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Potentiation by sevoflurane of the γ-aminobutyric acid-induced chloride current in acutely dissociated CA1 pyramidal neurones from rat hippocampus

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1 The effects of a new kind of volatile anaesthetic, sevoflurane (Sev), on γ-aminobutyric acid (GABA)-gated chloride current (I_{Cl}) in single neurones dissociated from the rat hippocampal CA1 area were examined using the nystatin perforated patch recording configuration under the voltage-clamp condition. All drugs were applied with a rapid perfusion system, termed the 'Y-tube' method.

2 When the concentrations were higher than 3×10^{-4} M, Sev, itself, induced an inward current (I_{Sev}) at a holding potential (V_H) of −40 mV. The concentration-response curve of I_{Sev} was bell-shaped, with a suppressed peak and plateau currents at high concentrations (above 2×10^{-3} M). The reversal potential of I_{Sev} (E_{Sev}) was close to the theoretical Cl^- equilibrium potential, indicating that I_{Sev} was carried mainly by Cl^-.

3 I_{Sev} was reversibly blocked by bicuculline (Bic), an antagonist of the $GABA_A$ receptor, in a concentration-dependent manner with a half-inhibitory concentration (IC_{50}) of 7.2×10^{-7} M. But I_{Sev} was insensitive to strychnine (Str), an antagonist of the glycine receptor.

4 At low concentrations (between 3×10^{-4} and 10^{-3} M), Sev markedly enhanced the 10^{-6} M GABA induced current (I_{GABA}) but reduced the I_{GABA} with accelerating desensitization accompanied by a 'hump' current after washout at high concentrations (higher than 2×10^{-3} M).

5 Sev, 10^{-3} M potentiated the current induced by low concentrations of GABA (between 10^{-7} and 3×10^{-6} M) but reduced the current induced by high concentrations (higher than 10^{-5} M) of GABA with a clear acceleration of I_{GABA} desensitization.

6 Sev, like pentobarbitone (PB), pregnanolone (PGN) or diazepam (DZP), potentiated the 10^{-6} M GABA-induced response without shifting the reversal potential of I_{GABA}.

7 I_{Sev} was augmented by PB, PGN, or DZP at concentrations that maximally potentiated I_{GABA}, suggesting that Sev enhanced I_{GABA} at a binding site distinct from that for PB, PGN, or DZP.

8 It is concluded that Sev acts on the $GABA_A$ receptor complex mimicking the GABA-induced chloride current at high concentrations. At low concentrations, Sev enhances GABA-gated chloride current at a binding site independent of the allosteric modulator sites of barbiturates, benzodiazepines or neurosteroids. The reversible potentiation of the inhibitory $GABA_A$ receptor-mediated Cl^- current may result in the depressing of postsynaptic excitability and may, at least in part, underlie the anaesthetic action of Sev.

Keywords: Hippocampal neurone; patch-clamp; volatile anaesthetic; sevoflurane; γ-aminobutyric acid; $GABA_A$ receptor; chloride current

Introduction

Despite almost a century of research, the mechanism of action of many anaesthetic agents remains obscure (Seeman, 1972; Franks & Lieb, 1982; 1984; 1987; 1994; Matthews, 1992). It is well known that the potency of anaesthetics correlates well with their lipid solubility, implicating a nonspecific membrane-perturbing mechanism of action (Koblin & Eger, 1986; Ueda & Kamaya, 1984; Richter, 1989). However, some investigators have suggested more specific actions of the anaesthetics on membrane receptors or ion channels, particularly, the $GABA_A$ receptor-Cl^- channel complex on the postsynaptic membrane has been considered as a major target site for most general anaesthetics. (Richards & Smaje, 1976; Halsey, 1984; Franks & Lieb, 1994; MacIver *et al.*, 1989). For example, several classes of general anaesthetics, including the barbiturates, steroids, and etomidate, share the property of potentiating $GABA_A$ receptor operated Cl^- currents (I_{Cl}) at low concentrations; at higher concentrations, these compounds show $GABA_A$-mimetic properties (Barker & Ransome, 1978; Evans & Hill, 1978; Nicoll & Wojtowicz, 1980; Akaike *et al.*, 1985; Barker *et al.*, 1987). The enhancement of GABA-induced I_{Cl} was produced by the volatile anaesthetics as well (Gage & Robertson, 1985a; Wakamori *et al.*, 1991; Jones *et al.*, 1992; Yang *et al.*, 1992). The potentiation of the GABA responses could contribute to anaesthetic-induced central nervous system (CNS) depression by shunting excitatory synaptic currents or by depressing postsynaptic excitability (MacIver *et al.*, 1991; Jones *et al.*, 1992).

Sevoflurane (Sev), a new kind of volatile anaesthetic, provides several advantages over other recently available potent inhaled anaesthetics such as halothane and isoflurane (Wallin *et al.*, 1975; Holaday & Smith, 1981; Scheller *et al.*, 1990; Brown & Frink, 1992; Frink *et al.*, 1992). Sev has a higher aqueous solubility than isoflurane or halothane, for more rapid uptake elimination and adjustment of depth, as well as more rapid recovery from anaesthesia (Yasuda *et al.*, 1991; Binstock *et al.*, 1994). Furthermore, Sev offers a relatively stable heart rate even at doses up to 2.0 MAC (Ebert *et al.*, 1994; Malan *et al.*, 1994), and at concentrations exceeding 1 MAC, sevoflurane produces less respiratory irritation than other volatile anaesthetics (Frink *et al.*, 1992; Holaday &

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Smith, 1981). By the end of 1993, more than 1,000,000 patients had received Sev in Japan, where it is approved for clinical use (Eger II, 1994). However, the mechanism by which Sev acts on the CNS to produce anaesthesia is still unknown. The aim of the present study was to observe the effects of Sev on GABA-induced I_{Cl} in pyramidal neurones freshly dissociated from the rat hippocampal CA1 region using the nystatin perforated patch-clamp recording configuration. Specifically, we focused on whether Sev enhanced GABA-gated I_{Cl} and, if present, whether this potentiating action of Sev was mediated by binding sites for benzodiazepines, barbiturates, or neurosteroids on the $GABA_A$-receptor-Cl^- channel complex. Some of these results have been presented in abstract form (Wu *et al.*, 1994b).

Methods

Preparation

The dissociation technique for rat hippocampal CA1 neurones has been described previously (Wu *et al.*, 1994a). Briefly, 2-week-old Wistar rats were decapitated under ether anaesthesia. The brain was sliced at a thickness of 400 μm with a microslicer (D.S.K., DTK-1000). The brain slices were pre-incubated in an incubation solution bubbled with 95% O_2 : 5% CO_2 gas at room temperature for 60 min. Thereafter, slices were treated with 1 mg pronase 6 ml^{-1} at 31°C for 30 min. After the enzyme treatment, the slices were washed with incubation solution. The CA1 area of the hippocampus was micro-punched out from slices and transferred into a culture dish filled with well-oxygenated standard external solution. The pyramidal neurones were dissociated mechanically with a fire-polished micro-pasteur pipette under the phase-contrast microscope (Nikon, TMS-1). The cells usually adhered to the bottom of the culture dish within 30 min. In the present experiments, we used the pyramidal neurones that maintained their original morphological features, such as the pyramidal-like somata and dendritic processes.

Solutions

The incubation solution had the following composition (in mM): NaCl 124, KCl 5, KH_2PO_4 1.2, $MgSO_4$ 1.3, $CaCl_2$ 2.4, $NaHCO_3$ 24 and glucose 10. The pH was adjusted to 7.4 with 95% O_2 : 5% CO_2 gas. The standard external solution contained (in mM): NaCl 150, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10 and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 10. The pH was adjusted to 7.4 with Tris [hydroxyethyl] aminomethane (Tris-OH). The perforated patch-pipette solution was (in mM): KCl 150 and HEPES 10. The pH was adjusted to 7.2 with Tris-OH. Nystatin was dissolved in acidified methanol and the stock solution was diluted with the internal (patch-pipette) solution to a final concentration of 200 μg ml^{-1}. For examining the reversal potential of the Sev-induced current, KCl in the internal solution was replaced with equimolar CsCl.

Nystatin perforated patch recording

Electrical recordings were made on dissociated pyramidal neurones using the nystatin perforated patch recording configuration under the voltage-clamp condition. The patch-pipettes were made from glass tubes (Narishige, G-1.5) in two stages on a vertical pipette puller (Narishige, PB-7). The resistance between the patch pipette filled with the internal solution and the reference electrode in the standard external solution was 4–6 MΩ. The current and voltage were measured with a patch-clamp amplifier (Nihon Kohden, CEZ-2300), and monitored on both a storage oscilloscope (Tektronix, 5111A) and a thermal-head pen recorder (Nippondenki San-ei, RECTI-HORIZ-8K21), and stored on video tapes after digitization with a PCM processor (Nihon Kohden, PCM501ESN). All experiments were performed at room temperature (21–23°C).

Drugs

The drugs used in the present experiments were pronase (Hoechst); sevoflurane (Sev) (Maruishi); γ-aminobutyric acid (GABA) (Tokyo Kasei); pentobarbitone sodium (PB) (Ishizu); diazepam (DZP) (Yoshitomi); and pregnanolone (PGN), strychnine (Str), bicuculline (Bic), tetrodotoxin (TTX) (Sigma). For preparing a solution with a desired Sev concentration, an amount of the agent calculated from the respective specific gravity (1.52) and molecular weight (200.1) was injected into 55 ml of external solution in a tightly capped glass flask. The solution was then sonicated for a few minutes until the Sev droplet had completely dispersed. The minimum alveolar concentration (MAC) of Sev for anaesthesia is known to be 1.48–2.03%, which give the concentrations of $3.4-4.7 \times 10^{-4}$ M in the blood phase (Katoh *et al.*, 1987; Scheller *et al.*, 1988; Lerman *et al.*, 1990; Nakajima *et al.*, 1993; Inomata *et al.*, 1994; Kimura *et al.*, 1994). Sev was applied every 3 min, at which interval the desensitization of the Sev-induced current was completely removed and constant responses could be repeatedly evoked for more than 1 h. Rapid application of the drugs was performed with the 'Y-tube' method, as described elsewhere (Wu *et al.*, 1994a). By this technique, the external solution surrounding a neurone could be exchanged within 20 ms.

Statistics

Experimental values are represented as mean ± standard error of the mean (s.e.mean) and significance was tested by Student's t test, and a P value of less than 0.05 was considered to be significant. For evaluation of the half-maximal effective concentration (EC_{50}) and the Hill coefficient (n) of the concentration-response curve, data were fitted to the Michaelis-Menten equation using a least-squares fitting, $I = (I_{max} \times C^n)/(C^n + K_d^n)$ (1), where I is current, I_{max} is the maximum current, C is the agonist concentration, and n is the Hill coefficient. The equation for concentration-inhibition curve is the mirror image of the Michaelis-Menton equation, $I = K_d^n/(C^n + K_d^n)$ (2) (Assuming that the value of I_{max} is 1), where C is the antagonist concentration. The data for the concentration-inhibition curve were fitted to (2) by the use of a least-squares fitting to obtain the half-maximal inhibition concentration (IC_{50}).

Results

Sev acts on the $GABA_A$ receptor complex and mimics GABA-induced chloride current

The dissociated CA1 pyramidal neurones were perfused with the standard external and internal solutions, and clamped to a holding potential (V_H) of −40 mV. Figure 1a shows the currents induced by Sev (I_{Sev}) at various concentrations. Sev elicited inward currents consisting of a transient peak and a successive steady-state components. At high concentrations (higher than 2×10^{-3} M), Sev always induced a transient inward 'hump' current after washout (Figure 1a arrowhead, also see Wu *et al.*, 1994a). The concentration-response relationships of transient peak and plateau components of the Sev-induced currents are shown in Figure 1b, in which all responses were nornalized to the peak response induced by 2×10^{-3} M Sev. The threshold concentration of the Sev-induced response was approximately 3×10^{-4} M. However, with increasing concentrations, the Sev-induced peak and plateau current components were reduced, leading to bell-shaped concentration-response curves (Figure 1b). The current-voltage (I-V) relationship for Sev-induced current (peak component) was examined by using the ramp voltage-clamp technique. A depolarizing voltage step of 50 mV was applied before and

during the application of Sev. Since the voltage-dependent Na^+, Ca^{2+} and K^+ channels were blocked by adding 10^{-7} M TTX and 10^{-5} M La^{3+} in the external solution and by replacing K^+ in the internal solution with Cs^+, the intersection of *I-V* curves before and during Sev application is a direct indication of the reversal potential of the I_{Sev} (Figure 1c, dashed line). The reversal potential estimated with the ramp-wave for the Sev-induced response (E_{Sev}) was -1.2 ± 0.7 mV ($n=4$). This value was close to the theoretical Cl^- equilibrium potential (E_{Cl}) of -1.8 mV calculated with the Nernst equation based on the extra- and intracellular Cl^- activities. These results suggest that the Sev-induced current is carried through Cl^- channels.

Application of bicuculline (Bic), a specific antagonist of the $GABA_A$ receptor, reversibly inhibited the Sev-induced I_{Cl} in a concentration-dependent manner with IC_{50} of 7.1×10^{-7} M (Figure 2a (i)). However, strychnine (Str), a specific antagonist of the glycine receptor, did not inhibit Sev-induced I_{Cl} even when the concentration was increased to 10^{-5} M (Figure 2a (ii)). Figure 2b summarizes the effects of Bic and Str on Sev-induced I_{Cl}. These results indicate that Sev induces I_{Cl} by acting on the $GABA_A$-receptor Cl^- channel complex but not on the glycine receptor.

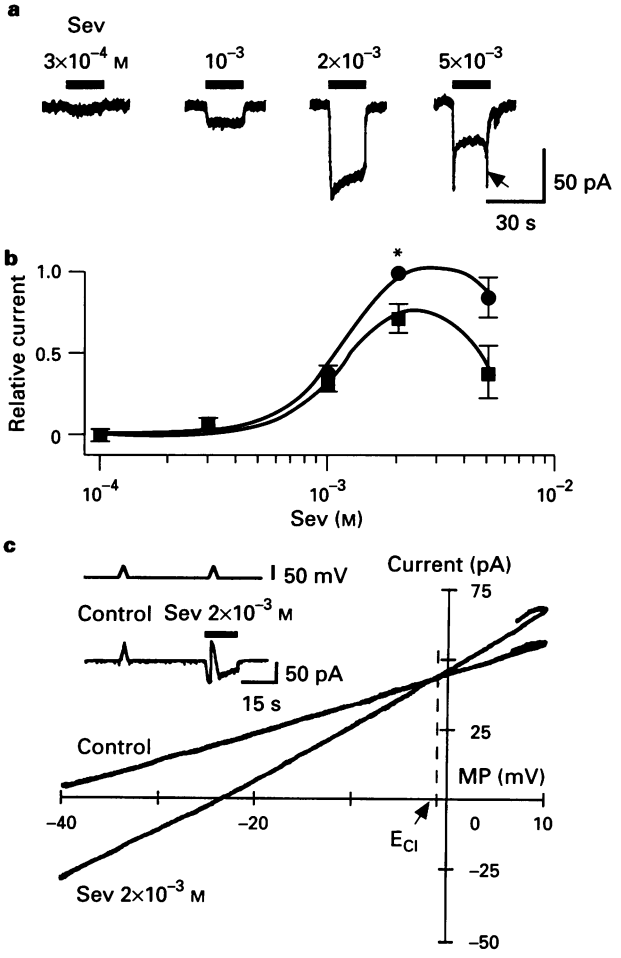

Figure 1 Ionic currents elicited by sevoflurane (Sev) in dissociated hippocampal CA1 pyramidal neurones. (a) Various concentrations of Sev induce inward currents at a holding potential (V_H) of -40 mV. Horizontal bar above each response indicates the application period of Sev. Arrowhead indicates the 'hump' current that appears with washout. (b) Concentration-response curves for Sev-induced peak (●) and plateau (■) currents. The amplitudes of the transient peak and plateau components at the onset of application were normalized to that induced by 2×10^{-3} M Sev (asterisk). Each point is the average of 4 neurones with the s.e. The continuous curves for Sev were fitted by eye. (c) Reversal potential of the inward current (peak component) induced by Sev. The reversal potential was measured by the ramp-wave method (see the inset to (c)). A 50 mV depolarizing voltage pulse was applied before (control) and during application of Sev. The duration of the ramp-wave was 3 to 4 s. The current-voltage (*I-V*) curve for the Sev-induced current shows the reversal potential (dashed line) close to the theoretical equilibrium potential (arrowhead) of Cl^- calculated with the Nernst equation based on the extra- ($[Cl^-]_o$) and intracellular Cl^- concentrations ($[Cl^-]_i$) of 161 and 150 mM, respectively. MP stands for membrane potential.

Sev potentiates the GABA-induced response

GABA binds to the $GABA_A$ receptor increasing Cl^- flux (Olsen, 1982). Since previous studies have shown that the volatile anaesthetics have a binding affinity for the $GABA_A$ receptor (Huidobro-Toro *et al.*, 1987), we examined the interaction between Sev and GABA-induced I_{Cl}. Figure 3a shows the 10^{-6} M GABA-induced current response (I_{GABA}) with only a steady-state plateau without desensitization at a V_H of -40 mV. Pretreatment with Sev enhanced the I_{GABA} in a narrow concentration range between 10^{-4} and 10^{-3} M. The degree of potentiation by Sev is summarized in Figure 3b. The amplitude of I_{GABA} was increased to $206.0\pm13.1\%$ (10^{-6} M I_{GABA} as 100%, $n=4$, $P<0.01$) or $352\pm49\%$ ($n=4$, $P<0.01$) in the presence of 3×10^{-4} or 10^{-3} M Sev, respectively. When the Sev concentration was increased to 2×10^{-3} M, the amplitude of I_{GABA} was $115\pm10.2\%$ ($n=3$, $P>0.05$) but clear desensitization of I_{GABA} was observed, and also an inward hump current appeared after washout. Further increase of Sev concentration to 5×10^{-3} M, dramatically accelerated the de-

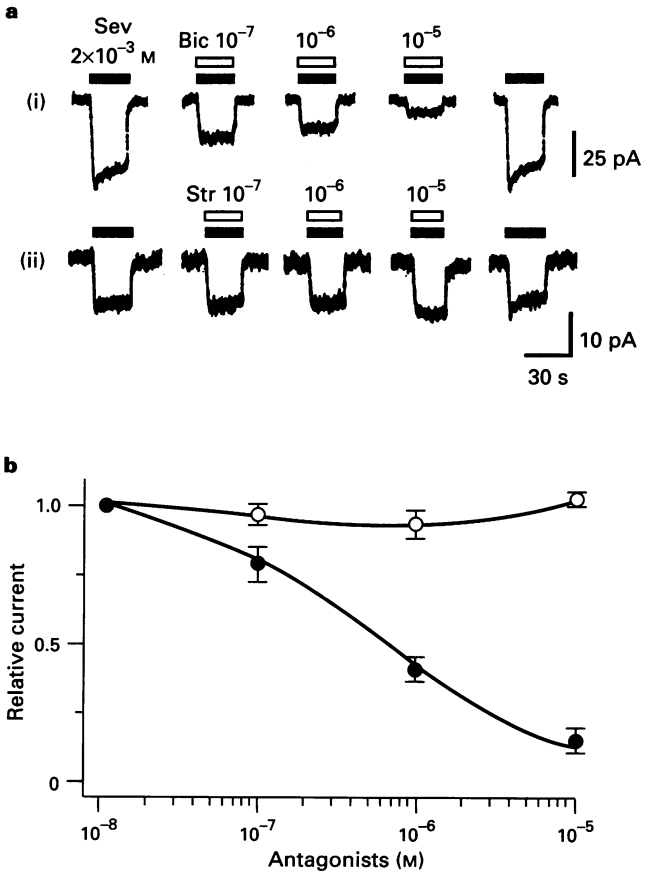

Figure 2 Effects of bicuculline (Bic) and strychnine (Str) on I_{Sev}. (a) (i): I_{Sev} was blocked by Bic in a concentration-dependent manner. (a) (ii): I_{Sev} was insensitive to Str. Horizontal bars above all responses show the application periods of 2×10^{-3} M Sev (solid bars) and various concentrations of antagonists (open bars). (b) Concentration-inhibition curves of the Sev-induced responses in presence of Bic (●) or Str (○). The concentration of Sev was 2×10^{-3} M. Each point is the mean of 4–6 neurones with s.e.

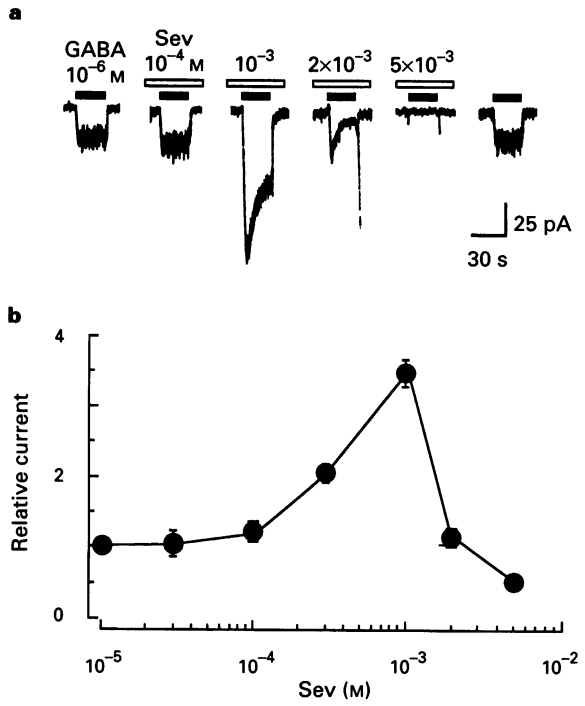

Figure 3 Potentiation of GABA-gated chloride current (I_{Cl}) by various concentrations of Sev. V_H was of −40 mV. (a) Horizontal bars above each response show the application of 10^{-6} M GABA (solid bars) and various concentrations of Sev (open bars). Neurones were pretreated with Sev for 10 s. (b) Facilitation ratio of GABA-gated I_{Cl} by Sev (ordinate scale), plotted against log Sev concentrations (abscissa scale). The ratio was calculated according to the formula: $I_{GABA+Sev}/I_{GABA}+I_{Sev}$. The amplitudes were measured at the transient peaks following agonist applications. Each point is the average of 4–6 neurones with the s.e.

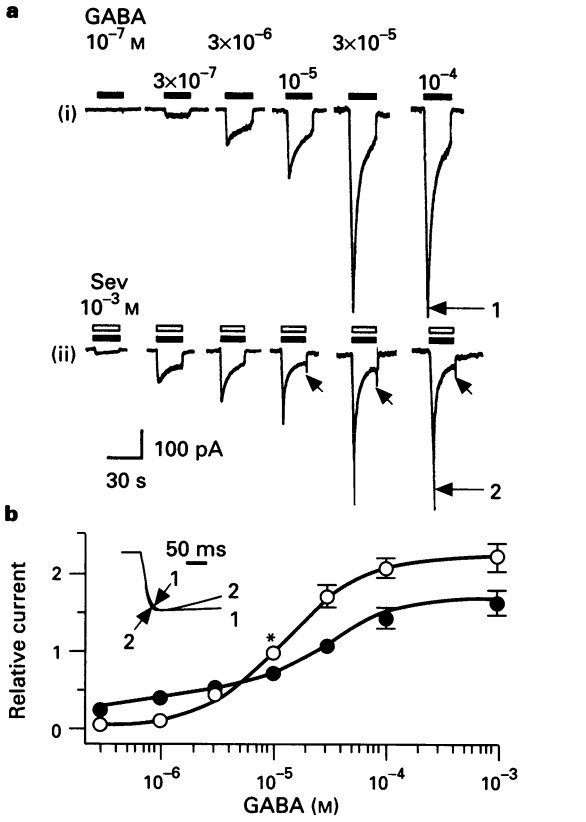

Figure 4 Effect of Sev on the concentration-response relationship of the GABA-induced I_{Cl}. (a (i)) I_{Cl}s elicited by GABA at various concentrations. (a (ii)) I_{Cl}s elicited by various concentrations of GABA plus 10^{-3} M Sev. Neurones were exposed to GABA alone (solid horizontal bars) or GABA in the presence of Sev (open bars) for periods indicated by horizontal bars above all responses. Recordings in (a (i)) and (a (ii)) were obtained from the same neurone. Arrowheads indicate 'hump' currents that appear with washout of GABA. (b) Concentration-response curves for GABA in absence (○) or presence of Sev (●). Amplitudes of the peak current components were normalized to that induced by 10^{-5} M GABA alone (asterisk). Inset to (b) shows the superimposed I_{Cl} induced by 10^{-4} M GABA (1) and GABA plus 10^{-3} M Sev (2). The numbers correspond to those in (a). The start of GABA application and the peak amplitudes were aligned so that the differences in activation and desensitization become more evident. Each point is the average of 4–8 neurones and the vertical bars represent ± s.e.

sensitization of I_{GABA} (the steady-state component decreased to zero) and the amplitude of I_{GABA} was reduced to 52.3 ± 2.5% ($n=3$, $P<0.05$).

Figure 4a (i) shows the concentration-dependent GABA responses. Below 3×10^{-6} M, GABA elicited only a steady-state plateau current (also see Figure 3a). With higher concentrations, GABA induced a transient peak response, which decayed to a steady-state plateau level due to desensitization during a sustained application of GABA. Co-application of 10^{-3} M Sev increased the amplitude of GABA-induced I_{Cl} for GABA concentrations between 10^{-7} M and 10^{-6} M with the facilitatory ratio greatest with a GABA concentration of 3×10^{-7} M. When concentrations of GABA were higher than 10^{-5} M, the co-application of Sev also caused the peak of I_{Cl} to fall more quickly to the plateau level compared with that induced by GABA alone (Figure 4a (ii)). Figure 4b illustrates the concentration-response curve of the peak I_{Cl}s induced by GABA in the presence or absence of Sev in 4–8 neurones. In the presence of 10^{-3} M Sev, the low concentration GABA-induced responses were potentiated (3×10^{-7} M = 444.4 ± 47.6%, 10^{-6} M = 354.5 ± 40.5%, $P<0.01$; 3×10^{-6} M = 106.3 ± 7.5%, $P>0.05$), whereas the high concentration GABA-induced responses were significant reduced (10^{-5} M = 73.0 ± 7.1%, 10^{-4} M = 64.4 ± 10.5%, 10^{-3} M = 74.5 ± 17.5, $P<0.01$).

Sev, PB, DZP, or PGN potentiate the GABA-gated I_{Cl} without a shift of the reversal potential

GABA-gated I_{Cl} is characteristically augmented by allosteric modulators such as barbiturates, benzodiazepines, or neurosteroids, as shown in frog sensory neurones (Akaike *et al.*, 1985; 1990; Yakushiji *et al.*, 1989c), rat hippocampal neurones (Pearce *et al.*, 1989), and bovine cultured chromaffin cells (Peters *et al.*, 1988). The potentiating actions of these agents were examined here in freshly dissociated rat hippocampal pyramidal neurones. Pentobarbitone (PB) and 5β-pregnan-3α-ol-20-one (pregnanolone, PGN) are potent stimulators of barbiturate and neurosteroid binding sites, respectively (Yakushiji *et al.*, 1989b), and diazepam (DZP) if a full agonist at the benzodiazepine receptor (Yakushiji *et al.*, 1989a). As seen in Figure 5a and b, in the presence of 10^{-3} M Sev, 3×10^{-5} M PB, 10^{-6} M DZP or 10^{-7} M PGN, 10^{-6} M GABA-induced responses were reversibly enhanced ($P<0.01$), respectively.

Reversal potentials (E_{GABA}s), during potentiation of the GABA response by Sev, PB, DZP, or PGN, were measured using the ramp-wave method. E_{GABA}s in the presence of Sev, PB, DZP, or PGN showed no detectable shift and remained close to E_{Cl} (Figure 5c), indicating that all potentiating effects were induced at the receptor level, and not as a shift in the reversal potential by opening of additional channels.

Augmentative interactions between Sev and PB, DZP, or PGN

The interactions of Sev on the PB-, DZP- or PGN-binding sites were examined. Sev (10^{-3} M)-induced response was markedly enhanced by 3×10^{-5} M PB, 10^{-6} M DZP, or 10^{-7} M PGN,

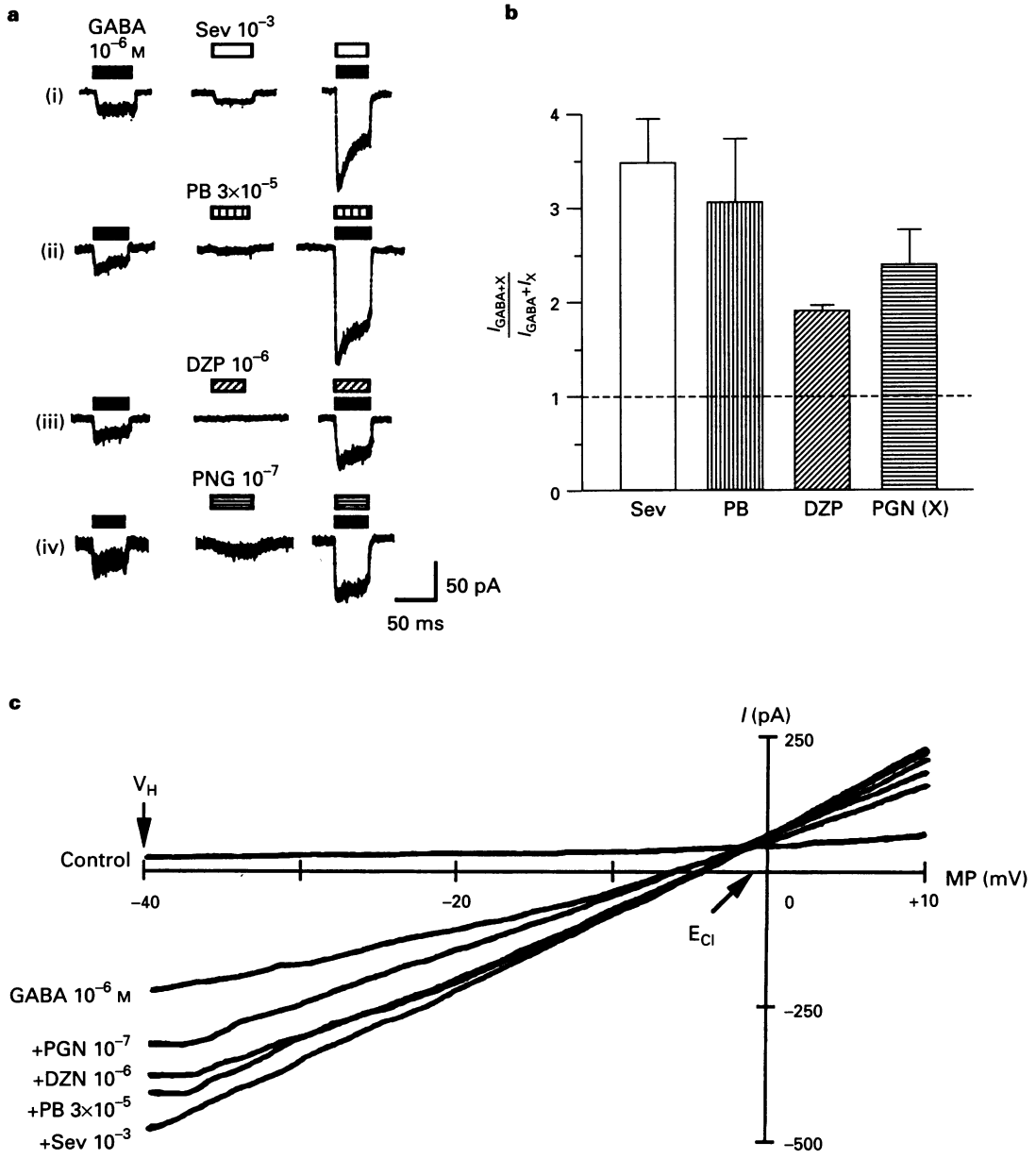

Figure 5 Enhancement of GABA-gated I_{Cl} by Sev, PB, DZP, or PGN without a shift of reversal potential. (a (i)) I_{Cl}s were first elicited either by 10^{-6}M GABA or 10^{-3}M Sev alone, and successively by the co-application of GABA plus Sev; a (ii–iv): Potentiating effects by 3×10^{-5}M PB, 10^{-7}M PGN, or 10^{-6}M DZP. Data are obtained from different neurones. (b) Each column represents the mean value of the facilitatory ratio, calculated with the formulae shown on the ordinary scale. X represents Sev, PB, PGN, or DZP. Number of neurones was 4–5 and s.e. is shown. (c) *I-V* relationship of GABA-induced I_{Cl}, potentiated by Sev, PB, PGN, or DZP in the same neurone. $[Cl^-]_o = 161$, $[Cl^-]_i = 150$ mM, $V_H = 40$ mV. Ramp-waves were applied before and during GABA reponses in presence or absence of Sev, PB, PGN, or DZP.

respectively (Figure 6). The enhancing actions were completely reversible. Since the concentrations of PB, DZP and PGN that were used could induce the maximal potentiation on GABA-induced response (Akaike & Oomura, 1985; Itabashi *et al.*, 1992; Yakushiji *et al.*, 1993; Yakushiji *et al.*, unpublished data), the augmentation of the Sev-induced response by these agents suggests that Sev affects the $GABA_A$-receptor Cl^- channel complex at a binding site independent from those of PB, DZP, or PGN.

Discussion

This study has shown that Sev, at clinically relevant concentrations, acts on the $GABA_A$-receptor Cl^- channel complex potentiating the GABA-induced response by an independent binding site from those of PB, DZP, or PGN, in which Sev allosterically modifies the function of the $GABA_A$ receptor-Cl^- channel complex.

$GABA_A$ receptor-channel complex as a major target for anaesthetics

Although the molecular and cellular mechanisms of anaesthesia induced by anaesthetics are still controversial, there has been an explosion of studies over the past decade on the influence of anaesthetics on putative targets in the CNS. More and more evidence supports the hypothesis that anaesthetics might act by potentiating inhibitory synaptic transmission, and the $GABA_A$ receptor channel complex has been established as a prime anaesthetic target by recent electrophysiological studies (Franks & Leib, 1994).

Most anaesthetics are very effective at potentiating responses to GABA. For instance, it has been suggested that

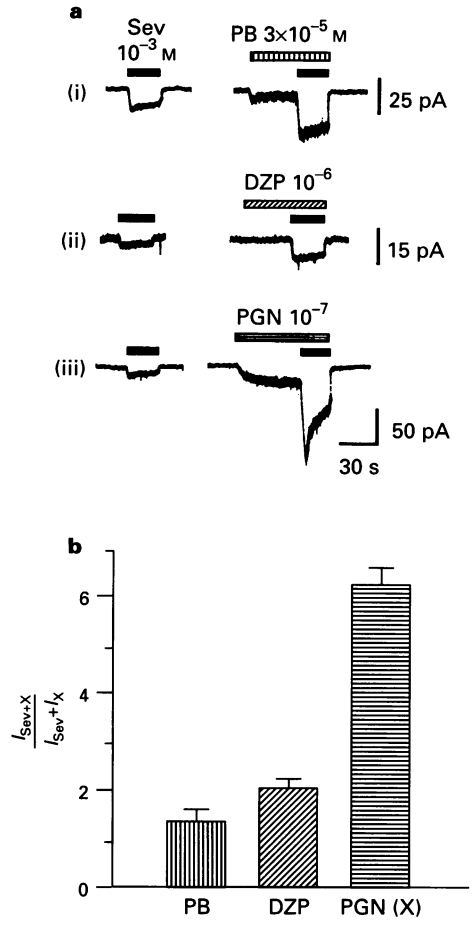

Figure 6 Enhancement of Sev-induced I_{Cl} by maximally effective concentrations of PB, PGN, or DZP. (a (i–iii)) Data were obtained from different neurones: (i) I_{Cl} was elicited by 10^{-3} M Sev alone, followed by Sev in the continuous presence of 3×10^{-5} M PB. (ii) and (iii) Enhancement of Sev represented by 10^{-7} M PGN and 10^{-6} M DZP, respectively. (b) Each column represents the mean value of the facilitatory ratio, calculated with the formulae shown on the ordinate scale. X represents PB, PGN, or DZP. Number of neurones was 4–5 and ± s.e. are shown.

barbiturates, benzodiazepines, steroids and propofol bind to the $GABA_A$ receptor-channel complex and consequently augment the inhibitory $GABA_A$ receptor-mediated responses via allosteric modulation of receptor function (Nicoll & Wojtowicz, 1980; Study & Barker, 1981; Barker *et al.*, 1987; Itabashi *et al.*, 1992; Hara *et al.*, 1994). A similar picture is also emerging for volatile anaesthetics. Nakahiro *et al.* (1989) reported that the volatile anaesthetics showed the GABA-potentiating properties at low concentrations but the residual steady-state current following desensitization by high levels of GABA was inhibited rather than enhanced. At high concentrations, Yang *et al.* (1992) reported that the volatile anaesthetics, like several other general anaesthetics, had a GABA-mimetic effect. Prolongation of an inhibitory postsynaptic current by volatile anaesthetics was reported in slices of guinea-pig olfactory cortex (Scholfield, 1980) and rat hippocampus (Gage & Robertson, 1985b). Wakamori *et al.* (1991) examined the effects of volatile anaesthetics on inhibitory and excitatory amino acid responses and found that the volatile anaesthetics, at clinically relevant concentrations, markedly augmented the GABA-gated I_{Cl}, but only slightly decreased the glutamate-induced responses. Jones *et al.* (1992) reported that three volatile anaesthetics, enflurane, halothane, and isoflurane enhanced GABA-activated Cl^- currents in rat cultured hippocampal neurones and concluded that $GABA_A$ receptors appear to be a target for a number of structurally diverse general anaesthetic molecules. Single channel study with volatile agents (Yeh *et al.*, 1991) showed no change in channel conductance, but the channel open time increased, consistent with the prolongation of postsynaptic current, suggesting that the molecular basis for the potentiation of GABA action by anaesthetics is an increased open probability for the $GABA_A$ receptor channel.

Sev potentiates the GABA-induced I_{Cl}

In the present study, low concentrations (3×10^{-4}–10^{-3} M) of Sev potentiated the low concentration GABA-induced currents (3×10^{-7}–10^{-6} M), but reduced the currents induced by high concentrations of GABA (higher than 10^{-5} M) with a dramatic acceleration of the I_{GABA} desensitization, suggesting that Sev, like other volatile anaesthetic agents, potentiates the GABA-induced Cl^- currents. Since Sev enhances low concentrations of GABA-induced I_{Cl} without shifting the reversal potential (Figure 5c), the most simple explanation is that Sev increases the apparent affinity of GABA for the $GABA_A$ receptor (Wakamori *et al.*, 1991; Franks & Lieb, 1994). In high concentrations GABA-induced I_{Cl}s (higher than 3×10^{-6} M), Sev clearly speeds up desensitization (Figure 4a (ii)); this may reflect the extent to which the channels are functionally active and it would be a secondary consequence of the increased affinity for GABA (Franks & Lieb, 1994). On the other hand, high concentrations of Sev reduced GABA-induced I_{Cl}. We sought to ascribe the apparent decrease of the peak amplitude to two factors. Firstly, Sev accelerates the desensitization to GABA, as shown in Figure 3a and 4a (ii). While Sev accelerated the activation, it also greatly facilitated the desensitization, thereby reducing the peak duration of GABA-induced I_{Cl} (Figure 4b). The onset of desensitization would effectively contribute to the apparent decrease of the peak amplitude. The second factor is the putative channel blocking action of high concentrations of Sev, indicated by the presence of a hump current after washout (Figures 3a, 4a (ii); also see Wu *et al.*, 1994a). Sev at concentrations over 3×10^{-3} M may itself block the Cl^- channel and reduce the amplitude of the GABA-induced I_{Cl}. The washouts of Sev and GABA remove the external solution containing the high concentrations of Sev, and the released molecules that once blocked the Cl^- channels may activate the un-desensitized $GABA_A$ receptor to produce the hump current. The same hump current has been observed during washout of high concentrations of acetylcholine in frog endplate (Adams, 1975), pentobarbitone in rat paratracheal ganglion cells (Itabashi *et al.*, 1992), and strychnine in rat hippocampal CA1 neurones (Ebihara *et al.*, 1992). Furthermore, the high concentration (2×10^{-3} M) Sev-induced I_{Cl} was blocked in a concentration-dependent manner by Bic, a specific antagonist of the $GABA_A$ receptor, but not by Str, a specific antagonist of the glycine receptor. These results strongly indicate that Sev acts on the $GABA_A$-receptor-Cl^- channel complex but not on the glycine receptor. Also these data suggest that the action of Sev in the CNS is rather specific, which supports the hypothesis of a more specific action on receptor proteins or ion channels of the volatile anaesthetics (Richards 1976; Halsey, 1984; Franks & Lieb, 1994; MacIver *et al.*, 1989).

Sev potentiates the $GABA_A$ *receptor-mediated response with a different binding site from other allosteric modulators*

The $GABA_A$ receptor complex is a multisubunit ligand-gated ion channel. GABA binding increases Cl^- flux and this response can be modified by several classes of drugs including the barbiturates, benzodiazepines, and steroids (Barker & Ransome 1978; Akaike & Oomura, 1985; Akaike *et al.*, 1985; Hattori *et al.*, 1986; Barker *et al.*, 1987; Yakushiji *et al.*, 1989a,b,c; Akaike *et al.*, 1990). Sedative hypnotic barbiturates, such as pentobarbitone, in electrophysiological studies, enhance the actions of GABA by increasing the mean channel open time (Study & Barker, 1981; Akaike *et al.*, 1990) and the

channel opening frequency by promoting burst-like activity of the GABA-gated single channel current (Mathers, 1985; 1987). This idea confirms the biochemical findings that barbiturates enhance the binding of GABA to its receptor and decrease the dissociation rate (Willow & Johnston, 1980; 1981; Willow, 1981; Olsen, 1982). Electrophysiological experiments have indicated that benzodiazepines, such as diazepam or flunitrzepam, enhance the action of GABA at the $GABA_A$ receptor by increasing the frequency of Cl^- channel opening (Suria & Costa, 1975; Macdonald & Barker, 1977; Study & Barker, 1981). Biochemical experiments demonstrated the existence of specific high-affinity binding sites for benzodiazepines on brain membranes, which are closely associated with $GABA_A$ receptors (Olsen, 1982). Several steroids, like barbiturates, were found to enhance GABA-induced Cl^- conductance in rat brain by prolonging the open time of the Cl^- channel (Peters *et al.*, 1988). In addition, these compounds enhanced the binding affinity of the $GABA_A$ agonist [^{3}H]-muscimol or of the benzodiazepine receptor agonist [^{3}H]-flunitrazepam to the receptor (Gee, 1988; Gee *et al.*, 1988). These data provide evidence for a separate site of action of barbiturates, benzodiazepines, and steroids at the $GABA_A$-receptor complex. In the present study, Sev modified GABA-gated I_{Cl} seemed to be at a binding site distinct from those for $GABA_A$, PB, DZP, or PGN for the following reasons: (1) at high concentrations, Sev-induced a I_{Cl} similar to the GABA-gated I_{Cl} (GABA-mimetic action) (Figures 1 and 2) and I_{Sev} was blocked by bicuculline in a concentration-dependent manner but not by strychnine (Figure 3), indicating that Sev binds to the $GABA_A$ receptor complex. (2) At low concentrations, Sev enhanced GABA-gated I_{Cl} markedly (Figure 3). Furthermore, I_{Sev} was never blocked by strychnine (even when the concentration was increased to 10^{-5} M) (Figure 2) but GABA-induced I_{Cl} by binding to the $GABA_A$ receptor binding site was also blocked by high concentrations of strychnine (Shirasaki *et al.*, 1991), suggesting the potentiation of GABA-gated I_{Cl} by Sev at a binding site which was different from the $GABA_A$ binding site. (3) Sev-induced I_{Cl} was enhanced by maximally effective concentrations of PB, DZP, or PGN (Figure 6), demonstrating that Sev has a binding site in the $GABA_A$ receptor complex different from that of other allosteric modulators.

Significance of the present studies

This is the first study of the interaction of Sev with GABA-induced currents at the single cell level using patch-clamp perforated whole-cell recording method. The results clearly show that Sev, like other volatile anaesthetics, potentiates the GABA-induced response. In the present experiments, the concentration for Sev-induced maximal inward current was 2×10^{-3} M (Figure 1a,b), and for the maximal potentiated effect of Sev on 10^{-6} M GABA-induced response was 10^{-3} M (Figure 3). In clinical practice, the minimum alveolar anaesthetic concentration (MAV) of Sev is 1.48–2.03%, depending on patient age and methodology (Katoh *et al.*, 1987; Scheller *et al.*, 1988; Lerman *et al.*, 1990; Nakajima *et al.*, 1993; Inomata *et al.*, 1994 and Kimura *et al.*, 1994). Thus, above the MAC of Sev used in clinical practice is given a concentration of $3.4–4.7 \times 10^{-4}$ M in blood phase. In this range of concentrations, Sev (3×10^{-4} M) increased the amplitude GABA-induced current to 205% (Figure 3b). In addition, Sev is seldom used as the sole agent during anaesthesia in the operating room. Usually, Sev induces anaesthesia in a mixture of N_2O and O_2, which will decrease the concentration of Sev. Recently, Kimura *et al.* (1994) reported that anaesthesia induction followed by tracheal intubation can be accomplished in adults when sevoflurane is administered as a sole anaesthetic, but in excess of 8% end-tidal concentrations, which is two times higher than the 4.52% concentration of Sev required for tracheal intubation (MAC_{EL}). These data indicate that 2.0 MAC is necessary to produce anaesthesia if Sev is used as the sole agent to produce anaesthesia in the clinic. The 2.0 MAC of Sev ($6.8–9.4 \times 10^{-4}$ M) is very close to the maximal effect of Sev (10^{-3} M) necessary to potentiate the GABA-induced response in the present study.

In summary, we have found that sevoflurane at clinically relevant concentrations acts on the $GABA_A$ receptor-channel complex mimicking or potentiating the GABA-induced Cl^- current with a binding site distinct from that for barbiturates, benzodiazepines, or neurosteroids. Sevoflurane's allosteric modulation of the postsynaptic $GABA_A$ receptor-channel complex may, at least in part, contribute to its mechanism of anaesthesia.

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