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$GABA_A$ receptor activation and open-channel block by volatile anaesthetics: a new principle of receptor modulation?☆

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Abstract

The rapid application of solutions containing the volatile anaesthetics isoflurane or sevoflurane induced inward currents in human embryonic kidney (HEK293) cells carrying rat recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptor assemblies. The responses evoked by the anaesthetics applied via a fast delivery system were recorded using the patch-clamp technique in the whole-cell mode. The anaesthetics induced a fast inward current which was followed by a prominent tail current upon the rapid withdrawal of the agent. These currents were simulated using a kinetic scheme embodying two agonist-like binding steps required for receptor activation, and one binding step by which the anaesthetic induces an open-channel block. According to this model of a biphasic receptor modulation, the open-channel block delays the ion flux through the ligand-gated receptors and, thus, prolongs the overall duration of the current response. Open-channel blocks might also be operative in other ligand-gated ion channels to modulate synaptic strength.

Keywords: $GABA_A$ receptor; Isoflurane; Patch-clamp; Kinetic modelling; Open-channel block

1. Introduction

The potentiating effect of the volatile anaesthetic isoflurane on γ-aminobutyric acid (GABA)-mediated synaptic transmission has been attributed to an enhancement of $GABA_A$ receptor-mediated chloride currents (Tanelian et al., 1993; Banks and Pearce, 1999). Recent studies show that isoflurane also evokes an open-channel block of $GABA_A$ receptors (Adelsberger et al., 1998; Neumahr et al., 2000; Hapfelmeier et al., 2001a,b). It was suggested that such an open-channel block of postsynaptic GABA receptors by isoflurane can prolong GABA-mediated synaptic responses independent of an increase in transmitter release (Jones and Harrison, 1993; Hapfelmeier et al., 2001b). A voltage-dependent open-channel block by acetylcholine, which prolongs postsynaptic responses in zebrafish neuromuscular junctions, has been reported recently (Legendre et al., 2000). This effect, which was suggested to modulate the neuromuscular transmission also in mammals, gains with an increase in agonist concentration (Drapeau and Legendre, 2001).

In postnatal rat hippocampal neurons, isoflurane induces a GABA-independent chloride current that is assumed to contribute to a decrease in neuronal excitability (Yang et al., 1992). In the present study, we used a rat recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptor assembly, supposedly the predominant subunit combination in mammalian brain (Möhler et al., 1996), expressed in human embryonic kidney (HEK293) cells to further characterize this GABA-independent receptor activation, and to investigate the possibility that the anaesthetics isoflurane and sevoflurane exert an influence via an open-channel block at $GABA_A$ receptors. Our results uncover a new principle in the modulation of a transmitter-gated ion channel.

☆ This study was performed at the Max-Planck-Institute of Psychiatry, Munich, Germany.

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2. Materials and methods

2.1. Cell preparation

Human embryonic kidney cells (HEK293, DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) were maintained in minimum essential medium (MEM), supplemented with 10% fetal calf serum, 4 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin, in an atmosphere of 5% CO_2, 95% air, and 100% relative humidity at 37 °C.

Transfection was performed, using an electroporation system (Biotechnologies and Experimental Research, San Diego, CA, USA). The cells were co-transfected with plasmids containing cDNAs encoding for rat α_1, β_2, and γ_{2L} $GABA_A$ receptor subunits, respectively. cDNA encoding for green fluorescent protein (GFP), as an expression marker, was co-transfected. After harvesting, the cells were suspended in a buffer used for transfection (in mM, 50 $K_2HPO_4 \times 3\ H_2O$, 20 K^+-acetate, 25 $MgSO_4 \times 7\ H_2O$, pH 7.35). Plasmids containing cDNAs encoding for the $GABA_A$ receptor subunits (5 μg for each subunit), and for GFP (10 μg), were added to the cell suspension. Electroporation was performed at 290 V and 1 mF with a pulse time of 30–45 ms. Transfected cells were placed in 10 × 35 mm culture dishes with supplemented medium and incubated (5% CO_2, 95% air, and 100% relative humidity, 37 °C) for 12–18 h before the experiments.

2.2. Electrophysiology

For the experiments, performed at 20–23 °C, the medium was replaced by extracellular buffer containing (in mM) 142 NaCl, 5.3 KCl, 0.67 Na_2HPO_4, 0.22 KH_2PO_4, 2 $CaCl_2$, 15 HEPES, 5.6 glucose, pH 7.4 adjusted with NaOH. The standard patch-clamp technique was used to measure anaesthetic-evoked chloride currents under whole-cell voltage-clamp (− 30 mV) conditions. Borosilicate glass pipettes (GC150TF-10, Clark Electromedical Instruments, Pangbourne Reading, UK) were pulled, using a two-step horizontal puller (Zeitz Instruments, Augsburg, Germany), and heat polished. The resulting tips had a series resistance of 4–9 MΩ. Pipettes were filled with intracellular solution containing (in mM) 140 KCl, 11 EGTA, 10 HEPES, 10

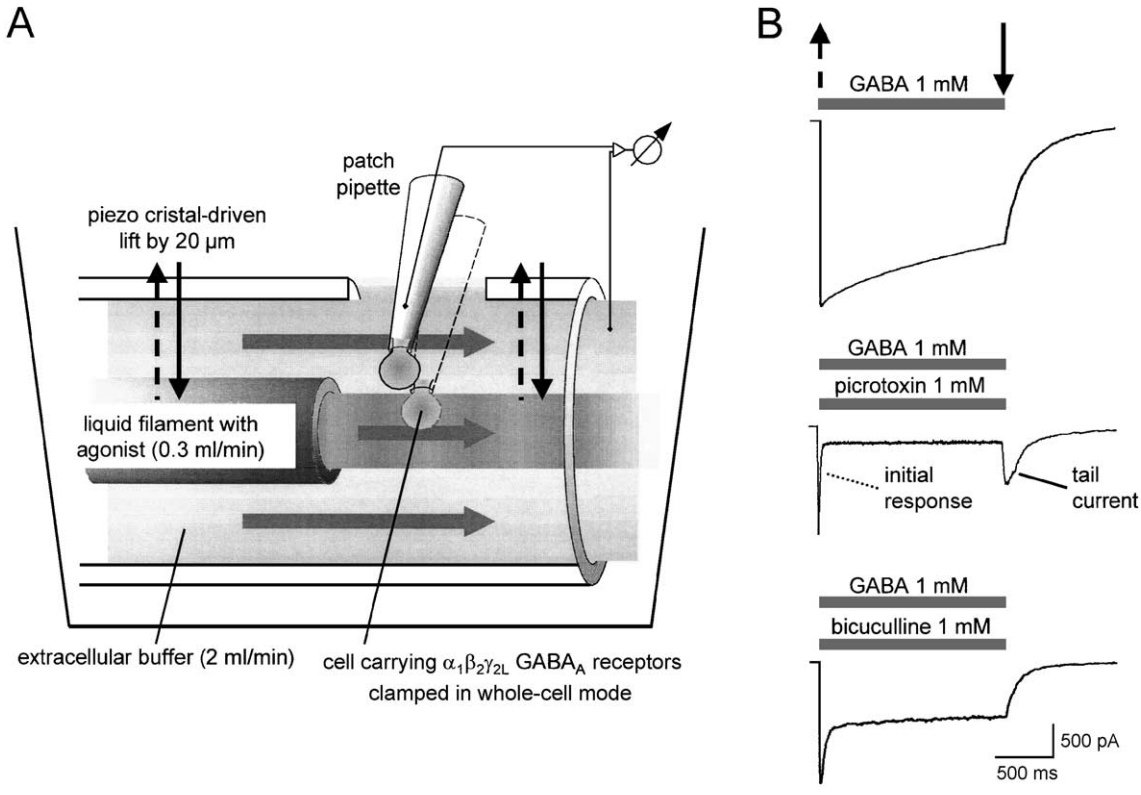

Fig. 1. (A) A piezo-driven 'liquid filament' switch was used to achieve exchange of solutions in the vicinity of the cell, clamped in the whole-cell mode, within < 1 ms. Agonist solution and extracellular buffer form a laminar-flow system. The piezo device shifts the tube upward (≈ 20 μm) to immerse the cell in the 'liquid filament' (dashed upward arrow). The downward shift of the 'liquid filament' upon piezo inactivation terminates the agonist application (downward arrow). (B) GABA (1 mM), applied to HEK293 cells carrying $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, induced an inward current, which was inhibited by the non-competitive $GABA_A$ receptor antagonist picrotoxin (reduced response, dashed line), and the competitive antagonist bicuculline. The rapid withdrawal of picrotoxin induced a tail current (line), which might result from the offset of an open-channel block. In contrast, the withdrawal of bicuculline did not induce any tail current.

glucose, 2 $MgCl_2$, 1 $CaCl_2$, pH 7.3 adjusted with KOH. Currents were recorded with an Axopatch 200B patch-clamp amplifier, low-pass filtered at a cut-off frequency of 2 kHz, and then digitized at 10 kHz with a digidata 1200 A/D converter, performed with pClamp 6.0 software (all from Axon Instruments, Foster City, CA, USA). Cell membrane capacitance was compensated by >90%, serial resistance was compensated by >50% by the patch-clamp amplifier. Nonspecific linear leak currents were negligible.

2.3. Drug application

To enable for rapid application and withdrawal of substance, a piezo-driven system for fast exchange of solutions was used (Fig. 1; Franke et al., 1987). Solutions of isoflurane (Forene™; Deutsche Abbott, Wiesbaden, Germany) or sevoflurane (Maruishi Pharmaceutical, Osaka, Japan) were applied to the cell via a 'liquid filament', that is, a tiny jet of solution, discharged from a borosilicate glass tube (inner diameter 0.15 mm) inside the recording chamber, which was perfused by extracellular solution (Fig. 1A). This technique enables a complete exchange of solutions in the vicinity of the cell within 1 ms. This rapid exchange of solutions was measured in separate experiments in which voltage-sensitive whole-cell currents were induced by high-potassium (40 mM) solutions (data not shown). The vehicle in the 'liquid filament' was extracellular solution which contained the indicated concentrations of isoflurane or sevoflurane.

A saturated solution of isoflurane or sevoflurane was prepared by adding a surplus of the anaesthetic to the extracellular solution, and stirring it in an airtight glass bottle for at least 3 h. At room temperature, the maximum concentration of isoflurane or sevoflurane in the extracellular solution was 15 or 5 mM, respectively, measured by gas chromatography. The trial concentrations of the anaesthetics were prepared from the saturated solutions immediately before application. To control the concentrations of isoflurane or sevoflurane, prepared and applied under our experimental conditions, some probes were passed through the application system, collected, and analyzed by gas chromatography. The differences between the calculated and the measured concentrations were less than 15% (Scheller et al., 1997). The range of isoflurane or sevoflurane concentrations used in this study was 0.15–15 mM or 0.5–5 mM, respectively. All solutions were freshly prepared and used within 20 s. No change of pH was observed after addition of any agent to the extracellular solution. Mechanical stability of the whole-cell recordings was not impaired during drug application.

2.4. Data analysis

Peak current and time to peak (10–90% rise time) were measured using automated detection algorithms (AxoGraph software for MacOS). The decay of the current was fitted (AxoGraph software) with an exponential function in the form:

$$I(t) = I_0 \exp(-t/\tau_0)$$

where I_0 is the component of the current decay and τ_0 is the time constant. Data are presented as means ± S.E.M. with the number of experiments indicated.

2.5. Kinetic modelling

Simulations of kinetic schemes and sets of rate constants were performed using a program for macroscopic current modelling (BIOQ–Biochemical Equations software) kindly provided by Prof. Hanna Parnas (Hebrew University, Jerusalem, Israel). This software for solving sets of differential equations was developed for analytical description of synaptic currents (Parnas et al., 1989) and is available for downloading from the web page , http://www.ls.huji.ac.il/~parnas/Bioq/bioq.html.

To match simulation and experimental data, the time course of the open probability of the receptor following the application of isoflurane was plotted. Kinetic schemes, which are based on the 'three-state kinetic model' theory (Del Castillo and Katz, 1957), were simulated with sets of rate constants. Based on established theories of kinetic modelling (Colquhoun and Hawkes, 1981; Colquhoun and Sakmann, 1985), the kinetic scheme and the rate constants were optimized by fitting the experimental data.

3. Results

Patch-clamp recordings in the whole-cell mode were employed to record $GABA_A$ receptor-mediated inward currents. The solutions were applied in the vicinity of the cell via an application system (Fig. 1A) that enables both rapid application and rapid withdrawal of solutions. Fig. 1B depicts the fast inward currents induced by GABA (1 mM) applied to cells carrying $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptor assemblies. This response to GABA decayed rapidly in the presence of the non-competitive $GABA_A$ receptor antagonist picrotoxin, which supposedly binds to the pore of the channel (Gurley et al., 1995). The tail current, induced by the rapid withdrawal of picrotoxin, might result from the offset of an open-channel block. In contrast, no tail current was induced by the rapid withdrawal of the competitive $GABA_A$ receptor antagonist bicuculline (Fig. 1B, bottom). This is in line with the assumption that blocked channels remain inactive even when the competitive antagonist dissociates.

3.1. Currents induced by volatile anaesthetics via the activation of $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptor assemblies

Application of isoflurane (3 mM) to HEK293 cells expressing recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptor channels

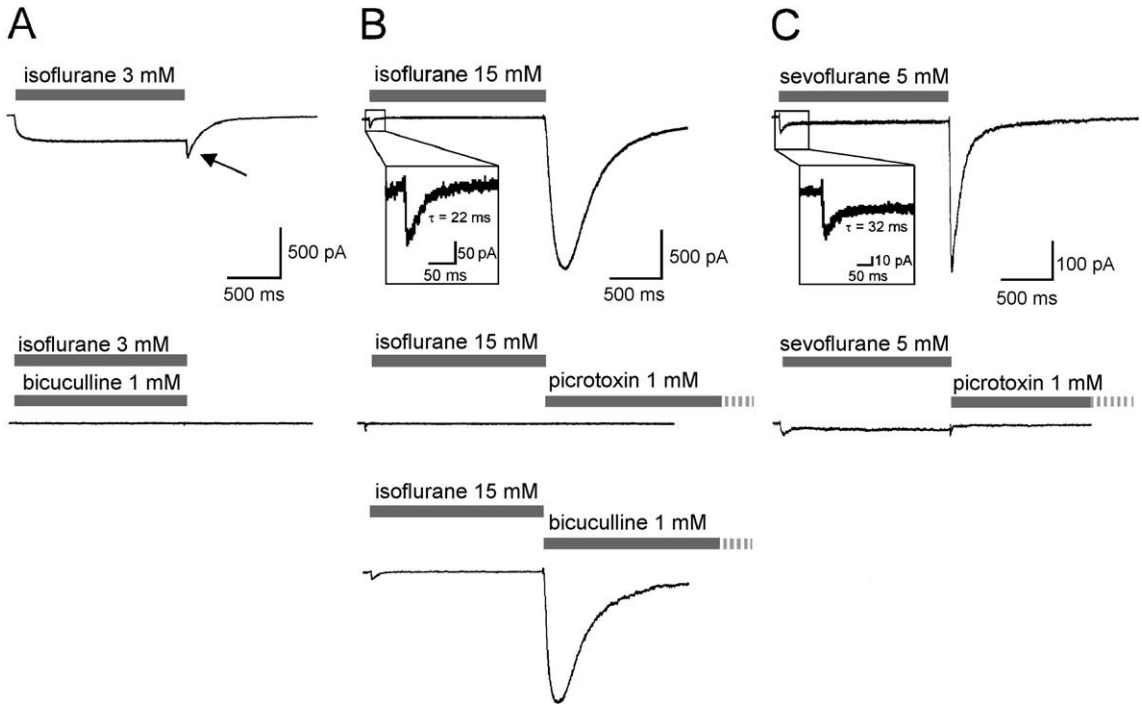

Fig. 2. (A) Application (grey bar) of isoflurane (3 mM) to HEK293 cells carrying $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors induced an inward current that transiently increased (so-called tail current; arrow) upon rapid discontinuation of isoflurane. This biphasic response was blocked by the competitive $GABA_A$ receptor antagonist bicuculline. (B) The five-fold isoflurane concentration (15 mM) induced a small initial response with a rapid decay (see inset). The rapid withdrawal of isoflurane evoked a prominent tail current, which was inhibited by picrotoxin, but not by bicuculline. (C) The response to isoflurane (15 mM) was mimicked by application of sevoflurane (5 mM).

induced a biphasic response: an initial rapid inward current with a plateau is followed by a tail current when isoflurane is rapidly withdrawn. The competitive $GABA_A$ receptor antagonist bicuculline (1 mM) blocked this effect (Fig. 2A). At higher concentrations, isoflurane (15 mM) induced a rather small initial response with a rapid decay ($\tau = 51 \pm 26$ ms, $n = 6$; Fig. 2B, inset). The prominent tail current, which was evoked by the rapid withdrawal of 15 mM isoflurane, was inhibited by the channel blocker picrotoxin, but not by bicuculline (Fig. 2B). The volatile anaesthetic sevoflurane (5 mM) evoked similar responses (Fig. 2C).

The current–voltage relationship of the isoflurane-induced responses is shown in Fig. 3. Isoflurane (3 mM) was applied under clamp potentials ranging from -30 to $+30$ mV. The $GABA_A$ receptor-mediated currents reversed near the chosen chloride equilibrium potential (0 mV). Neither the initial responses nor the tail currents showed any rectification (Fig. 3). This indicates that receptor activation and induction of tail currents are voltage-independent mechanisms.

Fig. 4 depicts the dose–response relationship of isoflurane-induced whole-cell currents. Stepwise increased concentrations of isoflurane (0.15–15 mM) were applied. Current traces recorded from one representative cell are shown in Fig. 4A. The dose-response curve of the plateau current was bell shaped, with a maximum of -35 ± 19 pA at 3 mM isoflurane ($n = 5$; Fig. 4B, top). The slope of the

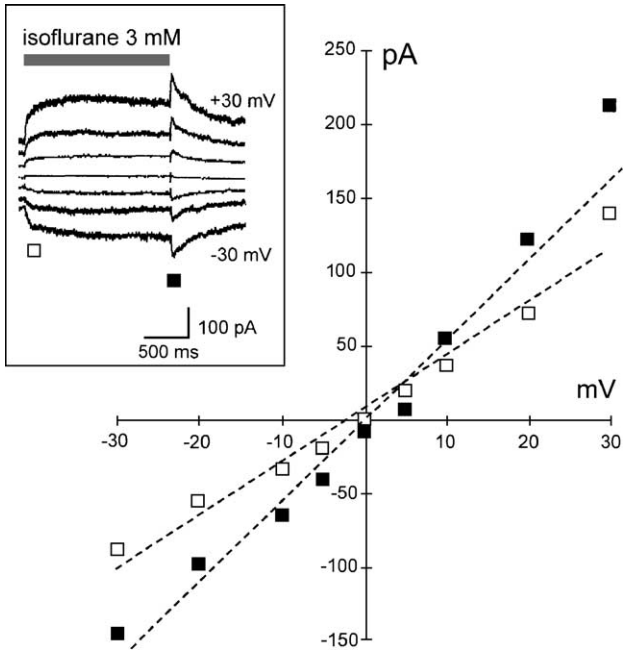

Fig. 3. Current–voltage relationship of $GABA_A$ receptor activation by isoflurane (3 mM). The inset shows currents recorded from one representative cell, clamped at holding potentials from -30 mV to $+30$ mV in 10 mV steps. The initial responses (open squares) and the tail currents (filled squares) were plotted versus the holding potential. The currents reversed near the (chosen) chloride equilibrium potential at 0 mV. Neither initial responses nor tail currents showed rectification.

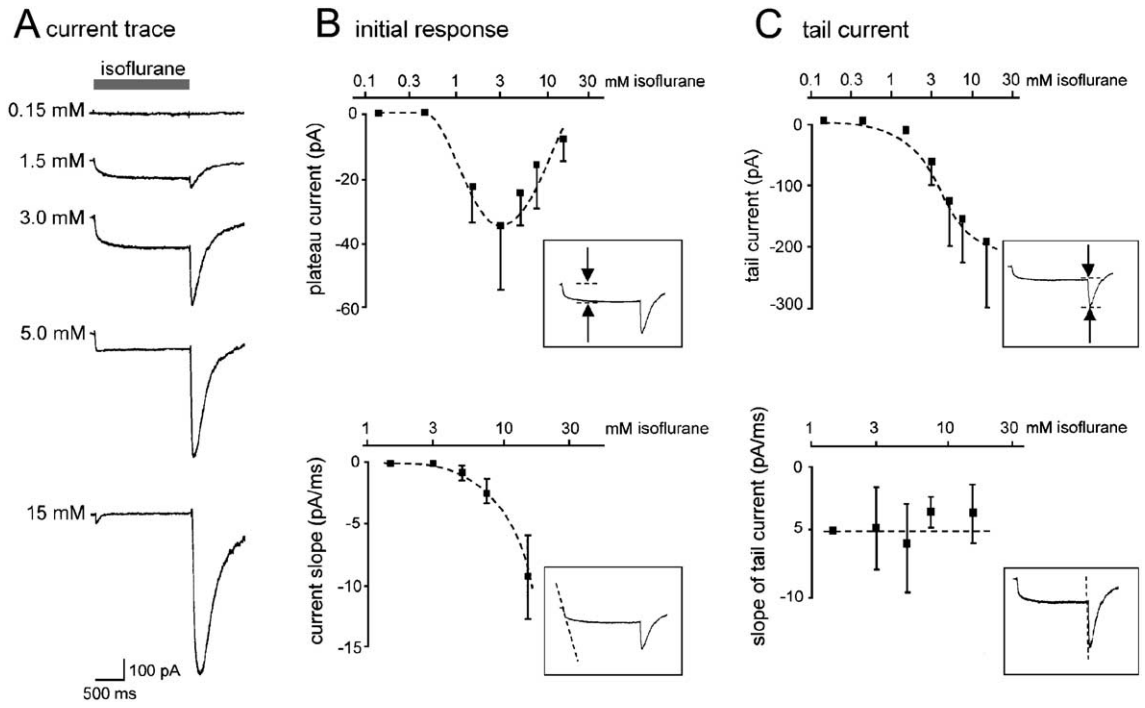

Fig. 4. (A) Increasing isoflurane concentrations (0.15–15 mM) were applied (grey bar) to induce $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptor-mediated whole-cell currents. The current traces show the concentration-dependent increase in the tail current amplitude. (B) The plateau current (at $t = 0.5$ s of isoflurane application, top) and the slope of the initial response (bottom) were plotted versus the isoflurane concentration (means ± S.E.M., $n = 4–6$, note the insets depicting the respective parameter). The dose-response curve of the plateau current was bell shaped. The slope of the initial response increased with increasing isoflurane concentrations. (C) The amplitude of the tail current markedly increased with increasing isoflurane concentrations (top), whereas its slope was concentration-independent (means ± S.E.M., $n = 4–6$, bottom).

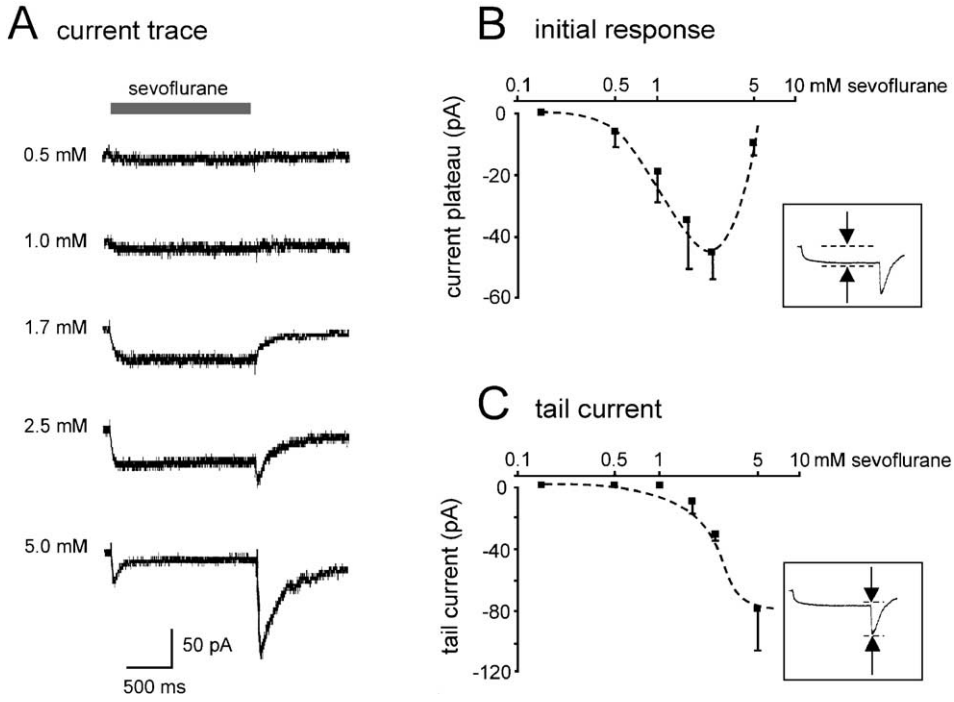

Fig. 5. Application of the volatile anaesthetic sevoflurane (0.5–5 mM). (A) Traces recorded from one representative cell carrying $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. (B) Pooled data (means ± S.E.M., $n = 4$) also revealed a bell-shaped dose-response curve for the plateau current (B) and a concentration-dependent increase in the tail current (C).

initial response increased with increasing isoflurane concentrations (Fig. 4B, bottom). The tail current amplitude also markedly increased with increasing isoflurane concentrations (Fig. 4C, top), whereas the slope of the tail currents was concentration independent (Fig. 4C, bottom).

The application of sevoflurane (0.5–5 mM) to $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors (Fig. 5A depicts current traces from one representative cell) also revealed a bell-shaped dose-response curve for the plateau current, with a maximum of -45 ± 8 pA at 2.5 mM sevoflurane ($n = 4$; Fig. 5B). Fig. 5C depicts the tail current amplitude versus the sevoflurane concentration.

3.2. Kinetic modelling of the interaction between isoflurane and the $GABA_A$ receptor

We based our kinetic scheme of the interaction between isoflurane and the $GABA_A$ receptor on the "three-state kinetic model" theory (see discussion). The experimental data suggest that isoflurane exerts both agonistic and antagonistic effects at the $GABA_A$ receptor. The tail current, which is induced by the withdrawal of isoflurane, reflects the ion flux discharged by the dissociation of isoflurane from a binding site involved in blocking of an open channel. Such a mechanism of tail current induction is in line with the finding that the slope of these tail currents was independent of the isoflurane concentration (see Fig. 4C, bottom). Thus, the model that best simulated the isoflurane-induced responses embodies two agonist-like binding steps required for channel gating, and one additional binding step to block an open channel (Fig. 6A). The rate constants were optimized by fitting simulated and experimental data on dose-response curves and kinetics of initial responses and tail currents. The rate constant K_{off} of isoflurane dissociation was adjusted to a value of 40 s^{-1} to fit the decay of the tail current. The concentration-independent rate constant K_{off} *block* (7 s^{-1}) determines the offset of the isoflurane-induced open-channel block and, thus, the concentration-independent slope of the tail currents (see also Fig. 4C, bottom).

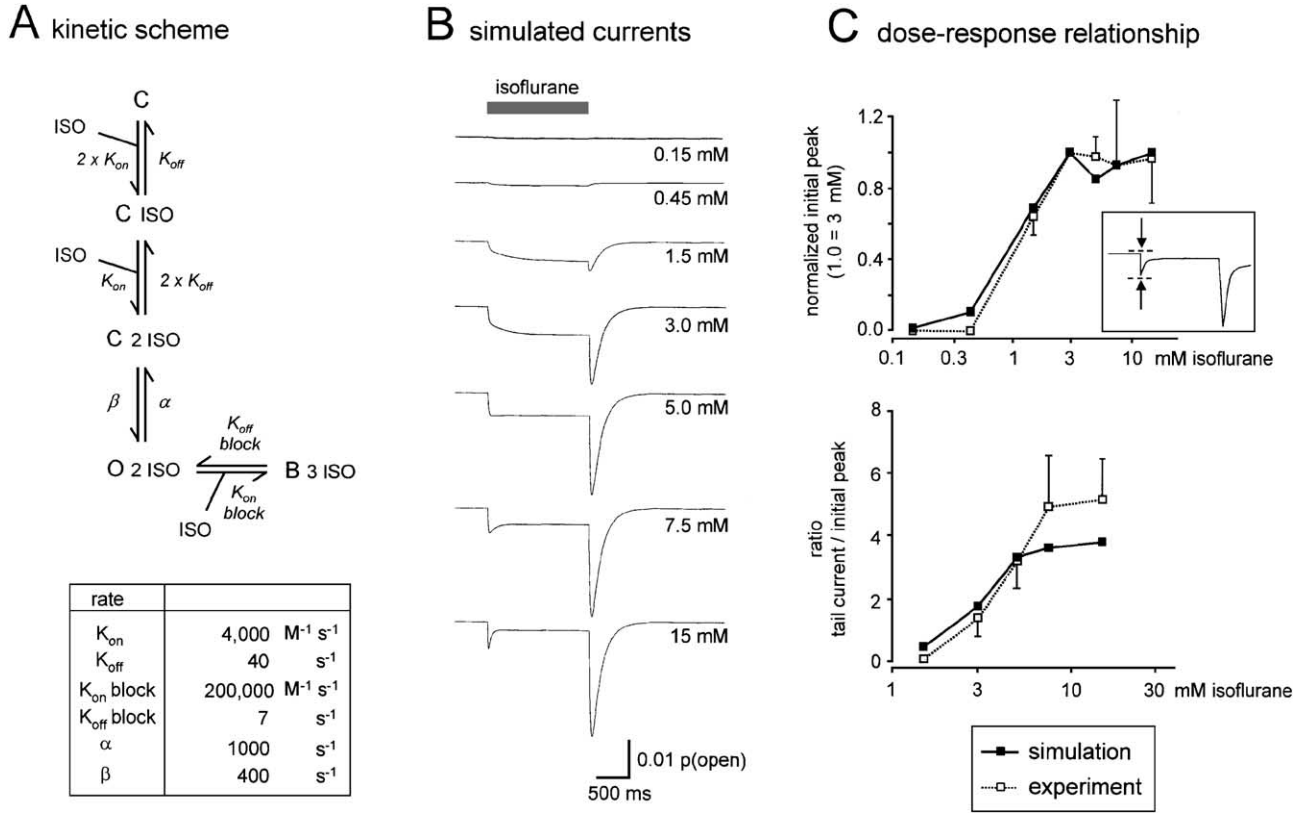

Fig. 6. Kinetic modelling of the interaction between isoflurane (ISO) and the $GABA_A$ receptor. (A) A kinetic scheme of ligand-gated ion channels usually describes receptor activation by agonist binding to the receptor in the closed state (C). After one or more agonist binding steps, the receptor–channel complex can attain the open state (O). The model that best simulated the isoflurane-induced currents embodies two agonist-like binding steps required for channel gating, and one additional binding step operating an open-channel block (blocked state, B; the binding site for an open-channel blocker is usually assumed to reside in the channel pore). The values of the rate constants (box) were optimized by fitting the simulated data to our experimental data. (B) The kinetic scheme, with the rate constants denoted, was used to generate the simulated currents by plotting the time course of the probability of the open state (O 2 ISO). These simulated currents (B) mimic the isoflurane-induced responses (see Fig. 4A). (C) Concentration-response curve of the initial peak (top) and the tail current (bottom). The curve for the initial peak plotted versus the isoflurane concentration is polyphasic (no Hill function), which results from the reciprocity of channel gating and channel block. More than two agonist-like binding steps would not improve the match between simulated and experimental data (data not shown).

The kinetic scheme, with the rate constants depicted (Fig. 6A), was used to generate the simulated currents by plotting the time course of the probability of the open state (O 2 ISO). These simulated currents (Fig. 6B) mimicked the isoflurane-induced responses (see Fig. 4A) in the amplitude of initial response and tail current plotted versus the isoflurane concentration (Fig. 6C).

4. Discussion

The present data suggest that the volatile anaesthetics isoflurane and sevoflurane evoke chloride currents by directly activating mammalian $GABA_A$ receptors. Similar to the non-competitive $GABA_A$ receptor antagonist picrotoxin, isoflurane and sevoflurane also induced an open-channel block at this receptor. Isoflurane and picrotoxin may share a common binding site in the channel pore of mammalian $GABA_A$ receptors, as was suggested for invertebrate GABA receptors (Edwards and Lees, 1997). The finding that picrotoxin suppressed the induction of tail currents on the withdrawal of isoflurane (Fig. 2B, mid) is also in line with the open-channel block mechanism of picrotoxin. In contrast, bicuculline lacked this effect (Fig. 2B, bottom) which favours the idea that bicuculline competitively blocks receptor activation by isoflurane (see also Fig. 2A).

The major aim of the present study was the detailed characterization and modelling of the interaction between a rat recombinant $GABA_A$ receptor and the volatile anaesthetic isoflurane, which had apparently a dual action at $GABA_A$ receptors. We based our kinetic modelling on the "three-state kinetic model" theory first put forward by Del Castillo and Katz (1957). The isoflurane-induced currents were well (with some tolerance) simulated by a kinetic model that embodies two agonist-like binding steps required for channel gating, and another to mediate an open-channel block. We took advantage of a fast piezo-driven application system that allows an almost instantaneous application and withdrawal of solutions to measure tail currents. Therefore, these fast current transients may be considered to reflect processes associated with open-channel block mechanisms. An open-channel block has already been postulated as a mechanism involved in the regulation of cholinergic transmission at the neuromuscular junction in zebrafish (Legendre et al., 2000). In contrast to the open-channel block of $GABA_A$ receptors by isoflurane, which is voltage-independent supposedly due to the non-polar nature of isoflurane, the block of acetylcholine receptors by acetylcholine is voltage dependent (Legendre et al., 2000).

The relevance of open-channel block mechanisms for synaptic signalling in vivo is still unclear. Nevertheless, there is already evidence that an open-channel block can prolong postsynaptic responses (Jones and Harrison, 1993; Legendre et al., 2000). Furthermore, it has been reported that *m*-chlorophenylbiguanide, a specific agonist of the ligand-gated 5-hydroxy-tryptamine$_3$ receptor, also induces an open-channel block at its receptor (Lankiewicz et al., 1998). These findings suggest that a concomitant open-channel block by receptor agonists may be operative also in other transmitter systems.

Is the observed activation of $GABA_A$ receptors evoked by volatile anaesthetics clinically relevant? Isoflurane concentrations of 0.5 mM are assumed to be reached under clinical conditions (Firestone et al., 1986; Jones and Harrison, 1993). The effective concentrations of isoflurane in the brain or even in the synaptic cleft are still obscure. Thus, the extent of the contribution of the GABA-independent receptor activation by isoflurane to its anaesthetic effect in vivo remains to be shown. We suggest that direct $GABA_A$ receptor activation by isoflurane, which has been reported also by others (Yang et al., 1992), and the open-channel block could act in concert to prolong GABAergic synaptic transmission.

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