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Modulation of a recombinant invertebrate γ-aminobutyric acid receptor-chloride channel complex by isoflurane: effects of a point mutation in the M2 domain

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1 Inhalational anaesthetics modulate ligand-gated ion channels at clinical concentrations. In this paper we address submolecular mechanisms for γ-aminobutyric acid (GABA) receptor modulation by isoflurane.

2 Wild-type *Drosophila melanogaster* homo-oligomeric GABA receptors were characterized and compared with an ion-channel mutant (alanine substituted to a serine in M2) by means of two-electrode voltage-clamp in membrane-invariant *Xenopus* oocytes.

3 Both channel receptor isoforms generated outwardly rectifying, bicuculline-insensitive currents with reversal potentials characteristic of a chloride current.

4 As previously shown, the point mutation in the M2 domain conferred a profound resistance to the blocking action of 10 μM picrotoxinin (PTX): *circa* 7 fold reduction at the GABA EC_{20}.

5 Isoflurane, 195–389 μM, enhanced GABA conductance in both receptor variants by significantly increasing the affinity of the agonist for its receptor without changing Hill slope or maximal response. Relative potencies were statistically indistinguishable.

6 Isoflurane concentration-response curves (on *circa* GABA EC_{25}) demonstrated that enhancement was effected at around 100–195 μM for both receptor subtypes, but a dramatic divergence was evident at concentrations above 400 μM: wild-type receptors exhibited concentration-dependent block, whilst mutant conductances continued to increase over the same concentration range, showing no tendency to saturate (up to 3330 μM).

7 The above divergence was not attributable to differential desensitization: neither wild-type nor mutant conductance desensitized significantly ($P>0.05$) in the absence or presence of anaesthetic.

8 This work demonstrates that modulatory sites for anaesthetic are present on a relatively primitive insect ion channel.

9 The depression of GABA response at high isoflurane concentrations, in WT receptors, (typical of a variety of anaesthetic agents) may reflect low affinity channel block via the PTX site.

10 The non-saturable enhancement of chloride conductances, when the PTX site is mutated, is not consistent with topical proposals that inhalational anaesthetics (stereoselectively) occupy a finite number of sites on these membrane spanning proteins.

Keywords: *Drosophila* recombinant GABA receptor; chloride channel, isoflurane; point mutation in M2; site within chloride channel lumen; *Xenopus* oocytes

Introduction

γ-Aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the mammalian brain where it is present at up to one third of all synapses (Bloom & Iversen, 1971). The $GABA_A$ receptor is a member of the ligand-gated ion channel superfamily (Schofield *et al*., 1987), and associated ionotropic chloride currents have been shown to be sensitive to a broad range of general anaesthetic compounds (reviewed by Tanelian *et al*., 1993). Cloning and functional expression of several subunits of the $GABA_A$ receptor have proven useful: to demonstrate isomerism in inhibitory receptors, to probe their gating mechanisms and to identify key domains on the proteins for the action of modulatory drugs (eg Wingrove *et al*., 1994; Wafford *et al*., 1991). By analogy with nicotinic receptors it is proposed that five subunits aggregate around an integral ion channel or pore. In vertebrates, six classes of $GABA_A$ subunits have been described (α, β, γ, δ, ε and ρ) each containing four hydrophobic transmembrane regions. The second of these, M2, is postulated to be an amphiphilic helix which actually lines the ion channel pore (Unwin, 1993) and is a highly conserved part of the protein sequence within the superfamily, for both cation and anion selective channels (Betz, 1990).

In this study we looked at wild- and mutant-type (WT and MUT, respectively) homo-oligomeric GABA-activated receptors from *Drosophila* called *Rdl*. The WT has been described as a novel GABA receptor subunit in that it can not be pharmacologically assigned to any of the present classes (Chen *et al*., 1994). This allele was originally cloned from the mutated gene, naturally occuring in *Drosophila*, named *Rdl* due to its resistance to dieldrin (ffrench-Constant *et al*., 1993b). *Rdl* is widely distributed across the *Drosophila* central nervous system (Aronstein & ffrench-Constant, 1995) and the mutation is found globally in resistant *Drosophila* populations (ffrench-Constant *et al*., 1993a). The MUT allele consists of an amino acid substitution at position 302, of an alanine to a serine, which falls in the M2 region of the sequence (ffrench-Constant *et al*., 1993b). These experiments also showed that cyclodiene pesticides, like dieldrin, are potent at the WT receptor and ineffective at the MUT homologue. Mutation of recombinant rat receptors suggests that the convulsant drug, picrotoxinin (PTX), binds in the channel lumen (Gurley *et al*., 1995) and radioligand studies have established that PTX competes for the same site as the cyclodienes and a variety of halogenated in-

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secticides, in the $GABA_A$ receptor/chloride channel complex (Matsumura & Ghiasuddin, 1983). This has been corroborated *in vivo* as insects share a tolerance or 'cross-resistance' to cyclodienes and PTX (Kadous *et al*., 1983) and also in ligand binding studies, which showed *circa* 90% reduction in the affinity for PTX in cyclodiene-resistant strains, relative to susceptible strains (Matsumura *et al*., 1987). Thus the single amino acid at position 302 is implicated as having an important role in the action of these drugs in insects, but not in mammalian receptor subunits (where resistance to PTX is conferred by a mutation of other residues within M2) (Gurley *et al*., 1995).

The cage convulsant *t*-butylbicyclophosphorothionate (TBPS) also binds to the PTX site (Squires *et al*., 1982) and it has been shown that GABA-induced responses at the WT receptor are blocked by TBPS (Buckingham *et al*., 1994). Volatile anaesthetics, including isoflurane, can displace radiolabelled TBPS from its binding site on neuronal membranes (Moody *et al*., 1988), which could be interpreted either as evidence for overlapping recognition sites or for allosteric coupling. Almost 100 years ago Meyer (1899) and Overton (1901) first pointed out the general rule that the potency of an anaesthetic molecule is directly proportional to its lipid solubility or lipid:water partition coefficient. My own (GL) unpublished data on bicyclo-octane congeners (experimental insecticides which interact with the chloride channel site) also suggested that potency *in vitro* was strongly correlated with calculated octanol: water log P.

Because *Rdl* generates robust currents as a homo-oligomer, pharmacological results derived from expression studies can be interpreted with relative ease. It has already been shown that WT currents are highly sensitive to potentiation by the barbiturate, pentobarbitone, not as sensitive as vertebrate $GABA_A$ receptors to the neurosteroid, 5 α-pregnan-3α-ol-20-one, and insensitive to bicuculline, flunitrazepam and zinc (Chen *et al*., 1994). Expression studies carried out in the invariant membranes of *Xenopus* oocytes, infer that differences in pharmacology can be ascribed to variations in protein composition, even for highly lipid soluble molecules (Ffrench-Constant *et al*., 1993a). In this study we examined the actions of a volatile anaesthetic on an invertebrate GABA-activated chloride channel and the implications of a channel directed point mutation (conferring insensitivity to PTX), on the modulation of the inhibitory currents by isoflurane.

Methods

RNA synthesis

Samples of the *Rdl* clones (WT and MUT), in absolute ethanol, were provided by Dr Alison Chalmers (Head of Biology Research, Rhone Poulenc Ag Co., North Carolina, U.S.A.; the clones are licensed to Cornell University and were released by kind permission of Dr Richard Rousch). They were contained within the pNB40 plasmid, which includes an insert for ampicillin resistance (the β-lactamase gene). Large scale plasmid preparation and purification of each sample was carried out, by standard techniques, to increase DNA quantities before transcription reactions could be performed. Concentration and quality of the newly made DNA were assessed by u.v. spectrophotometery and by running samples on a mini-agarose gel.

Aliquots of the DNA plasmids (10 μg) were linearized with the restriction enzyme Not I, at 37°C for 1 h. Proteinase K, 1 μl of 1 mg ml^{-1}, was utilized, per tube, to remove the enzyme and any RNAases. Phenol-chloroform extracted DNA was ammonium acetate (5 M)/ethanol precipitated, resuspended in Tris-EDTA buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA) and assessed by u.v. spectrophotometry. Transcription was performed by use of a kit (Ambion mMessage mMachine Capping Kit). Linearized, proteinase K-treated DNA (*circa* 1 μg per tube) was incubated at 37°C with SP6 RNA polymerase. An RNAase-free DNAase step was carried out to remove the DNA template and the RNA was extracted, precipitated and resuspended in Tris-EDTA buffer. The concentration was estimated and the RNA was stored as aliquots at −80°C until required.

Oocyte preparation

Adult female *Xenopus* laevis (imported from African Xenopus Facility c.c., South Africa) were kept in a standard glass tank with a 12/12 h light/dark cycle. The water temperature was thermostatically controlled at *circa* 23°C and recirculated through a filter. Feeding was carried out daily and consisted of standard fish pellets. Donor frogs were anaesthetized by immersion in 2–3 cm of 0.4% 3-amino-benzoic acid ethyl ester (tricaine) before ovarectomy by use of standard surgical techniques. Viable donor frogs were not re-used within 6 weeks of this procedure. Ovarian lobes were cut and collected into calcium-free saline (82 mM NaCl, 2 mM KCl, 5 mM HEPES and 1 mM $MgCl_2$, pH 7.5). The lobes were washed in a culture dish, by use of the calcium-free solution, before being immersed in an aliquot (3 ml) of collagenase buffer (2 mg ml^{-1}) and digested for 10 min. The collagenase was removed and the oocytes were washed 4–5 times. Stage V and VI oocytes were manually dissected from their loosened epithelial and thecal layers under a low-powered microscope (12.5x + 2.5x). Stripped oocytes were re-soaked in collagenase for 3–5 min to remove remaining follicular cells, the enzyme action being quenched by immersion in albumin (15 mg 10 ml^{-1}) for 5–10 min. Finally, cells were transferred into filtered (0.2 μm, Gelman Sciences) Modified Barth's saline (MBS: 88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 2.5 mM $NaHCO_3$ and 10 mM HEPES, pH 7.4, autoclaved and supplemented with gentamicin (100 mg l^{-1}), theophylline (90 mg l^{-1}), penicillin (10,000 u l^{-1}) and streptomycin (10 mg l^{-1})).

A pre-prepared aliquot of each cRNA (1 μg μl^{-1}) was taken from the freezer (−80°C) and placed on ice. An auto-claved micropipette (glass capillary, Laser, Southampton, U.K.), with tip size ranging from 10–20 μm, was back-filled completely with heavy white mineral oil (Sigma, U.S.A.) and attached to the automatic Drummond microdispenser (Laser, Southampton, UK). Oocyte nuclei were injected blind (20 nl per oocyte) and transferred to 96-well plates containing filtered, supplemented MBS. Cells were incubated at 18–22°C.

Electrophysiological recording

Electrodes (GC150F-10; Clark Electromedical Instruments, Berkshire, UK) were back-filled (4–5 mm) with warmed 1% agar in 2 M KCl solution. When required for use, they were back-filled with liquid 2 M KCl and broken back to between 0.5 MΩ and 3 MΩ (current and voltage electrodes, respectively). Agar bridges (bath ground) were made with 2 M KCl and connected to the pre-amplifier via reference wells containing 2 M KCl. Oocytes were placed within a narrow channel of a perspex bath (30 μl) before impalement. Cells were clamped at between −10 and −100 mV: for conductance measurements holding potentials between −40 mV and −20 mV were used to limit the driving force for evoked currents. Results were measured either as a current or as change from baseline conductance in response to a −20 mV, 200 ms voltage jump, applied at 2.5 Hz via a stimulus isolator (Harvard Apparatus, Kent, UK). Conductance was measured as the resultant current shift in response to these jumps (depicted as height of dense black bars in traces). Subtraction of the resting G_m from the evoked response was used to determine evoked G_{GABA}. Currents were inadvertently inverted at the chart recorder throughout (inward currents are upward deflections and *vice versa*). A GeneClamp 500 Amplifier (Axon Instruments, California) was used. Frog Ringer solution (115 mM NaCl, 2.5 mM KCl, 10 mM HEPES and 1.8 mM $CaCl_2$, pH 7.2) constantly perfused the clamped cell (*circa* 10 ml min^{-1}), through 2 mm tubing by a gravity-feed mechanism. Various drug concentrations were held in 50 ml or 100 ml syringes and selected to flow, as required. A digital

oscilloscope (RadioSpares, U.K.) was used to maximize clamp gain and stability and to ensure that the frequency response of the chart recorder was sufficient to monitor accurately steady-state conductance changes. Real-time recordings were made on a Graphic 1002 Chart Recorder (Llyod Instruments, Hampshire, U.K.). GABA was applied for enough time to elicit peak responses (5–30 s). Modulatory drug responses were assessed at equilibrium (sufficient time was allowed for the conductance response to peak, ie attain a new steady state in the presence of anaesthetic): peak current or conductance being presented as a percentage of control GABA response. Saturated isoflurane solutions were diluted in Ringer to the stated nominal concentration (confirmed by gas chromatography: $n=6$). Isoflurane solutions were delivered from glass reservoirs covered with polyethylene (high density) floats to retard loss by evaporation and perfused through Teflon lines (Franks & Lieb, 1991). To ascertain the degree of loss during preparation and handling of anaesthetic solutions, gas chromatography was employed with a gas-phase assay based on established methods (Eger & Eger, 1985) and published gas partitioning coefficients over a range of temperatures (Smith *et al*., 1981). The mean ($\pm$s.e.mean) loss of isoflurane from float covered cylinders, over a period of 2–3 h was $11.3\pm2.8\%$ ($n=6$, from 2 different anaesthetic concentrations and cylinders) and a mean ($\pm$s.e.mean) $87.7\pm3.2\%$ of the stated isoflurane concentration reached the cell, up to 2.5 h after start of experimentation ($n=6$). In this paper we cite nominal calculated isoflurane concentrations and consider the negligible loss only in the discussion. PTX was dissolved in dimethylsulphoxide, then diluted 1:1000 in Ringer to yield the experimental solutions. All experiments were conducted at room temperature (22–24°C).

Sources of chemicals

Isoflurane from Abbott Laboratories (Kent, U.K.), antibiotics from Gibco/Life Technologies (Scotland, U.K.) and all other chemicals were purchased from Sigma Chemical Co. (Dorset, U.K.).

Data analysis

Unless otherwise stated, data are given as mean$\pm$s.e.mean. Details of statistical tests (Prism software, Graphpad, U.S.A.) are given in the text; two-tailed *t* test or one-way ANOVA were used as appropriate. Log concentration-response curves were fit to a two- or three-term logistic equation by non-linear regression (Prism).

Results

Both the WT and MUT forms of the receptor were expressed rapidly, producing peak GABA-induced currents (in the μA range) within 20 h and were viable for pharmacological studies for a further 2–3 days. The apparent threshold for an inward current in response to GABA at -40 mV was between 0.01 μM and 10 μM GABA: currents were dose-dependent and saturated below 2 mM. *I*-V plots were generated (Figure 1a) between -100 mV and -10 mV for the GABA-induced currents: the reversal potentials derived from these were -28.4 ± 0.48 mV ($n=3$) for WT and -28.6 ± 2.69 mV ($n=4$) for MUT. These are consistent with the equilibrium potential for a chloride current in oocytes. The plots also show outward rectification (steeper slope) at the more positive potentials. The receptors were very similar in dose-dependence (Figure 1b): within any given batch of cells the EC_{50}s were within a few μM of each other for WT and MUT. However, over the entire suite of experiments (38 cells) GABA EC_{50}s ranged between 3 μM and 20 μM, demonstrating considerable batch-dependence. Hill slopes were not analysed in detail, but ranged between 1.5 and 3 for both WT and MUT, indicating co-operative activation by GABA.

Conductance measurements were more stable or reliable indicators of receptor activation than currents (prone to large fluctuations with repeated GABA application) and were generated from a holding potential between -20 mV to -35 mV for the following quantitative experiments.

The WT receptor was very sensitive to 10 μM PTX (Figure 2a), which almost completely blocked responses to <50 μM GABA and effected 75% block at the peak dose of 100 μM GABA ($n=6$). Even after extensive washing (*circa* 7 min) with drug free saline, the antagonistic action was only partially reversible (not shown). The MUT receptor was relatively insensitive to PTX, with *circa* 10% block of the response to 100 μM GABA ($n=6$). As shown in Figure 2b, PTX evoked a small and parallel right shift (Hill slopes were not significantly different, $P>0.05$, *t* test) and *circa* 10% block at saturation, this suggests a mixed blocking action for PTX on the MUT receptor. The point mutation reduced the relative potency of PTX (at the GABA EC_{20}) by *circa* 7 fold. It has been previously shown (ffrench-Constant *et al*., 1993a) that the WT receptor is insensitive to the diagnostic $GABA_A$ receptor antagonist bicuculline: ionotropic,

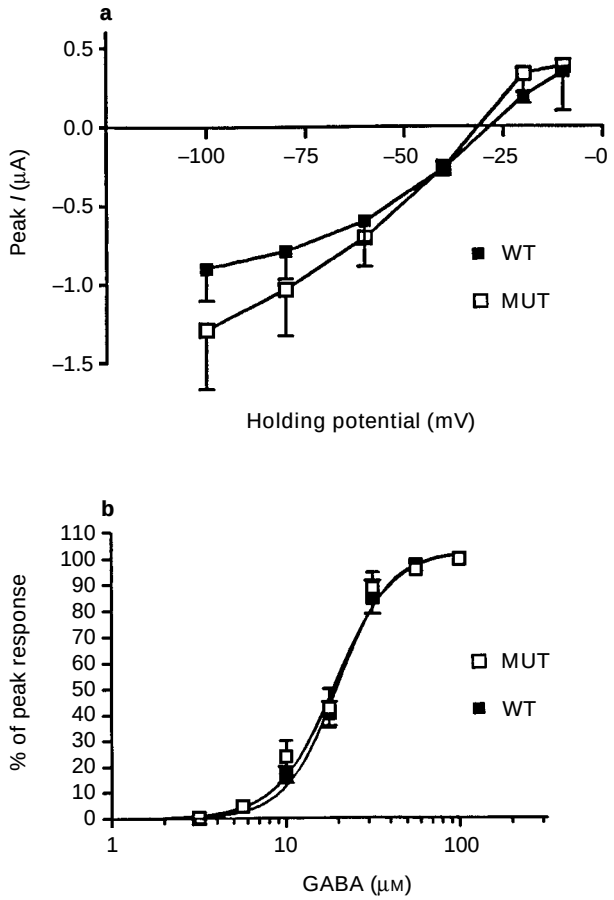

Figure 1 Current-voltage relationships and agonist profiles for both the WT and MUT receptors. (a) Graphical depiction of the current elicited by bath applied 10 μM GABA measured in oocytes injected with either WT or MUT *Rdl* receptors, at the following sequence of holding potentials: -100, -80, -60, -40, -20 and -10 mV. Data points represent the mean for 3–4 oocytes and are connected by point-to-point fit (vertical lines show s.e.mean). The mean reversal potentials were determined to be -28.4 ± 0.48 mV and -28.6 ± 2.69 mV for the WT and MUT receptors, respectively, which is typical of a chloride channel in oocytes. (b) Concentration-response curves, produced by bath application of GABA over the range 3–100 μM (logarithmic scale), where the responses to each concentration are normalized to the peak response for that oocyte. The change in base-line conductance (-20 mV jumps from a holding potential of between -35 mV and -30 mV) was measured in cells injected with either the WT or MUT cRNA. Data points represent the mean (with s.e.mean shown by vertical lines) from 6 oocytes and were fit to produce sigmoid curves (see Methods) with mean EC_{50} concentrations that were statistically indistinguishable ($P>0.05$, *t* test): 19.41 ± 1.05 μM and 19.01 ± 1.08 μM, respectively.

chloride selective, receptors of this nature have been classified as 'GABA$_c$' receptors (Johnston, 1996). Our experiments involved measuring peak current for 10 μM GABA (at -60 mV) before, during and after application of 100 μM bicuculline (not shown). We confirmed the WT response and also showed the MUT receptor to be similarly resistant to the blocking action of 100 μMbicuculline ($P>0.05$, t test for each control:test data).

Pilot experiments (not shown) indicated that isoflurane (195–389 μM) enhanced GABA currents and conductance and attained equilibrium within 4 min, and full or at least substantial reversal was generally attainable within 10 min ($n=3$ for each receptor). The modulatory effects of isoflurane were found to be voltage-dependent in that inward currents produced from holding potentials between -100 mV and -30 mV were more markedly potentiated than outward currents from between -30 mV and -20 mV (not shown). Isoflurane 389 μM enhanced inhibitory chloride conductance in all cells tested by increasing the affinity of GABA for its receptor (Figure 3). In two batches of cells the anaesthetic induced a significant parallel shift of both WT and MUT curves ($n=11-13$ per point, $P<0.05$) to the left by enhancing re-

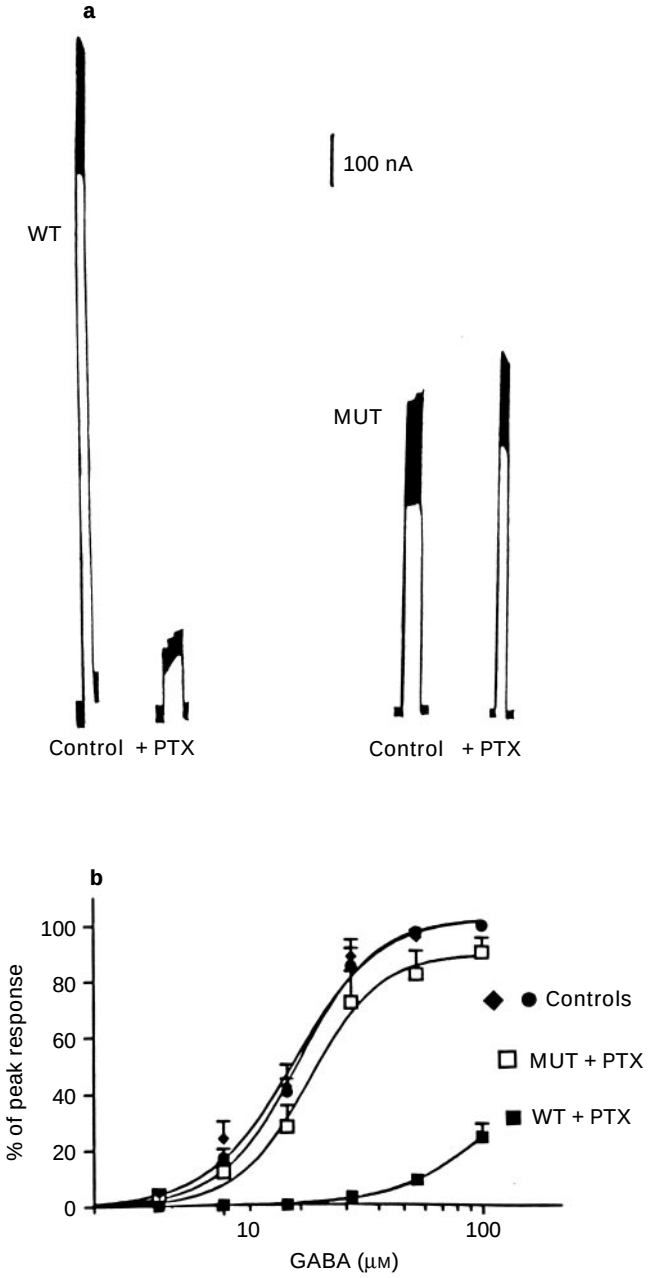

Figure 2 Picrotoxinin (PTX) selectively blocked chloride currents in oocytes injected with the WT *Rdl* subunit. Conductance was measured (-20 mV jumps from a holding potential between -35 mV and -30 mV) for bath applied GABA concentrations, in the absence (control) and presence (+PTX) of bath-applied 10 μM PTX in oocytes expressing either the WT or MUT *Rdl* receptor. (a) Representative examples comparing the effect of PTX on 56 μM GABA responses showing that there was almost complete block of the WT response and negligible inhibition of MUT conductance. (b) Compounded concentration-response data (fit as described in Methods) from 6 cells for each *Rdl* subunit. Points represent the mean (vertical lines show s.e. mean) and show profound block of the WT receptor and a markedly reduced antagonistic effect for the mutated receptor compared to their respective control curves.

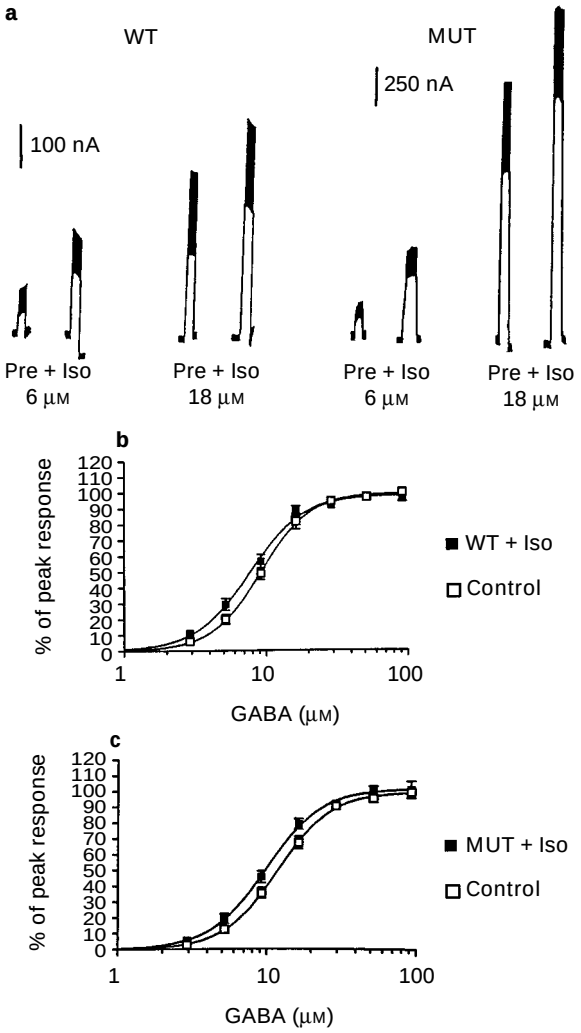

Figure 3 Potentiation of GABA-induced responses by 389 μM isoflurane (Iso) was similar for WT and MUT *Rdl* receptors. Conductance was measured (-20 mV jumps from holding potentials between -20 mV and -30 mV) pre- and post-equilibration (4 min) with Iso. (a) Representative examples of the potentiation seen for both WT and MUT receptors at the non-saturating concentrations of GABA (6 μM and 18 μM) in the presence of 389 μM Iso. Note that less, if any, enhancement was seen at the higher GABA concentrations. (b) GABA concentration-response curves of averaged data from oocytes injected with the WT receptor cRNA ($n=11-13$ per data point, means and s.e.mean are shown). The significant ($P<0.05$) parallel shift of the control curve to the left in the presence of Iso indicates potentiation of responses which give a change in affinity and a relative potency at the EC_{50} of 0.85x. (c) GABA concentration-response curves of averaged data for oocytes injected with the MUT receptor cRNA ($n=11-13$ per data point, means and s.e.mean are shown). Similar to the WT response to Iso, the significant parallel shift of the MUT control curve to the left when in the presence of 389 μM Iso presented a relative potency at the EC_{50} of 0.82x. These potency ratios were statistically indistinguishable ($P>0.05$, t test). Curves were fit as described in Methods.

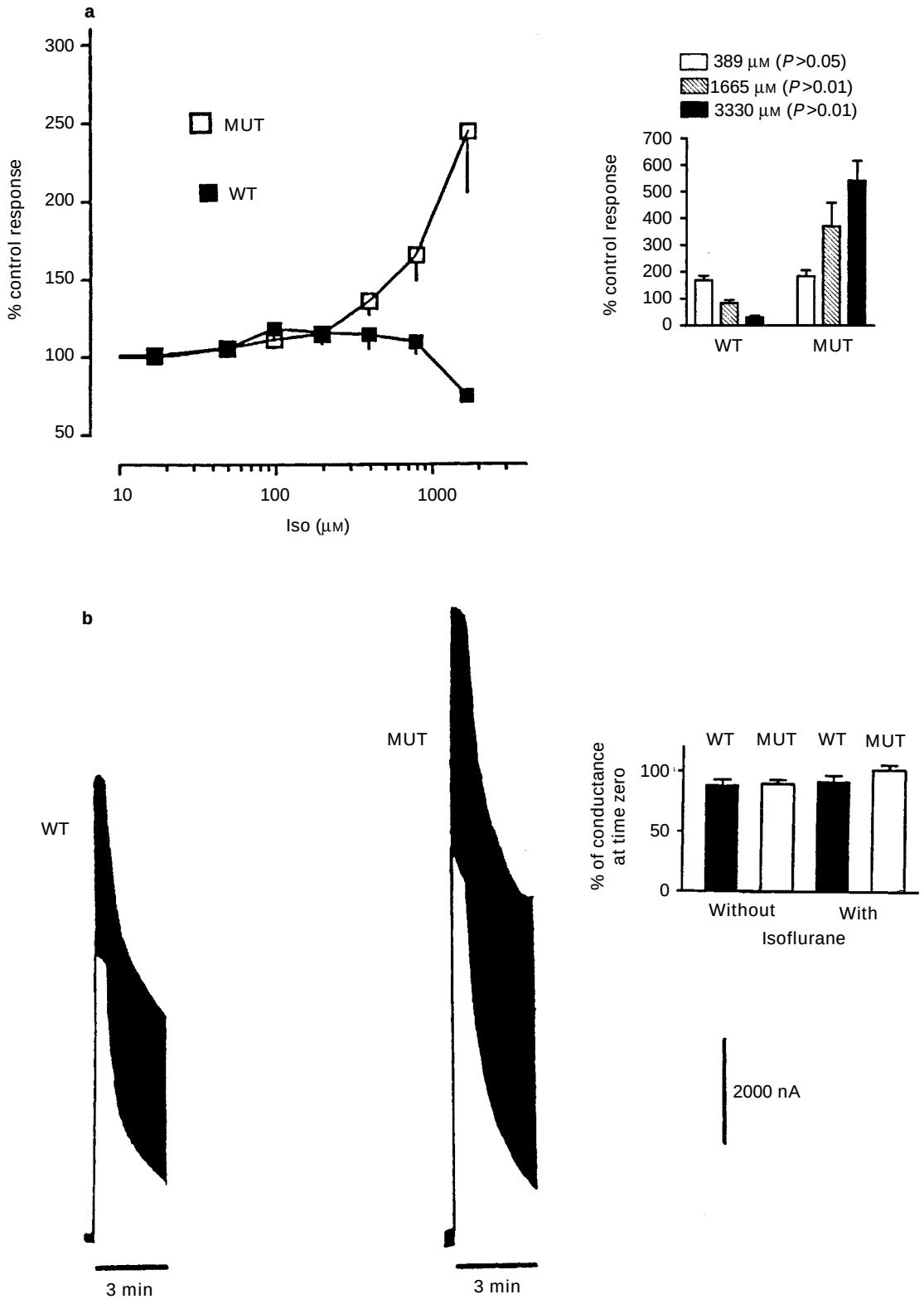

Figure 4 (a) A divergence was seen in the isoflurane (Iso) concentration-dependence of WT and MUT receptors. Conductance measurements (−20 mV jumps from holding potentials between −20 mV and −30 mV) on bath application of 5.6 μM GABA (*circa* EC_{25}) over the range 17–1665 μM Iso, were recorded. Graphs of averaged data (with vertical lines showing s.e.mean) for (6–10 oocytes) show that responses in both receptors were enhanced by Iso, with a threshold of around 100 μM. However, at 400–1665 μM Iso there were opposing effects: WT conductances were inhibited whilst MUT responses continued to be enhanced. Inset: averaged ($n=3-10$) conductance measurements (generated as for (a)) in the presence of 389 μM, 1665 μM and 3330 μM Iso, as a percentage of their own control response (ie GABA alone) in an attempt to demonstrate saturability. Even at 3330 μM, Iso effected an increasingly profound block of WT responses and further potentiated MUT responses. (b) There was no difference in desensitization profiles for each receptor in the absence or presence of 3330 μM Iso. Representative examples from an oocyte injected with WT or MUT *Rdl* cRNA show little fade in conductance throughout a 3 min challenge with a maximal concentration of GABA (2 mM) after equilibration with 3330 μM Iso. Inset: compounded data (mean±s.e.mean) confirming this negligible desensitization for both the WT and MUT receptors ($n=5-7$, $P>0.05$, one-way ANOVA).

sponses to subsaturating concentrations of GABA (maximal conductance was not significantly affected). At 389 μM, potentiation of currents was most pronounced (up to 220%) at low agonist concentrations. Isoflurane modulated the GABA EC_{50}s by 0.85x (95% CI = 0.78 – 0.93) for the WT and 0.82x (0.76 – 0.88) for the MUT. These relative potencies were statistically indistinguishable.

Having established that the receptors were equally susceptible to modulation by clinical concentrations of anaesthetic, we examined the effects of varying the concentration of isoflurane over the nominal range 17 – 1665 μM, at a fixed GABA concentration (*circa* EC_{25}, 5.62 μM). The apparent threshold for potentiation of GABA conductance was similar for both receptor subtypes (*circa* 100 μM), as was the weak enhancement seen up to 389 μM isoflurane, but a drastic divergence in modulatory effect was evident at higher concentrations (Figure 4a). WT receptors exhibited concentration-dependent block by 770 – 1665 μM isoflurane, whereas MUT conductances continued to increase across this range. These observations were extended in independent experiments (Figure 4a inset): at 1665 μM this trend was confirmed and at 3330 μM WT responses were depressed *circa* 5 fold whilst MUT conductances were potentiated to a similar degree and showed no tendency to saturate. In the absence of GABA, isoflurane perfusion (17 – 3330 μM) did not induce conductance changes so the anaesthetic was unable to gate directly either the WT or MUT inhibitory channels. In an attempt to seek a mechanism for the divergent modulation, desensitization profiles for the two receptors were characterized. Neither WT or MUT receptors desensitized significantly ($P > 0.05$, $n = 5 – 7$) when challenged for 3 – 4 min with saturating GABA (2 mM) in the presence or absence (inset) of 3330 μM isoflurane (Figure 4b).

Discussion

The WT and MUT homo-oligomeric receptors appeared to be physiologically equivalent, with almost superimposable dose-response curves, similar EC_{50}s and Hill slopes and indistinguishable reversal potentials. To validate that the point mutation was functionally expressed in response to RNA injection, we confirmed the results of ffrench-Constant *et al*., (1993a) by demonstrating the dramatic difference in the antagonistic efficacy of PTX at each receptor. The profound block of GABA response seen in the WT was not exhibited in the less sensitive MUT, where the parallel rightwards shift indicates an apparent competitive action of PTX and the reduction in maximal response is indicative of a non-competitive effect. Thus, PTX appears to behave like a mixed-antagonist at the MUT receptor, as found by Constanti (1978) in lobster muscle. Our own interpretation of the antagonistic actions at the WT receptor is constrained by the limited range of GABA concentrations tested: the effect is at least partially surmountable with a depressed Hill slope. We also compared the effect of the classical competitive antagonist bicuculline. There was no difference between subunit response to 100 μM bicuculline in that both were totally resistant even to this very high concentration.

Our results suggest that the MUT and WT receptors desensitize only slowly and marginally in response to prolonged exposure to saturating GABA concentrations. In contrast, Zhang *et al*., (1994) describe WT *Rdl* currents, expressed in cultured *Drosphila* neurones, which desensitize rapidly whilst MUT currents desensitize much more slowly and to a lesser extent (98.4% fade for WT cf 68% for MUT). Our experiments differ in that we measured conductance, as opposed to current. Fading of very large currents in stage V – VI oocytes appears to reflect ionic redistribution or erosion of ionic gradients: our data suggest that conductance is a more stable and reliable indicator of activation and status of the ion channel pore.

Anaesthetics appear to modulate GABA receptors by increasing affinity and promoting desensitization (eg Nakahiro *et al*., 1989; Wakamori *et al*., 1991). Our experiments do not indicate that WT or MUT receptors are significantly differentially prone to desensitization, even in the presence of mM concentrations of isoflurane. It is still controversial as to whether anaesthetic action upon the nervous system involves changes in signalling by modifying the lipid matrix of biological membrane lipids (Miller, 1985) or by a more direct mechanism involving hydrophobic interactions with proteins within this lipid matrix (Franks & Lieb, 1990). Several laboratories have addressed these issues directly, and an increasing number of experiments indicate a specific, stereo-selective action upon proteins (Tas *et al*., 1987; Moss *et al*., 1991; Moody *et al*., 1993), with ion channels within the ligand-gated superfamily appearing to be particularly sensitive targets (Franks & Lieb, 1994). A recent elegant study by Forman *et al*., (1995) combined site-directed mutagenesis of the M2 domain of the nicotinic acetylcholine receptor with anaesthetic pharmacology: a change in one or two amino acids to the more hydrophobic residue isoleucine, within the amphiphilic helix, increased the sensitivity to isoflurane by about 9 fold. Our own experiments on recombinant human receptors (unpublished data) with isoflurane suggest that these are modulated in a more pronounced manner (7 – 10 fold by 75 – 150 μM isoflurane, at GABA concentrations below the EC_{25} for various heteromeric isoforms). At up to 389 μM, a concentration in the clinical range for mammals (Franks & Lieb, 1994), WT and MUT *Rdl* GABA responses were potentiated to a much lesser degree (on average a 2 fold increase in either case). The mutant *Rdl* channel bears a relatively polar hydroxylated amino acid compared to an aliphatic substituent in the WT receptor. This appears to overcome the apparent antagonistic action of isoflurane observed in wild-type receptors at higher drug concentrations. It is noteworthy that this amino acid occurs spontaneously at an analogous site in mammalian $GABA_A$ receptor γ-subunits (Gurley *et al*., 1995), which may underpin their enhanced susceptibility to modulation (noted above). Similar bell-shaped responses to diverse structural classes of anaesthetic have been observed (eg Lin *et al*., 1992; Mihic *et al*., 1994; Sanna *et al*., 1995). However, a unifying mechanistic explanation has not, as yet, been proposed. Elegant studies with barbituates (Rho *et al*., 1996) demonstrate that at high concentrations they effect a flickering block of the receptor channel: this occurs at higher concentrations than those required to potentiate GABA responses or produce direct gating of the channel.

Even taking into account the small loss of anaesthetic by our experimental and perfusion methods, divergent effects were observed in the high clinical range for isoflurane: such low affinity responses are often used to discount the relevance or validity of a proposed molecular target. It is conceivable that this reflects a secondary channel blocking action at or near the picrotoxinin site within the channel lumen. Certainly, when the picrotoxinin site is modified, responses of MUT receptors are enhanced across a very broad range of concentrations and show no tendency to saturate. This is not consistent with GABA receptor enhancement being secondary to occupancy of a finite number of sites on a target protein.

To summarize, in this study we have demonstrated that relatively primitive invertebrate receptors are sensitive to modulation by clinical concentrations of isoflurane and further that the possibility of channel block at high drug concentrations was overcome by a point mutation at a domain within the channel lumen. This was observed within the invariant lipid bilayer of the *Xenopus* oocyte and re-inforces the importance of protein composition for anaesthetic recognition and net effect. Recent work within the same superfamily suggests that the amphiphilic M2 domain may regulate anaesthetic potency/efficacy (Forman *et al*., 1995). Our work re-inforces the importance of this helix, which has a pivotal role in channel gating (Unwin, 1995), and suggests that anaesthetics may concurrently potentiate $GABA_A$ currents and, at higher doses, effect channel block via an interaction with the picrotoxinin recognition site. The mutated domains may be important in anaesthetic recognition but at this stage an allosteric mechanism cannot be discounted.

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