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Inhibition of excitatory amino acid-activated currents by trichloroethanol and trifluoroethanol in mouse hippocampal neurones

[1,2]Robert W. Peoples & [1]Forrest F. Weight

[1]Laboratory of Molecular and Cellular Neurobiology, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, Maryland 20892-8115, USA

1 The effects of the active metabolite of chloral derivative sedative-hypnotic agents, 2,2,2-trichloroethanol (trichloroethanol), and its analog 2,2,2-trifluoroethanol (trifluoroethanol), were studied on ion current activated by the excitatory amino acids *N*-methyl-D-aspartate (NMDA) and kainate in mouse hippocampal neurones in culture using whole-cell patch-clamp recording.

2 Both trichloroethanol and trifluoroethanol inhibited excitatory amino acid-activated currents in a concentration-dependent manner. Trichloroethanol inhibited NMDA- and kainate-activated currents with IC_{50} values of 6.4 and 12 mM, respectively, while trifluoroethanol inhibited NMDA- and kainate-activated currents with IC_{50} values of 28 and 35 mM, respectively.

3 Both trichloroethanol and trifluoroethanol appeared to be able to inhibit excitatory amino acid-activated currents by 100 per cent.

4 Concentration-response analysis of NMDA- and kainate-activated current revealed that trichloroethanol decreased the maximal response to both agonists without significantly affecting their EC_{50} values.

5 Both trichloroethanol and trifluoroethanol inhibited excitatory amino acid-activated currents more potently than did ethanol. The inhibitory potency of trichloroethanol and trifluoroethanol appears to be associated with their increased hydrophobicity.

6 The observation that trichloroethanol inhibits excitatory amino acid-activated currents at anaesthetic concentrations suggests that inhibition of excitatory amino acid receptors may contribute to the CNS depressant effects of chloral derivative sedative-hypnotic agents.

Keywords: Alcohol; anaesthetic; chloral hydrate; receptor; ion channel; neuronal excitability; glutamate receptor; NMDA receptor

Introduction

Although the mechanism of action of general anaesthetics has not been established, ligand-gated ion channels have recently emerged as possible sites of anaesthetic action for at least some anaesthetics (Tanelian *et al*., 1993; Franks & Lieb, 1994). As γ-aminobutyric acid (GABA) acting upon $GABA_A$ receptor-ion channels mediates the majority of fast inhibitory neurotransmission in the brain, it has been proposed that enhancement of the function of the $GABA_A$ receptor may contribute to the anaesthetic state (Tanelian *et al*., 1993). The potential importance of the $GABA_A$ receptor in anaesthesia is suggested by observations that its activity can be enhanced by a very diverse assortment of CNS depressants, including benzodiazepines, barbiturates, anaesthetic steroids, ethanol, and inhalational anaesthetics (Tanelian *et al*., 1993; Franks & Lieb, 1994). The apparent importance of the $GABA_A$ receptor-ion channel in anaesthesia, however, does not exclude the possibility of other important sites of anaesthetic action in the nervous system. For example, because excitatory amino acid-activated ion channels subserve the majority of excitatory neurotransmission in the CNS (Mayer & Westbrook, 1987), these ion channels are also possible sites of action of anaesthetic agents. In this regard, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate glutamate receptors are inhibited by anaesthetic concentrations of barbiturates (Weight *et al*., 1992; Marszalec & Narahashi, 1993), and NMDA receptors are potently inhibited by ethanol and other alcohols (Lovinger *et al*., 1989; Hoffman *et al*., 1989), as well as by dissociative anaesthetics such as phencyclidine and ketamine (Anis *et al*., 1983). Although dissociative anaesthesia differs considerably from general anaesthesia (Winters, 1976), a possible role of the NMDA receptor in contributing to the anaesthetic state is suggested by the observation that NMDA receptor antagonists increase the potency or duration of action of various anaesthetics (Scheller *et al*., 1989; Daniell, 1990). Similarly, the AMPA/kainate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX) increased the duration of anaesthesia produced by hexobarbital (Dall *et al*., 1993), and reduced the anaesthetic concentration of halothane by nearly 60% (McFarlane *et al*., 1992). Thus, while excitatory amino acid receptor inhibition by itself may not be sufficient to account for the phenomenon of anaesthesia, these receptors may nevertheless, at least in certain instances, be among several sites acting in concert to produce the anaesthetic state.

The primary metabolite of chloral derivative sedative/hypnotic compounds, trichloroethanol, is believed to be responsible for the pharmacological effects of these agents (Hobbs *et al*., 1996). Recent studies have shown that trichloroethanol can enhance $GABA_A$ receptor-mediated current and synaptic transmission in CNS neurones (Lovinger *et al*., 1993; Peoples & Weight, 1994), and 5-HT_3 receptor-mediated current in neurones (Lovinger & Zhou, 1993) and *Xenopus* oocytes (Downie *et al*., 1995). In the present study, we investigated the action of trichloroethanol, and its analog trifluoroethanol, on excitatory amino acid-activated currents

[2] Author for correspondence.

in hippocampal neurones. Some of the results presented here have been reported previously in preliminary form (Peoples & Weight, 1991).

Methods

Cultures of hippocampal neurones were prepared from 15- to 17-day fetal mice as described previously (Peoples & Weight, 1994). Neurones were cultured for 1 to 4 weeks prior to use in experiments.

Patch-clamp recording of whole-cell currents was performed in hippocampal neurones at room temperature using a List EPC-7 (List Medical), Axopatch-1D (Axon Instruments), or Axopatch 200 (Axon Instruments) patch-clamp amplifier. Gigaohm seals were formed using electrodes with tip resistances of 2–5 MΩ, and series resistances of 4–15 MΩ were compensated by 40–80 per cent. Membrane potentials were held at −50 mV. Data were recorded using a Gould 2400S chart recorder and a microcomputer using a Labmaster TL-1 interface and AxoTape software (Axon Instruments).

Neurones were superfused at 1–2 ml min^{-1} in an extracellular medium containing (in mM): NaCl, 150; KCl, 5; $CaCl_2$ 1; HEPES, 10; glucose, 10; tetrodotoxin, 0.0002; picrotoxin, 0.1 (to block $GABA_A$ receptor-mediated chloride currents); pH was adjusted to 7.4 using NaOH and osmolality to 340 mosmol kg^{-1} using sucrose. In experiments using kainate, the medium also contained 1 mM $MgCl_2$. The patch-pipette (internal) solution contained (in mM): CsCl, 140; $MgCl_2$, 2; Mg_4ATP, 2; BAPTA, 10; HEPES, 10; pH was adjusted to 7.4 using CsOH and osmolality to 310 mosmol kg^{-1} using sucrose.

Solutions of agonists and drugs (all obtained from Sigma Chemical Co., St. Louis, MO, USA) were prepared in extracellular medium and were applied to neurones by gravity flow using a linear multi-barrel array (diameter of each pipette 200–300 μm) placed within 100 μm of the cell body to allow for rapid solution changes. Cells were constantly bathed in extracellular medium flowing from one pipette barrel (flow rate 2–3 μl s^{-1}), and treatment solutions were applied by opening a valve and moving the barrel array so that the desired solution bathed the cell. Solutions containing excitatory amino acids were applied at intervals of at least 90 s.

Hydrophobicity of various alcohols was compared using octanol : water partition coefficients. Octanol : water partition coefficient values were 0.479 for ethanol (McCreery & Hunt, 1978), 2.37 for trifluoroethanol (Leo *et al*., 1971), and 44.67 for trichloroethanol; the value for trichloroethanol was calculated by taking the mean log P for chloral hydrate minus the π value for the hydroxyl substituent (Leo *et al*., 1971).

Statistical analysis of concentration-response data was performed using the nonlinear curve-fitting program ALLFIT (DeLean *et al*., 1978), which uses an analysis of variance (ANOVA) procedure. Values reported for maximal response (E_{max}), concentration yielding 50 per cent of maximal response (EC_{50}; IC_{50} for inhibition), and slope factor (n) are those obtained by fitting the data to the equation:

$$y = \frac{E_{max} - E_{min}}{1 + (x/EC_{50})^{-n}} + E_{min}$$

where x and y are concentration and response, respectively, and E_{min} is the minimal response. In cases where the initial curve fits indicated that E_{min} was not significantly different from 0, and E_{max} was not significantly different from 100% inhibition, E_{min} and E_{max} were constrained to 0 and 100%, respectively, to obtain values for IC_{50} and *n*. All values are reported as the mean ± s.e.mean.

The care and use of animals in this study were approved by the Animal Care and Use Committee of the National Institute on Alcohol Abuse and Alcoholism (protocol no. LMCN-SP-01) in accordance with National Institutes of Health guidelines.

Results

Figure 1 illustrates the inhibition by trichloroethanol of currents activated by 25 μM NMDA and 100 μM kainate. In these neurones, 10 mM trichloroethanol inhibited NMDA-activated current by 71% (Figure 1a), and kainate-activated current by 44% (Figure 1b). On average, trichloroethanol at this concentration produced a decrease in amplitude of the currents activated by 25 μM NMDA and 100 μM kainate of

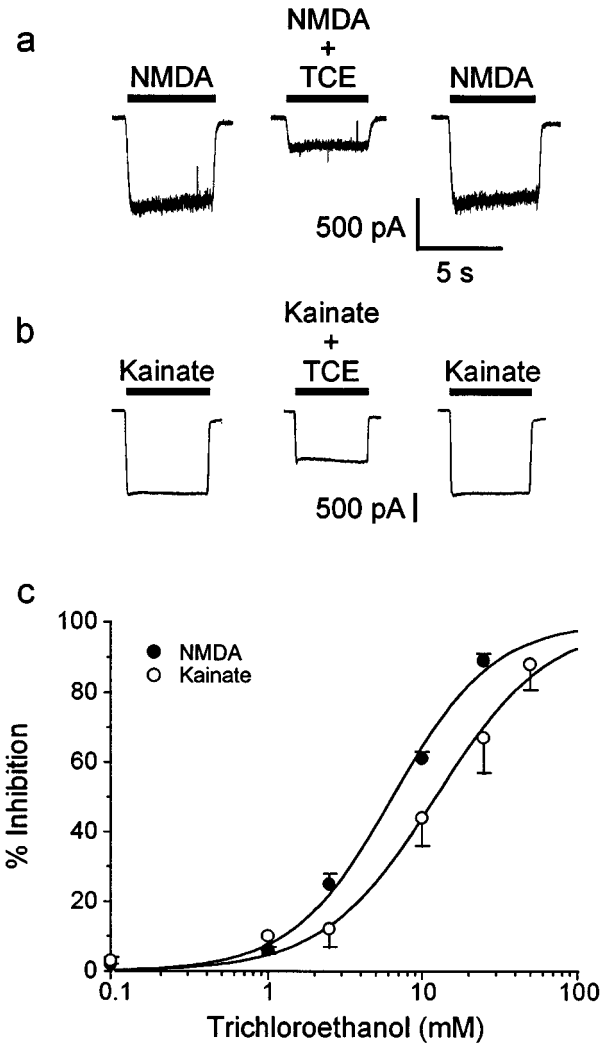

Figure 1 Trichloroethanol inhibition of NMDA- and kainate-activated current. (a) Current activated by 25 μM NMDA in the presence of 1 μM glycine and its inhibition by 5 mM trichloroethanol (TCE). (b) Current activated by 100 μM kainate and its inhibition by 5 mM trichloroethanol (TCE). Traces in *a* and *b* were obtained from different neurones; the time scale in *a* applies to both *a* and *b*. (c) Concentration-dependence of inhibition by trichloroethanol of current activated by 25 μM NMDA (●) and 100 μM kainate (○). Solutions of NMDA also contained 1 μM glycine. Data points are means ± s.e.mean of six neurones. Trichloroethanol inhibited NMDA-activated current with an IC_{50} of 6.4 ± 0.39 mM and a slope factor of 1.3 ± 0.1, and kainate-activated current with an IC_{50} of 12 ± 0.72 mM and a slope factor of 1.2 ± 0.1.

61 ± 2 and $44 \pm 8\%$, respectively. Trichloroethanol inhibition of excitatory amino acid-activated currents was concentration-dependent (Figure 1c). As is evident, the NMDA-activated current was more sensitive to inhibition by trichloroethanol than was the kainate-activated current. Trichloroethanol inhibited current activated by NMDA with an IC_{50} of 6.4 ± 0.39 mM, whereas it inhibited kainate-activated current with an IC_{50} of 12 ± 0.72 mM ($P < 0.001$; $n = 6$). In addition, trichloroethanol was apparently able to inhibit both NMDA- and kainate-activated currents by 100%, as the E_{max} values obtained in the initial curve fits were 117 and 103% inhibition for NMDA- and kainate-activated currents, respectively.

Trifluoroethanol inhibited currents activated by 25 μM NMDA or 100 μM kainate in a manner similar to that of trichloroethanol. In two individual neurones, the results from which are shown in Figure 2, 25 mM trifluoroethanol inhibited NMDA-activated current by 45% (Figure 2a), and kainate-activated current by 41% (Figure 2b). The average inhibition by 25 mM trifluoroethanol of currents activated by 25 μM NMDA and 100 μM kainate was 44 ± 2 and $37 \pm 1\%$, respectively. As we observed for trichloroethanol, trifluoroethanol inhibited excitatory amino acid-activated currents in a concentration-dependent manner (Figure 2c). Trifluoroethanol inhibited current activated by NMDA with an IC_{50} value of 28 ± 0.84 mM, and current activated by kainate with an IC_{50} value of 35 ± 1.2 mM ($P < 0.001$; $n = 6$). Furthermore, trifluoroethanol was apparently able to inhibit completely both NMDA- and kainate-activated currents, as initial curve fits yielded E_{max} values of 109 and 111% inhibition for NMDA- and kainate-activated currents, respectively.

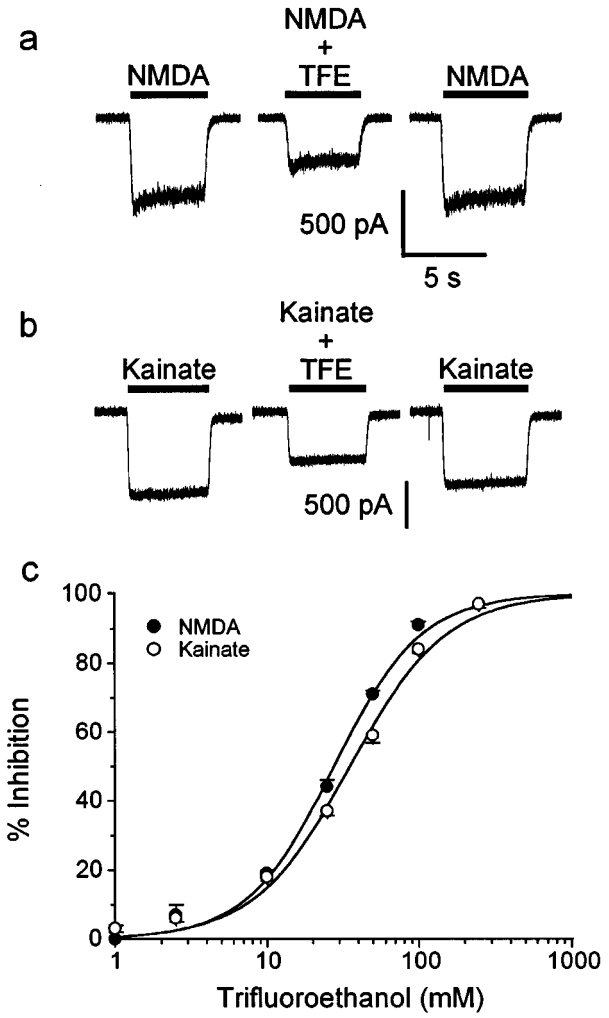

Figure 2 Trifluoroethanol inhibition of NMDA- and kainate-activated current. (a) Current activated by 25 μM NMDA in the presence of 1 μM glycine and its inhibition by 25 mM trifluoroethanol (TFE). (b) Current activated by 100 μM kainate and its inhibition by 25 mM trifluoroethanol (TFE). Traces in *a* and *b* were obtained from different neurones; the time scale in *a* applies to both *a* and *b*. (c) Concentration-dependence of inhibition by trifluoroethanol of current activated by 25 μM NMDA (●) and 100 μM kainate (○). Solutions of NMDA also contained 1 μM glycine. Data points are means ± s.e.mean of six neurones. Trifluoroethanol inhibited NMDA-activated current with an IC_{50} of 28 ± 0.84 mM and a slope factor of 1.5 ± 0.1, and kainate-activated current with an IC_{50} of 35 ± 1.2 mM and a slope factor of 1.4 ± 0.1.

To assess the possibility of an interaction of these agents with the agonist binding sites of the receptor-ion channels, we constructed concentration-response curves for NMDA and kainate in the absence and the presence of trichloroethanol. Figure 3a shows records of currents activated by three concentrations of NMDA and their inhibition by trichloroethanol in an individual hippocampal neurone. In this neurone, 5 mM trichloroethanol inhibited current activated by 10, 100, and 1000 μM NMDA by 57, 74, and 79%, respectively. Concentration-response curves for NMDA-activated current in the absence and presence of 5 mM trichloroethanol are shown in Figure 3b. Trichloroethanol at this concentration decreased the E_{max} of the NMDA concentration-response curve from 1.0 ± 0.036 to 0.25 ± 0.030 (normalized current; $P < 0.05$, $n = 3-8$). Although trichloroethanol appeared to decrease the EC_{50} of NMDA, this apparent change was not statistically significant (8.3 ± 1.2 μM in the absence of trichloroethanol vs 3.0 ± 1.5 μM in the presence of trichloroethanol; $P > 0.05$). Figure 3c shows records of currents activated by three concentrations of kainate and their inhibition by trichloroethanol in a hippocampal neurone. In this neurone, 5 mM trichloroethanol produced 54, 32, and 27% inhibition of current activated by 10, 100, and 1000 μM kainate, respectively. Concentration-response analysis of the effect of trichloroethanol on kainate-activated current (Figure 3d) revealed that 5 mM trichloroethanol decreased the E_{max} of the kainate concentration-response curve from 5.1 ± 0.12 to 3.7 ± 0.11 (normalized current; $P < 0.05$; $n = 4$). Trichloroethanol did not have a statistically significant effect on the EC_{50} of kainate (73 ± 5.1 μM in the absence of trichloroethanol vs 86 ± 7.1 μM in the presence of trichloroethanol; $P > 0.05$).

Discussion

Trichloroethanol, the active metabolite of chloral derivative sedative-hypnotic agents, has been previously reported to potentiate GABA responses mediated by $GABA_A$ receptors (Lovinger *et al.*, 1993; Peoples & Weight, 1994) and 5-HT responses mediated by $5\text{-}HT_3$ receptors (Lovinger & Zhou, 1993; Downie *et al.*, 1995). In the present study, trichloroethanol inhibited NMDA-activated current over a concentration range similar to the concentration range over which it potentiates $GABA_A$ and $5\text{-}HT_3$ receptor-mediated responses, and inhibited kainate-activated current at somewhat higher concentrations. Trifluoroethanol, a fluorinated analog of ethanol, also inhibited NMDA- and kainate-activated currents, but with a lower potency than trichloroethanol.

Ethanol and other alcohols have been shown previously to inhibit responses mediated by NMDA receptors in a wide variety of experimental preparations (Weight, 1992). A role of

NMDA receptors in mediating some of the behavioral effects of alcohols is consistent with several lines of evidence. For example: NMDA receptor inhibitory potency is correlated with intoxicating potency for several alcohols (Lovinger *et al.*, 1989; Fink & Göthert, 1990; Peoples & Weight, 1995); the noncompetitive NMDA receptor antagonists MK-801, PCP, and ketamine can substitute for ethanol in drug discrimination studies in mice, rats, and pigeons (Grant *et al.*, 1991; Sanger, 1993); and NMDA antagonists can block ethanol withdrawal seizures in dependent animals (Grant *et al.*, 1990; Liljequist, 1991). AMPA/kainate receptors may have a less important role in producing the behavioral effects of ethanol, as these receptors in neurones are inhibited only by high concentrations of ethanol (Lovinger *et al.*, 1990). However, recombinant non-NMDA glutamate receptor subunits have been reported to be more sensitive to ethanol when expressed in HEK 293 cells (Lovinger, 1993) or *Xenopus* oocytes (Dildy-Mayfield & Harris, 1995). In addition, slight to moderate inhibition of AMPA/kainate receptor-mediated excitatory synaptic responses that are minimally above the threshold for action potential firing could have profound effects on overall CNS excitability. Concentrations of trichloroethanol associated with anaesthesia may reach the low millimolar range (Owen

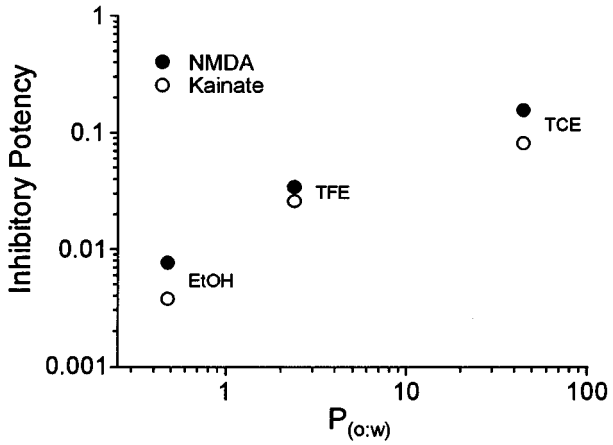

Figure 4 Potency (IC_{50}^{-1}) of ethanol (EtOH), trifluoroethanol (TFE), and trichloroethanol (TCE) for inhibition of current activated by 25 μM NMDA (●) and 100 μM kainate (○) plotted as a function of octanol : water partition coefficient ($P_{(o:w)}$). Solutions of NMDA also contained 1 μM glycine. The value for ethanol inhibitory potency of NMDA-activated current is from Peoples & Weight (1995); that for kainate-activated current is from Akinshola *et al.* (1996).

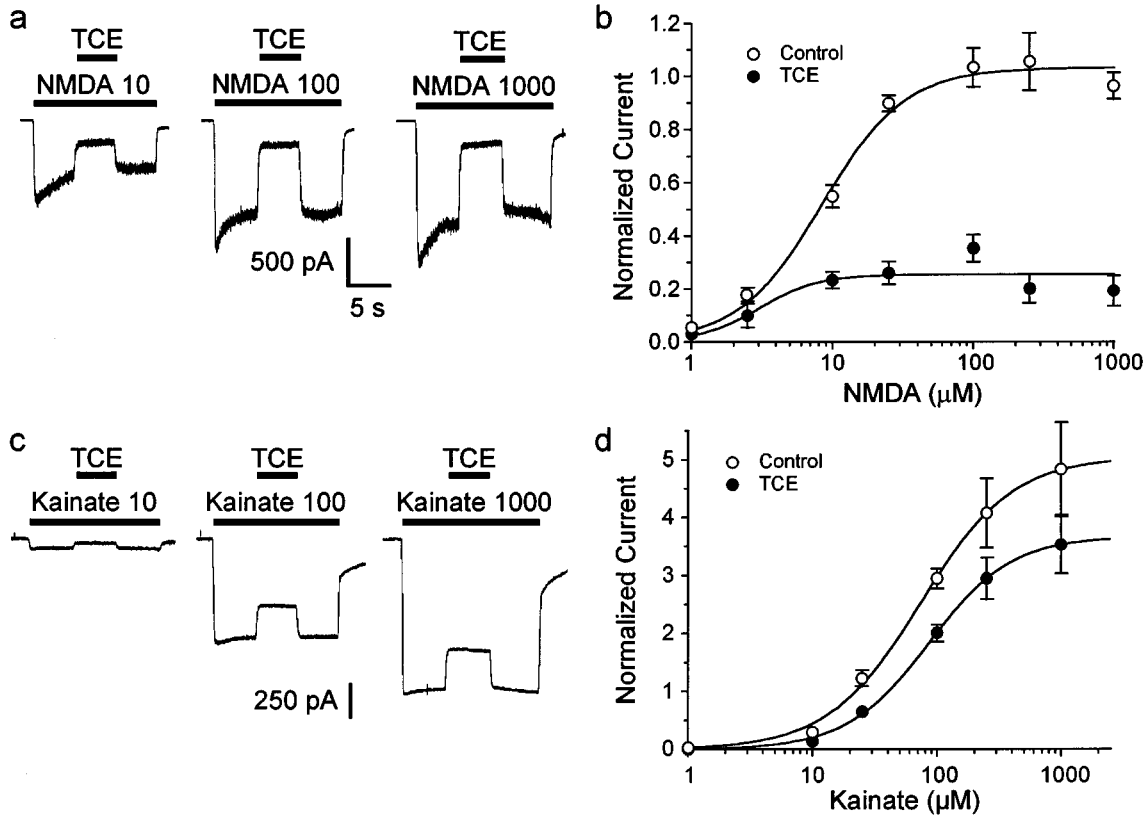

Figure 3 Effect of concentration of NMDA and kainate on inhibition by trichloroethanol (TCE). (a) Currents activated by 10, 100, and 1000 μM NMDA in the presence of 1 μM glycine and their inhibition by 5 mM trichloroethanol (TCE). (b) The graph plots current activated by NMDA (normalized to that activated by test pulses of 25 μM NMDA) vs NMDA concentration in the absence (○) and the presence (●) of 5 mM trichloroethanol. Trichloroethanol decreased the E_{max} of the NMDA concentration-response curve from 1.0 ± 0.036 to 0.25 ± 0.030 ($P < 0.05$), but did not significantly change the EC_{50} of NMDA (9.5 ± 1.8 μM in the absence of trichloroethanol vs 3.2 ± 2.3 μM in the presence of trichloroethanol; $P > 0.05$). Data points are means ± s.e.mean of three to eight neurones. Error bars not visible are smaller than the size of the symbols. (c) Currents activated by 10, 100, and 1000 μM kainate and their inhibition by 5 mM trichloroethanol (TCE). Traces in *a* and *c* were obtained from different neurones; the time scale in *a* applies to both *a* and *c*. (d) The graph plots current activated by kainate (normalized to that activated by test pulses of 25 μM kainate) vs kainate concentration in the absence (○) and the presence (●) of 5 mM trichloroethanol. Trichloroethanol decreased E_{max} of the kainate concentration-response curve from 5.1 ± 0.12 to 3.7 ± 0.11 ($P < 0.05$), but did not have a significant effect on the EC_{50} of kainate (73 ± 5.1 μM vs 85 ± 7.1 μM in the absence and the presence of trichloroethanol, respectively; $P > 0.05$). Data points are means ± s.e.mean of four neurones. Error bars not visible are smaller than the size of the symbols.

& Taberner, 1980); anaesthetic concentrations of trifluoroethanol have not been determined. Thus, the results of the present study, that the halogenated alcohol trichloroethanol can inhibit NMDA-activated current at pharmacologically-relevant concentrations, and kainate-activated current at somewhat higher concentrations, are consistent with previous reports on the effects of ethanol and other alcohols on these receptor-ion channels. Furthermore, the increased potency of the halogenated alcohols relative to ethanol may be associated with their increased hydrophobicity. For example, trifluoroethanol, which is approximately 4.9 times more hydrophobic than ethanol (Leo *et al*., 1971), was found to be 4.7 times more potent than ethanol in inhibiting NMDA-activated current (Figure 4). The observed NMDA receptor inhibitory potency of trichloroethanol deviated somewhat from its predicted value based on its hydrophobicity, as it is approximately 93 times more hydrophobic than ethanol but was found to be 20 times more potent than ethanol as an inhibitor of NMDA receptors. However, the relationship between hydrophobicity and NMDA receptor inhibitory potency of *n*-alcohols was found to be nonlinear for alcohols that are more hydrophobic than *n*-pentanol (Peoples & Weight, 1995), as is trichloroethanol. In this regard, it should be noted that the predicted NMDA receptor inhibitory potency of trichloroethanol obtained from a plot of hydrophobicity vs NMDA receptor inhibitory potency for *n*-alcohols (Peoples & Weight, 1995) was in good agreement with the experimentally determined value (6.2 vs 6.4 mM).

The observation that trichloroethanol decreased the E_{max} of the NMDA and kainate concentration-response curves without significantly affecting their EC_{50} values indicates that its mechanism of inhibition of both these receptor-channels is noncompetitive with respect to NMDA. Although previous studies have not addressed the inhibition by trichloroethanol of responses to NMDA and kainate, the findings of the present study are consistent with previous observations of the effect of ethanol on those responses. Ethanol has been reported to produce noncompetitive inhibition of NMDA-activated current in hippocampal neurones (Peoples *et al*., 1997), NMDA-evoked [^{3}H]noradrenaline or [^{3}H]dopamine release from cerebral cortical (Göthert & Fink, 1989; Gonzales & Woodward, 1990) or striatal slices (Woodward & Gonzales, 1990), NMDA-stimulated Ca^{2+} influx in cerebellar granule (Rabe & Tabakoff, 1990) or isolated brain neurones (Dildy-Mayfield & Leslie, 1991), NMDA-activated current in *Xenopus* oocytes injected with rat hippocampal mRNA (Dildy-Mayfield & Harris, 1992), and kainate-activated current in *Xenopus* oocytes expressing rat recombinant GluR3 or GluR6 AMPA receptor-channels (Dildy-Mayfield & Harris, 1995). Although the location of the site through which the halogenated alcohols inhibit NMDA- and kainate-activated current remains unclear, the results of the present study are not consistent with an action of these agents as competitive antagonists at the agonist binding sites of these receptor-channels. It should be noted that the site through which ethanol inhibits these receptor-channels, and whether other alcohols act at the same site, are not known at present (Peoples *et al*., 1997).

Numerous studies demonstrating enhancement of $GABA_A$ receptor-mediated responses by a wide variety of general anaesthetic and sedative/hypnotic agents have led to the hypothesis that potentiation of $GABA_A$ receptor-mediated neurotransmission contributes to the sedative/hypnotic actions of these agents (Tanelian *et al*., 1993; Franks & Lieb, 1994). The involvement of $GABA_A$ receptors in the CNS actions of trichloroethanol is suggested by previous studies that reported enhancement by trichloroethanol of GABA-mediated synaptic transmission (Lovinger *et al*., 1993) and GABA-activated current (Peoples & Weight, 1994) in central neurones. A role of excitatory amino acid receptors in mediating the behavioral effects of trichloroethanol may appear less likely because of a report that trichloroethanol is less potent in depressing excitatory synaptic responses than in enhancing inhibitory synaptic responses (Lovinger *et al*., 1993). However, as mentioned previously, a relatively small inhibition of excitatory amino acid receptor-mediated synaptic responses could result in a much greater effect on synaptically-activated spike firing in postsynaptic neurones. The possible involvement of NMDA receptors in the CNS depressant effect of trichloroethanol is suggested by the observation that the EC_{50} for trichloroethanol potentiation of GABA-activated current is 3.0 ± 1.4 mM (Peoples & Weight, 1994), and the IC_{50} for trichloroethanol inhibition of NMDA-activated current was found to be 6.4 ± 0.4 mM in the present study. Thus, at the EC_{50} for trichloroethanol potentiation of $GABA_A$ receptors, NMDA receptors would be inhibited by nearly 30%. Although trichloroethanol inhibits kainate-activated current less potently ($IC_{50} = 12 \pm 0.7$ mM), at the EC_{50} for trichloroethanol potentiation of $GABA_A$ receptors, AMPA/kainate receptors would be inhibited by over 15%. Thus, the inhibition of NMDA and AMPA/kainate receptor-channel function produced by trichloroethanol may not be sufficient to account completely for its sedative effect, but since it has been shown that NMDA and AMPA/kainate receptor antagonists can increase the potency or duration of action of anaesthetics (Scheller *et al*., 1989; Daniell, 1990; McFarlane *et al*., 1992; Dall *et al*., 1993), it seems very likely that these actions of trichloroethanol may contribute to its CNS depressant effects.

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