minutes were allowed for the cell interior to equilibrate with the internal solution prior to recording. Currents were filtered at 10 Hz (−3 dB, 8-pole Bessel filter). Series resistance was compensated by >75% for all recordings. Experiments were performed at room temperature (21–23°C).

### *Preparation and delivery of anaesthetic solutions*

The volatile anaesthetics were made up as fractions of saturated solutions at room temperature. The concentrations of the saturated solutions were taken to be: methoxyflurane, 9.1 mM (Seto *et al.*, 1992); enflurane, 11.9 mM (Seto *et al.*, 1992); isoflurane 15.3 mM (Franks & Lieb, 1991); halothane, 17.5 mM (Raventós, 1956); sevoflurane, 11.8 mM (Watts *et al.*, 1992). Reservoirs containing volatile anaesthetics were sealed with a rigid plastic float, and all tubing and valves were made of PTFE. With these precautions, losses of volatile agents from the perfusion system were found to be negligible when measured by gas chromatography (Hall *et al.*, 1994). The sources of the anaesthetics were as follows: isoflurane, enflurane, methoxyflurane, sevoflurane (Abbott Laboratories Ltd., Queenborough, Kent); halothane (ICI Ltd., Macclesfield, Cheshire). The two optical isomers of isoflurane were a gift from Anaquest Inc. (Murray Hill, New Jersey, U.S.A.). The chemical and optical purities of the S-(+)- and R-(−)-isoflurane isomers were determined to be >99% by chiral gas chromotography with an 80-metre Chiraldex G-TA column (Advanced Separation Technologies Inc., Whippany, New Jersey, U.S.A.).

### *Calculation of potentiations*

For the receptors expressed in *Xenopus* oocytes, the glycine-activated currents were found generally to increase during the time-course of the experiments. Consequently, throughout an experiment, a maximum dose of glycine was applied periodically so that all the observed currents could be appropriately normalized. For the anaesthetic experiments, controls were recorded before and after application of the anaesthetic, and these controls were then averaged to give the control current $I_0$. The percentage potentiation was calculated as $100 \times (I-I_0)/I_0\%$ where, $I$ is the average of the glycine-activated currents in the presence of anaesthetic. With the medullary neurones we often observed a similar 'run-up' in the currents. The percentage potentiations caused by the anaesthetics were calculated as described above, except for cases where the control currents after anaesthetic exposure differed markedly from the previous controls. In these cases the first controls were used to calculate potentiations. The anaesthetics on their own usually had no significant effect on the resting current. (Only for isoflurane at the highest concentrations used did we observe a very small inward current). Nonetheless, the anaesthetics were always pre-applied for 30–60 s before application of glycine to establish an accurate baseline.

Values throughout the paper are given as means± s.e.means.

## Results

### *The control glycine-activated current for recombinant receptors*

Glycine, in the concentration range 10 μM to 10 mM, induced inward currents (at a membrane potential of −50 mV) of between 1 nA and 3 μA. At low concentrations of glycine these currents were relatively slow to activate and did not desensitize with time (see Figure 1a). As the glycine concentration increased, the time to peak became shorter and the rate of desensitization became much greater. A 300 μM glycine-activated current could be completely (and reversibly, not shown) blocked by 100 nM of the selective

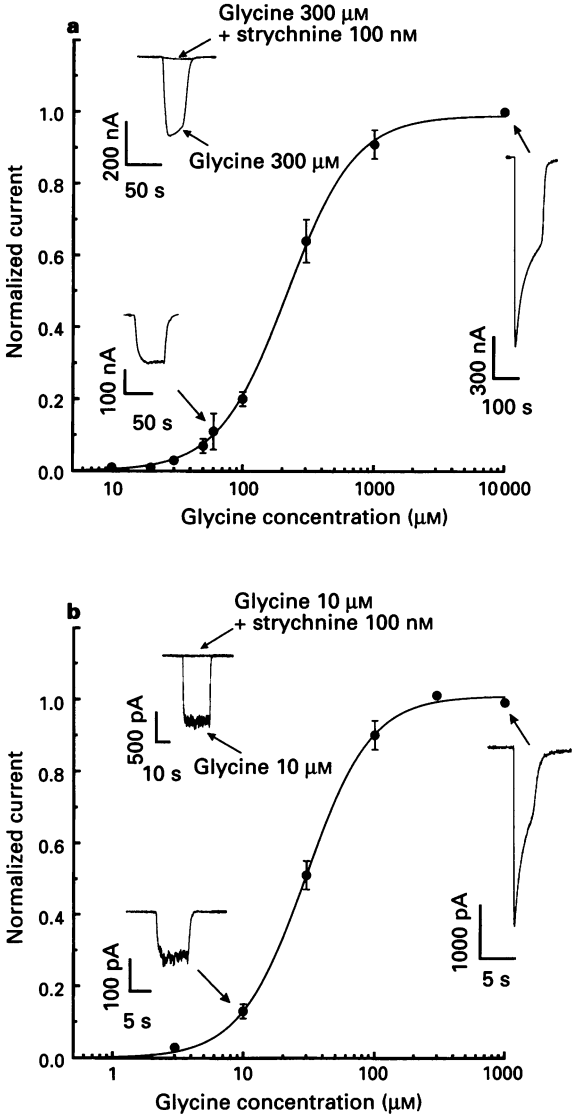


**Figure 1** Glycine concentration-response curves. (a) Data from receptors expressed in oocytes. The data points (●) give the mean peak currents as a function of glycine concentration. The errors are s.e.means and where not shown were smaller than the size of the symbols (except for the data point at 10 mM glycine which was normalized to unity). The line is an unweighted least squares fit of the mean responses (each from 3 to 5 cells) to a Hill equation, with $EC_{50}=215\pm5$ μM and Hill coefficient $=1.70\pm0.05$ (means±s.e. means). The insets on the lower left and on the right show typical current traces from cells (voltage-clamped at −50 mV) in response to 60 μM and 10 mM glycine, respectively. The current traces in the upper left inset, from another cell, show inhibition of the response to glycine (300 μM) by strychnine (100 nM), a selective antagonist at the glycine receptor. Strychnine was applied before addition of glycine and had no effect on its own. (b) Data from medullary neurones. As in (a) except that the mean of the data points at 0.3 and 1 mM glycine was normalized to unity. The line is an unweighted least squares fit of the mean responses (each from 3 to 11 cells) to a Hill equation, with $EC_{50}=30\pm1$ μM and Hill coefficient $=1.76\pm0.08$. The insets on the lower left and on the right show typical current traces from cells (voltage-clamped at −60 mV) in response to 10 μM and 1 mM glycine, respectively. The current traces in the upper left inset, from another cell, show inhibition of the response to glycine (10 μM) by strychnine (100 nM). Strychnine was applied before addition of glycine and had no effect on its own.

glycine antagonist, strychnine ($n=3$ cells; see inset to Figure 1a). The peak responses were concentration-dependent and could be accurately fitted by a Hill equation of the form $y=1/(1+(EC_{50}/c)^{n_H}$, where y is the current expressed as a fraction of the maximal current, c is the glycine concentration, $^{n_H}$ is the Hill coefficient, and $EC_{50}$ is the glycine concentration giving a half-maximal response (see Figure 1). The least squares fit gave an $EC_{50}=215\pm5$ $\mu$M and Hill coefficient $=1.70\pm0.05$, consistent with at least two agonist molecules being required for activation of the glycine receptor channel.

### *The control glycine-activated current for native receptors in medullary neurones*

Glycine, in the concentration range 3 $\mu$M to 1 mM, induced whole-cell currents (at a membrane potential of $-60$ mV) of between 25 pA and 9 nA. Qualitatively, the glycine-activated currents (see Figure 1b) behaved very similarly to those in the *Xenopus* oocytes in terms of changes in activation times and desensitization. The glycine-activated current could also be reversibly blocked by strychnine ($n=3$ cells). The peak responses, however, fitted a Hill equation with a very much smaller value of the $EC_{50}$ ($30\pm1$ $\mu$M), although with a similar value of the Hill coefficient ($1.76\pm0.08$).

### *Potentiation of glycine responses by volatile general anaesthetics*

The responses to low doses of glycine were markedly potentiated by clinically relevant concentrations of the volatile anaesthetics halothane, isoflurane, enflurane, methoxyflurane and sevoflurane. Figure 2 shows typical current traces from oocytes and medullary neurones exposed to anaesthetics at concentrations close to their minimum alveolar concentrations (MACs) in human subjects. For both oocytes and neurones, the glycine concentration was set roughly equal to a third of the control $EC_{50}$ (i.e. 60 $\mu$M for the oocytes and 10 $\mu$M for the medullary neurones). Table 1 gives values for the average potentiations observed from several cells. In all cases the potentiations observed with the oocyte currents were significantly greater (approximately 2 fold) than those found with the neurones, although there was a rough correspondence between the two systems in terms of the re-

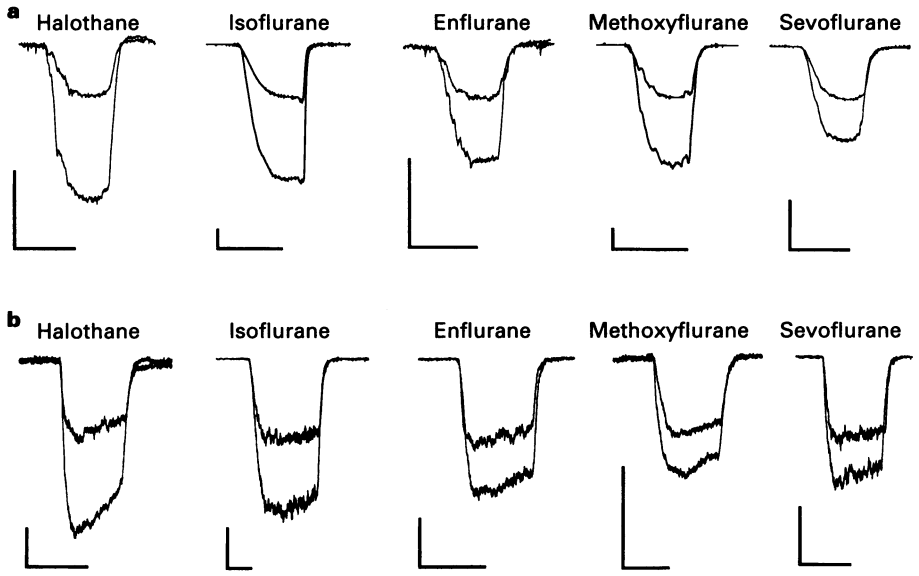


**Figure 2** Representative examples of the potentiation of glycine responses from receptors expressed in (a) oocytes and (b) medullary neurones by five volatile general anaesthetics: halothane, isoflurane, enflurane, methoxyflurane and sevoflurane. The concentration of each anaesthetic was close to its minimum alveolar concentration (MAC) in human subjects (see Table 1). In (a) the glycine concentration was 60 $\mu$M and the membrane potential was $-50$ mV, while in (b) the glycine concentration was 10 $\mu$M and the membrane potential was $-60$ mV. For each pair of traces, the control current is the smaller response. The calibration lines in (a) correspond to 50 s and 50 nA while those in (b) correspond to 5 s and 300 pA. Table 1 gives average potentiations observed for several cells.

**Table 1** Percentage potentiation of glycine currents by clinically relevant concentrations of inhalational general anaesthetics

| | | *% potentiation of currents from receptors expressed in* | | |
|---|---|---|---|---|
| *Anaesthetic* | *Concentration* (mM) | *Xenopus oocytes*[a] | *Medullary neurones*[b] | *Human MAC* (mM) |
| Halothane | 0.18 | $223\pm67$ | $76\pm19$ | 0.19[c] |
| Isoflurane | 0.31 | $177\pm39$ | $81\pm20$ | 0.27[c] |
| Enflurane | 0.48 | $163\pm54$ | $62\pm7$ | 0.49[c] |
| Methoxyflurane | 0.27 | $100\pm19$ | $38\pm11$ | 0.27[d] |
| Sevoflurane | 0.30 | $63\pm15$ | $44\pm8$ | 0.30[c] |

The percentage potentiations ($100\times(I-I_0)/I_0\%$) are given for each anaesthetic as means$\pm$s.e.means for between 3 to 10 cells. [a]The glycine concentration was 60 $\mu$M and the membrane potential was –50 mV. [b]The glycine concentration was 10 $\mu$M and the membrane potential was –60 mV. [c]Franks & Lieb (1996); [d]Franks & Lieb (1993).

lative effectiveness of the different anaesthetics (halothane ≈ isoflurane ≈ enflurane > methoxyflurane ≈ sevoflurane). We investigated the effects of isoflurane in more detail.

### *Potentiation of glycine responses by isoflurane optical isomers*

We tested the effects of the two optical isomers of isoflurane on glycine-activated currents in both oocytes and medullary neurones. At 305 μM (~1 MAC for racemic isoflurane) we found the **S**-(+)- and **R**-(−)-isomers were equally effective in potentiating the current induced by a low concentration of glycine (10 μM). The ratio of the percentage potentiation observed for the **S**-(+)-isomer, divided by that observed for the **R**-(−)-isomer, was 0.99±0.21 ($n$=4 cells) for the recombinant receptors in oocytes and 1.00±0.14 ($n$=4 cells) for the native receptors in medullary neurones.

### *Voltage-dependence of control and isoflurane-potentiated currents*

The potentiation of the glycine-activated currents was independent of membrane potential. This was found both for receptors expressed in oocytes (Figure 3a) and for those in medullary neurones (Figure 3b). The observed reversal potentials $V_{rev}$ were close to those expected for selective chloride conductances. The shapes of the current-voltage relationships for the two systems, however, were very different. The receptors in oocytes showed strong outward rectification (with $V_{rev}$ = −21±2 mV for the control and −20±2 mV in the presence of isoflurane), while the current-voltage relationship for the medullary neurones was essentially linear (in symmetrical $Cl^-$ solutions; $n$=3 cells) with a reversal potential that was close to zero (3.2±0.6 mV for the control currents and 2.8±0.3 mV for the currents in the presence of isoflurane).

### *Concentration-response curves for isoflurane*

In order to study the concentration-dependence of the isoflurane potentiation we investigated the effects of a range of isoflurane concentrations on the responses to a low concentration of glycine. The concentration-response curves are shown in Figure 4. At the glycine concentrations used (50 μM for the oocytes and 10 μM for the neurones), the potentiations observed (Figure 4) with the oocytes were generally larger than those found in the neurones, particularly at the lower isoflurane concentrations. As the concentration of isoflurane increased, there was a significant increase in the rate of desensitization of the glycine-activated current (data not shown). Although we did not investigate this effect in any detail, it appeared that the degree of desensitization could be accounted for in terms of the extent to which the current had been activated. In other words, control currents of an equivalent size showed similar rates of desensitization.

### *The effects of isoflurane on the glycine concentration-response curves*

Whereas clinically relevant concentrations of anaesthetics greatly potentiated the responses to low concentrations of glycine, this was not true at very high glycine concentrations. Indeed, at high glycine concentrations, isoflurane had no significant effect (see Figure 5) on either the peak current or the degree of desensitiation in both oocytes ($n$=5 cells) and medullary neurones ($n$=10 cells). We followed up this observation by determining the glycine concentration-response relationships at different concentrations of isoflurane. The results are plotted in Figure 5a for the recombinant receptors expressed in oocytes and in Figure 5b for native receptors in medullary neurones. These data show that the major effect of isoflurane was to shift the glycine concentration-response curve to the left in a dose-dependent manner. The left inset in Figure 5a shows the corresponding decrease in the glycine $EC_{50}$ for recombinant receptors with increasing isoflurane con-

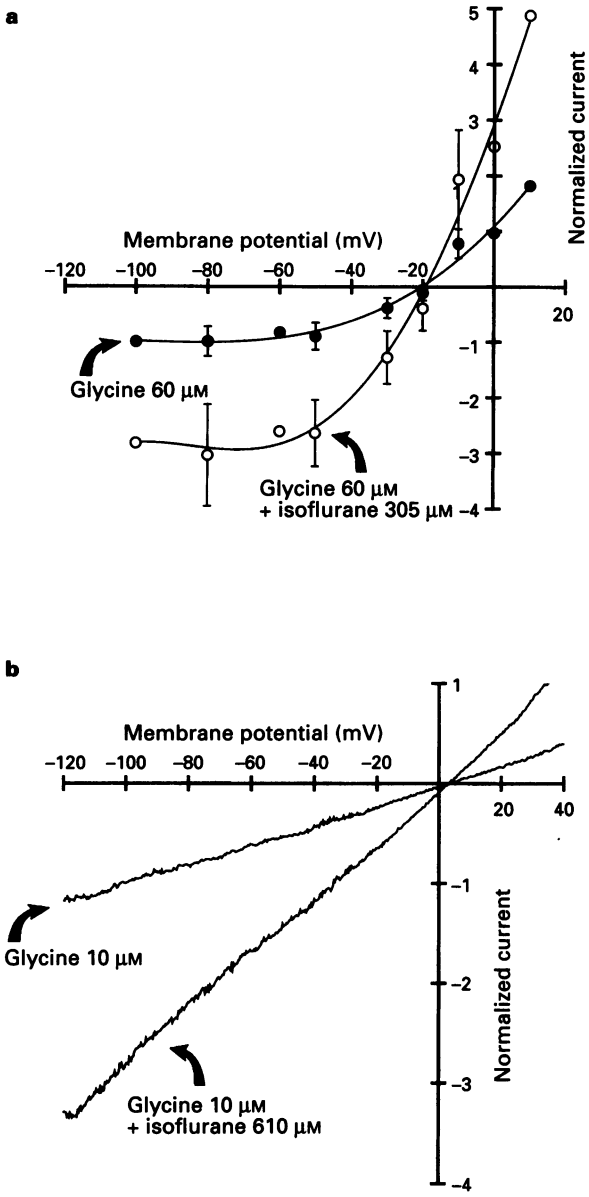


**Figure 3** Effect of membrane potential on control and isoflurane-potentiated currents activated by low, non-desensitizing concentrations of glycine. (a) Current-voltage relationships for (●) control (60 μM glycine) and (○) isoflurane-potentiated responses (305 μM isoflurane) from receptors expressed in oocytes. Data from different cells were combined by measuring the responses as fractions of the maximum response to glycine (see Figure 1a). The data shown have been renormalized by setting the control currents at −100 mV to an arbitrary value of −1. The errors are s.e.means of data from 4 cells and where not shown were smaller than the size of the symbols. The lines were drawn by eye and have no theoretical significance. Reversal potentials, calculated from linear fits to the data between −30 and 0 mV, were −21±2 mV for the control currents and −20±2 mV for the currents in the presence of anaesthetic. Notice that there is no significant voltage-dependence of the anaesthetic potentiation. (b) Current-voltage relationships for control (10 μM glycine) and isoflurane-potentiated responses (610 μM isoflurane) from receptors in medullary neurones (data for a typical cell). Data were obtained by ramping the membrane potential from −120 mV to +40 mV (at 15 mV $s^{-1}$) and were normalized by setting the control current at −100 mV to an arbitrary value of −1. Reversal potentials were 3.2±0.6 mV for the control currents and 2.8±0.3 mV for the currents in the presence of anaesthetic ($n$=3 cells). Notice that there is no significant voltage-dependence of the anaesthetic potentiation.

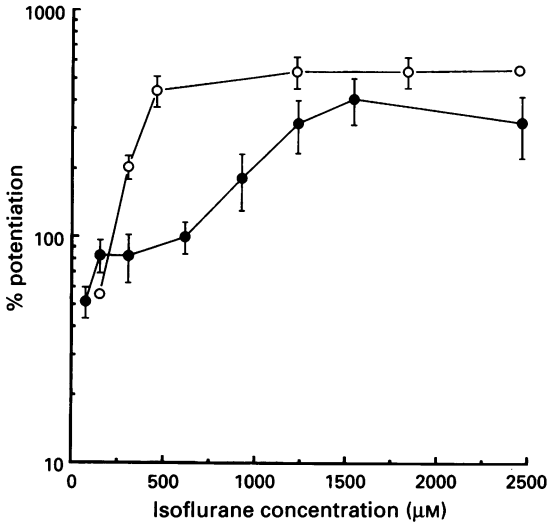


**Figure 4** Concentration-response relationships for the potentiation of responses to low concentrations of glycine by isoflurane. For receptors expressed in oocytes (○) the glycine concentration was 50 μM and the cells were voltage-clamped at −50 mV. The data show mean responses (for 3 to 4 cells). For receptors in medullary neurones (●) the glycine concentration was 10 μM and the cells ($n=6$ to 11 cells) were voltage-clamped at −60 mV. The errors are s.e.means and where not shown were smaller than the size of the symbols. Note that, because of the large range of potentiations observed, the data are plotted on a semi-logarithmic scale.

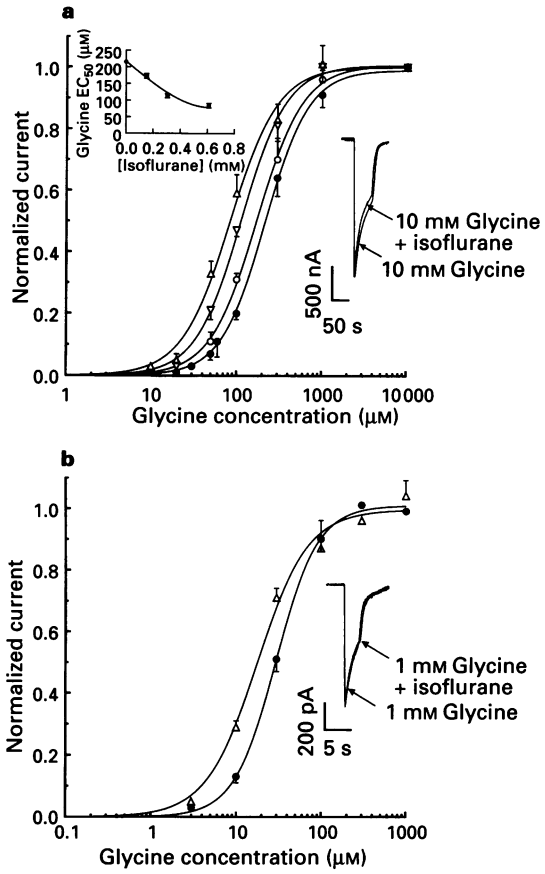


**Figure 5** Glycine concentration-response curves at different isoflurane concentrations: (●) 0 μM; (○) 150 μM; (▽) 305 μM; (△) 610 μM. (a) Data for receptors expressed in oocytes. The data are mean responses from 4 to 5 cells and the errors are s.e.means (except for the data points at 10 mM glycine which were normalized to unity), where not shown, the errors were smaller than the size of the symbols. The lines are unweighted least squares fits of the data to a Hill equation with $EC_{50}$ concentrations of 215±5, 172±7, 113±6 and 84±7 μM glycine and Hill coefficients of 1.70±0.05, 1.60±0.08, 1.64±0.12 and 1.55±0.18 for isoflurane concentrations of 0, 150, 305 and 610 μM, respectively. The inset on the left shows the decrease in the glycine $EC_{50}$ concentration with increasing concentrations of isoflurane. The error bars in the inset are s.e.means calculated from the fits to the Hill equation; the line was drawn by eye and has no theoretical significance. The inset on the right shows the lack of an effect of 610 μM isoflurane on a maximal response to 10 mM glycine. (b) Data for receptors in medullary neurones; same symbols as in (a). The data are the mean responses from 3 to 11 cells for the control curve and 5 to 9 cells for the isoflurane curve. The mean of the data points at 300 and 1000 μM glycine were normalized to unity. The lines are unweighted least squares fits of the data to a Hill equation with $EC_{50}$ concentrations of 29.6±0.8 and 18±2 μM glycine and Hill coefficients of 1.76±0.08 and 1.45±0.23 for isoflurane concentrations of 0 and 610 μM, respectively. The inset on the right shows the lack of an effect of 610 μM isoflurane on a maximal response to 1 mM glycine.

centration. The Hill coefficient, on the other hand, did not change significantly. A qualitatively similar picture was seen with the medullary neurones (Figure 5b), although the leftward shift in the $EC_{50}$ in the presence of an equivalent concentration of isoflurane was less pronounced.

### *The effects of isoflurane on the taurine concentration-response curve*

We studied the effects of 305 μM isoflurane (~1 MAC) on the concentration-dependence of the partial agonist, taurine. Figure 6 shows data recorded from *Xenopus* oocytes. Unlike the responses to glycine, responses at all concentrations of taurine were potentiated by isoflurane. The taurine concentration-response curve in the presence of isoflurane shows a significantly reduced $EC_{50}$ concentration and also a substantial increase in the maximum response to taurine.

## Discussion and conclusions

### *Control currents*

The glycine-activated currents that we have observed from recombinant receptors expressed in oocytes and native receptors in medullary neurones show both qualitative and quantitative differences (Figure 1). Most strikingly, the concentration of glycine required to produce a half-maximal response in the medullary neurones is approximately seven times lower than that required in oocytes, although the Hill coefficients are very similar. Our data are consistent with many previous studies which show that recombinant receptors expressed in oocytes (Grenningloh *et al.*, 1990*b*; Schmieden *et al.*, 1992; Kuhse *et al.*, 1993) are very much less sensitive to glycine than native receptors in neurones (Krishtal *et al.*, 1988; Akaike & Kaneda, 1989; Lewis *et al.*, 1991; Wakamori *et al.*, 1991; Trombley & Shepherd, 1994). This difference is unlikely to be due to the expression of different native receptor subtypes since glycine $EC_{50}$ concentrations vary little, if at all, with receptor subunit composition (Grenningloh *et al.*, 1990b; Pribilla *et al.*, 1992; Schmieden *et al.*, 1992; Bormann *et al.*, 1993; Kuhse *et al.*, 1993). Interestingly, glycine receptor subunits expressed in transfected mammalian cells (human embryonic kidney 293 cells) also show (Sontheimer *et al.*, 1989; Pribilla *et al.*, 1992; Bormann *et al.*, 1993) a much higher sensitivity to glycine than oocytes expressing the same subunits (Grenningloh *et al.*, 1990b; Schmieden *et al.*, 1992; Kuhse *et al.*, 1993). These differences in apparent glycine affinities between receptors in oocytes and those in mammalian cells might be explained in a number of different ways. It is just conceivable that the very different sizes of cells, and consequently the very different equilibration times for the application of the agonist, could lead to distortions of the concentration-response curves. However, it seems unlikely to us that this artefact could ac-

count for such a large difference in apparent affinities. Another explanation is that some second messenger activity in the oocytes might be influencing glycine binding. This is certainly a possibility since the activity of glycine receptors in *Xenopus* oocytes has been shown to be enhanced by endogenous protein kinase C (Nishizaki & Ikeuchi, 1995). Finally, and perhaps most interestingly, is the possibility that receptor-receptor interactions are involved. It has recently been shown that the $EC_{50}$ for glycine in oocytes decreases 5 to 6 fold at high receptor densities (Taleb & Betz, 1994). Moreover, a receptor-associated protein, gephyrin, which is thought to anchor or cluster glycine receptors in the postynaptic membrane, also causes a significant leftward shift in the glycine concentration-response curves (Takagi *et al.*, 1992) when co-expressed in mammalian cells. Thus the most likely explanation for the differences between the glycine apparent affinities for native and recombinant receptors may lie in differences in receptor densities and receptor-receptor interactions.

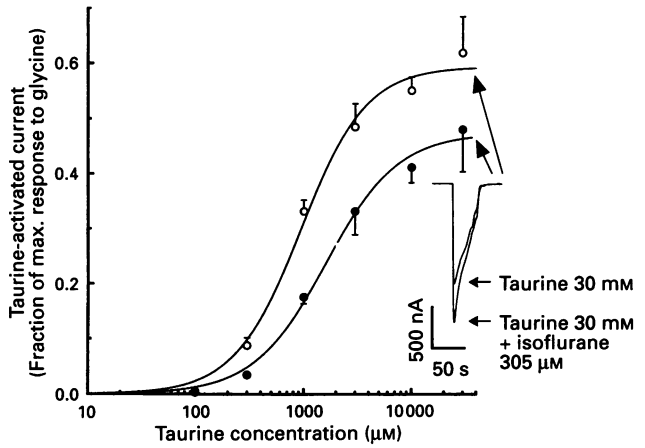


**Figure 6** The effect of isoflurane on the concentration-response curve of the partial agonist, taurine, acting on glycine receptors expressed in oocytes. The data points for taurine alone (●) and those for taurine in the presence of 305 μM isoflurane (○) are expressed as a fraction of the maximum response to glycine (see Figure 1a). The error bars are s.e.means (data from 4 to 5 cells) and where not shown were smaller than the size of the symbols. The lines are unweighted least squares fits of the data to a Hill equation, with $EC_{50}$ concentrations of 1.6±0.2 and 0.9±0.1 mM taurine, Hill coefficients of 1.3±0.2 and 1.4±0.2, and maximum responses of 47±2 and 59±2% for the control and anaesthetic-potentiated currents, respectively. The inset on the right shows current traces from a cell in response to 30 mM taurine with and without 305 μM isoflurane.

Another difference between the data from oocytes and those from medullary neurones is the shapes of the *I-V* curves (see Figure 3). We observed a linear current-voltage relationship for the native receptors, but a markedly non-linear relationship for the recombinant receptors. The strong outward rectification of the glycine-activated currents in oocytes has been reported several times (see, for example, Gundersen *et al.*, 1984; Schmieden *et al.*, 1989; Morales *et al.*, 1994), as has the linearity of *I-V* curves in mammalian cells, for both neurones (Krishtal *et al.*, 1988; Lewis *et al.*, 1991; Trombley & Shepherd, 1994) and transfected cells (Sontheimer *et al.*, 1989; Bormann *et al.*, 1993). There are, however, instances of slight (Wakamori *et al.*, 1991) or marked (Akaike & Kaneda, 1989) rectification in glycine responses in certain neurones. Since it is clear that the non-linearity cannot be accounted for in terms of the Goldman-Hodgkin-Katz constant-field equation (Akaike & Kaneda, 1989), the differences between the various *I-V* curves remain a puzzle.

### *Potentiation of glycine receptors by volatile anaesthetics*

All five of the volatile anaesthetics we tested potentiated the glycine-activated currents in both the *Xenopus* oocytes and medullary neurones at low glycine concentrations. At 1 MAC, each anaesthetic potentiated the glycine response between 40 to 80% in the neurones and 60 to 220% in the oocytes (in both cases with the glycine concentration fixed at about a third of its control $EC_{50}$). The potentiations we observed with halothane, isoflurane and enflurane at 1 MAC were, on average, somewhat larger than those we observed with methoxyflurane and sevoflurane (Table 1). This was true for both the native and recombinant receptors. Comparable glycine potentiations have been reported for halothane and enflurane acting on neurones from the nucleus tractus solitarius (Wakamori *et al.*, 1991) and for isoflurane acting on $\alpha_2$ subunits expressed in HEK 293 cells (Harrison *et al.*, 1993). We investigated the effects of isoflurane in more detail and found, as did Wakamori *et al.* (1991), that anaesthetics did not significantly affect the chloride reversal potential (Figure 3).

The concentration-response curves for isoflurane (Figure 4) acting on the native and recombinant receptors at low glycine concentrations were somewhat different. For the medullary neurones (but not for the oocytes), the effect of isoflurane

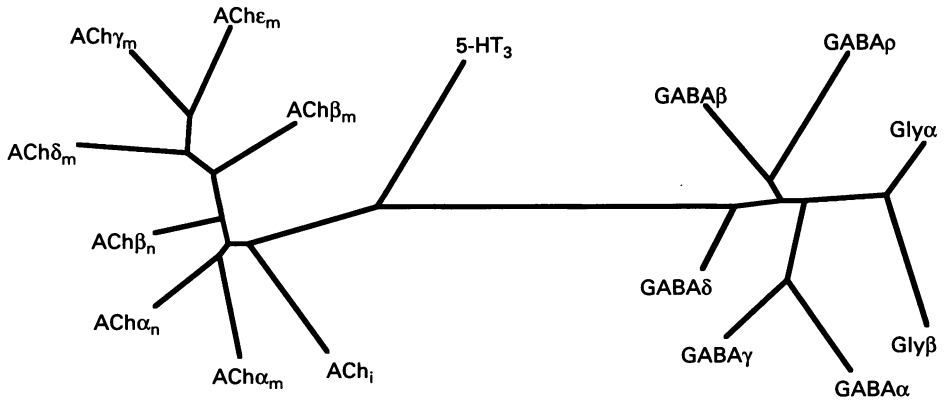


**Figure 7** Schematic diagram showing the proposed phylogenetic relationship between glycine receptor subunits and other members of the superfamily of fast neurotransmitter-gated receptor channels. The diagram is a much simplified version of the phylogenetic tree given by Ortells & Lunt (1995) and shows the principal subunit types grouped together. The distance between any two subunits in the diagram is a measure of the similarity of their DNA base sequences. GABA and glycine receptors (to the right of the diagram) are markedly potentiated by inhalational general anaesthetics (with the exception of GABA ρ homomers) while acetylcholine receptors (to the left) are inhibited. The 5-$HT_3$ receptor is strongly potentiated by some volatile agents, but not by others. See text for details. The subscripts m, n and i refer to muscle-type, neuronal-type and invertebrates, respectively.

appeared to be biphasic, with the potentiation at low concentrations appearing to level out somewhat before increasing again at higher concentrations. For both oocytes and medullary neurones the potentiations due to isoflurane were, however, only seen at low agonist concentrations. At saturating levels of glycine, isoflurane had little or no effect on the glycine-activated currents (see Figure 5). For both the native and the recombinant receptors, isoflurane caused a roughly parallel leftward shift in the glycine concentration-response curve, as has previously been observed for halothane and enflurane in nucleus tractus solitarius neurones (Wakamori *et al.*, 1991). Thus, a more appropriate measure of the sensitivity of the glycine receptor to isoflurane is the extent to which the glycine $EC_{50}$ concentration is reduced, rather than the percentage potentiation at any particular glycine concentration. At approximately 2 MAC isoflurane (610 $\mu$M), the $EC_{50}$ concentration for glycine was decreased to about 40% of its control value in oocytes but to only about 60% of its control value in medullary neurones. The greater anaesthetic sensitivity (at least at the lower isoflurane concentrations) of recombinant glycine receptors in oocytes compared to native receptors (Figures 2, 4 and 5) may be as a result of the same factors that affect the apparent affinity for glycine (see above).

Inhalational anaesthetics have previously been shown to cause a leftward shift in the agonist concentration-response curves for both GABA and 5-hydroxytryptamine acting on $GABA_A$ (Wakamori *et al.*, 1991) and 5-$HT_3$ (Machu & Harris, 1994; Jenkins *et al.*, 1996) receptors, respectively. It therefore seems likely that the underlying molecular mechanisms will prove to be similar for all members of the superfamily where this effect is observed. Whether this involves a change in the true agonist binding affinity, or a change in gating properties of the channel (or a combination of both), remains to be seen and will probably require single-channel analysis. Nonetheless, our observation that the maximal response of the partial agonist taurine is increased by isoflurane suggests that, at least in this case, the answer may not lie entirely with true agonist binding affinities. Downie *et al.* (1995) observed a similar effect with trichloroethanol acting on 5-$HT_3$ receptors, which increased the maximal response of the 5-$HT_3$ partial agonist 2-methyl 5-HT. Regardless of the exact molecular mechanism, if inhalational anaesthetics increase the apparent affinity of glycine for its receptors then one would anticipate that the corollary of this effect in intact synapses would be a prolongation of the inhibitory postsynaptic potential (Franks & Lieb, 1994).

### *Relevance to general anaesthesia*

Our results on the effects of volatile anaesthetics on glycine receptors are best viewed in the context of the anaesthetic sensitivity of other members of the superfamily to which the GlyRs belong. Phylogenetic trees can be constructed (see, for example, Ortells & Lunt, 1995) which describe the inter-relationships between the various receptor subunits in terms of differences and similarities in their DNA base sequences (Figure 7). At one extreme of the phylogenetic tree are the nicotinic acetylcholine receptors. These are known to be inhibited by volatile general anaesthetics (Arimura & Ikemoto, 1986; Firestone *et al.*, 1986; Dilger *et al.*, 1993; McKenzie *et al.*, 1995), showing a sensitivity, at least in some cases (McKenzie *et al.*, 1995), which makes them a plausible anaesthetic target, even though there is considerable uncertainty as to their physiological role in the brain (McGehee *et al.*, 1995; Sivilotti & Colquhoun, 1995). At the other extreme of the phylogenetic tree lie the glycine receptors, which appear to display the same broad sensitivity to volatile agents as $GABA_A$ receptors, to which they are most closely related. Indeed, one might anticipate a close structural homology between the volatile anaesthetic binding sites on the $GABA_A$ receptors and those on glycine receptors. The GABA $\rho$ subunit may well be an exception, since it has been reported to be insensitive to the presence of isoflurane (Harrison *et al.*, 1993). The 5-$HT_3$ receptor appears to occupy an intermediate position (both genetically and pharmacologically) between $GABA_A$ and nicotinic ACh receptors, being strongly potentiated by some volatile anaesthetics but only weakly by others (Jenkins *et al.*, 1996). In view of the relatively close genetic relationship between the inhibitory glycine and $GABA_A$ receptors, it is not too surprising that they share some similarities not only in their general pharmacology but also in their sensitivities to general anaesthetics.

This does not mean, however, that effects of inhalational agents on the glycine receptor necessarily contribute to the state of anaesthesia. One test for this is to determine if the stereoselectivity found with isoflurane enantiomers acting on whole animals matches that found *in vitro* for a particular putative target (Franks & Lieb, 1994). For example, for $GABA_A$ receptors (Jones & Harrison, 1993; Moody *et al.*, 1993; Hall *et al.*, 1994; Quinlan *et al.*, 1995), the S-(+)-isomer is significantly (up to twofold) more potent than the **R**-(−)-isomer. This is roughly in line with animal potency measurements (Harris *et al.*, 1992; Lysko *et al.*, 1994), although the *in vivo* stereoselectivity is not quite as marked as that found *in vitro*. A different picture, however, emerges with the glycine receptors, which display little or no stereoselectivity towards isoflurane (see Results). While this result rules out the idea that isoflurane causes anaesthesia by affecting the glycine receptor alone, it seems reasonable to suppose, particularly in view of the sensitivity we have observed, that effects of inhalational anaesthetics at glycine receptors nonetheless contribute to the anaesthetic end-point (MAC). Indeed, the fact that the isoflurane stereoselectivity observed at $GABA_A$ receptors is generally somewhat larger than that found with animals, leaves open the possibility of a contribution from a target which shows little or no stereoselectivity. Moreover, since many neurones respond to both glycine and GABA (Curtis *et al.*, 1968), it is easy to imagine that glycine receptors might play at least a supporting role in general anaesthesia, and this hypothesis should be explored further.

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