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Action of enflurane on cholinergic transmission in identified *Aplysia* neurones

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1 Effects of enflurane on the cholinergic transmission in *Aplysia* neurones were studied by current and voltage clamp methods. Acetylcholine (ACh) evoked three types of postsynaptic responses on different identified neurones: (1) a depolarizing response due to an increase in Na and K conductances (D-response), (2) a fast hyperpolarizing response due to an increase in Cl conductance (Cl-response), and (3) a slow hyperpolarizing response due to an increase in K conductance (K-response).

2 Enflurane altered neither the action potential nor the membrane resistance of the neurones but depressed the three ACh-induced responses, non-competitively, in a dose-dependent manner. The K-response was less suppressed than the other two.

3 Blockade of the closed state of ion channel was suggested by a reduction in the first ACh response evoked 1 min after administration of enflurane.

4 The anaesthetic facilitated the decay of the neurally evoked e.p.s.c. and i.p.s.c. in suggesting a reduction in the mean open time of the postsynaptic ion channel.

5 It is concluded that enflurane depresses excitatory and inhibitory cholinergic transmission by reducing the postsynaptic currents.

Introduction

The manner in which general anaesthetics exert their effects on the whole animal remains controversial. Although disruption of membrane lipids was once a favoured mechanism of anaesthetic action, some authors suggested multiple sites and more specific effects of anaesthetic agents (Halsey *et al.*, 1980; Simon & Bennet, 1980; Richards *et al.*, 1980). The importance of the membrane proteins in the mechanism of anaesthesia has been emphasized (Franks & Lieb, 1982).

Loss of perception and consciousness with anaesthesia is brought about by a reversible depression of neuronal activity in the central nervous system by the reduction of the excitatory transmission and/or enhancement of inhibitory transmissions (Judge, 1983; Richards, 1983). Block of excitatory transmission occurs at much lower anaesthetic concentrations than blockage of axonal conduction of action potentials (Larrabee & Posternak, 1952; Richards *et al.*, 1975; Richards & White, 1975). In molluscan preparations, both excitatory (Barker, 1975) and inhibitory (Cote & Wilson, 1980; Judge & Norman, 1982) synaptic responses may be depressed by anaesthetics. Volatile anaesthetics including enflurane have depressant effects at the neuromuscular junction (Gage & Hamill, 1976). Since enflurane is a commonly used general anaesthetic, we examined its effects on postsynaptic responses to neurally and iontophoretically released acetylcholine (ACh) in *Aplysia* ganglia.

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Methods

Aplysia oculifera weighing 40–200 g were collected from the beach of Shikanoshima Island in Fukuoka City, Japan. The abdominal and pleural ganglia were removed and one was pinned in a 0.5 ml Sylgard lined chamber. The connective tissue capsule surrounding the ganglia was dissected with razor blade chips and fine forceps. The ionic composition of artificial sea water was (mM): NaCl 450, KCl 10, $CaCl_2$ 10, $MgCl_2$ 55, N-2-hydroxyethylpiperazine -N'-2-ethansulphonic acid (HEPES) 10. The pH was adjusted to 7.8. Neurones were identified according to the maps of the ganglia, type of firing and responsiveness to iontophoretic ACh (Frazier *et al.*, 1967; Kandel, 1976). ACh induced an excitatory response (D-response) on R-15 and RB cells in the abdominal

ganglion (Blankenship *et al.*, 1971; Kandel, 1976). The right pleuro-abdominal connective was stimulated to elicit the excitatory postsynaptic response on R-15 with 0.3 ms rectangular voltage pulse at a frequency of 0.2 Hz. On neurones of L-2-6 of the abdominal ganglion and the medial cells of pleural ganglia, ACh elicited two phases of inhibitory response, which were attributed to a conductance increase for Cl ion (Cl-response) and to that for K ion (K-response) (Blankenship *et al.*, 1971; Kehoe, 1972a,b,c).

Identified cells were penetrated with two microelectrodes, one for monitoring the membrane potential through a high input impedance preamplifier (Nihon Kohden Co., MEZ-7101) and the other for passing current in both current and voltage clamp experiments. The first derivative (dV/dt) of the action potential was obtained with a differentiator which responded linearly to rate of change of voltage up to 500 V s^{-1}. The cells were voltage-clamped with a voltage clamp amplifier (Nihon Kohden Co., CEZ-1100). Filters for current measurements were set for 300 Hz cut-off in iontophoresis experiments and 3 kHz cut-off in experiments of neurally evoked postsynaptic currents. When dealing with the Cl-response to ACh, the current passing electrodes were filled with 0.6 M K_2SO_4 and the potential measuring ones with 3 M KCl. In some cases, both electrodes were filled with 0.6 M K_2SO_4. In other experiments, both electrodes were filled with 3 M KCl. The resistance of all electrodes was between 3 and 10 MΩ.

The synaptic membrane in *Aplysia* neurones is located on axonal branches in the neuropil (Kandel *et al.*, 1967), while in all present experiments the two microelectrodes were introduced into the somas of postsynaptic neurones. We checked the effective voltage control of the synapse with criteria similar to those described by Gardner & Stevens (1980).

Acetylcholine chloride (ACh) of 0.5 M in glass microelectrodes, resistance 50–100 MΩ, was used for iontophoretic application to the somatic membrane of neurones. Positive square currents of 100–300 ms duration and various amplitudes were applied to eject ACh every 2 min by use of a current pump (Dia Medical System Co., DIP-30A and DIP-30TC). The somatic membrane, which is devoid of any synaptic contact, is similarly sensitive to ACh and has pharmacological properties identical to those in the subsynaptic membrane (Gerschenfeld & Tauc, 1961). The

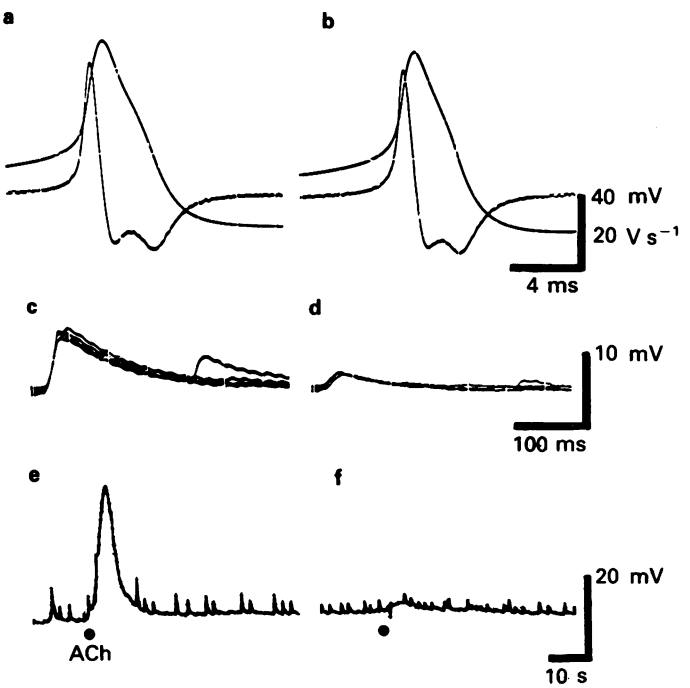

Figure 1 Effects of 2% enflurane on the action potential, e.p.s.p. and acetylcholine (ACh) D-response of R-15. Action potential (upper trace) and its dV/dt (lower trace) in control solution (a) and in the presence of 2% enflurane (b). E.p.s.ps were elicited by stimulation of the right pleuroabdominal connective every 5 s in control solution (c) and in 2% enflurane (d). The membrane potential was held at − 80 mV. ACh was applied iontophoretically (shown by dots) with 200 ms, 400 nA current pulses every 2 min in control solution (e) and in 2% enflurane (f). The neurone was held at − 80 mV. All recordings are from the same neurone, R-15.

low frequency was chosen because a slow recovery from desensitization after ACh administration was noted in *Aplysia* neurones (Tauc & Bruner, 1963). A retaining current was used to prevent leakage of ACh from the electrodes.

Enflurane was vapourized in compressed air flowing through an Enflurane Vapor 19.1 (Dräger). The enflurane 0.2–4% containing air was bubbled into the reservoir solution at room temperature for at least 30 min before and during the administration of the agent. All experiments were carried out at a room temperature between 18 and 21°C, with the range maintained within 0.5°C. Potentials and currents were displayed on a storage oscilloscope (Tektronix 5113) and photographed. In some experiments, a digital averager of eight bit resolution (Nihon Kohden Co., ATAC 250) was used. Tests of significance were performed using Student's t test, when appropriate. The differences were considered significant when the P value was less than 0.05.

Results

Current clamp experiments

Action potential and excitatory postsynaptic potential (e.p.s.p.) The neurones were silent, regularly firing or bursting after impalement with two microelectrodes. In current clamp experiments, the membrane potential was hyperpolarized to − 60 mV with a d.c. current injection which prevented action potential discharge. The neuronal action potentials were elicited by reducing the hyperpolarizing current or passing a small amount of depolarizing current. Figure 1 shows a typical experiment with an identified cell R-15 in the abdominal ganglion. The first action potentials after a rest period longer than 2 min were monitored (Figure 1a). Enflurane up to 4% did not affect the action potential amplitude or its dV/dt (Figure 1b). Figure 1c–d shows a marked depression of the e.p.s.p. by 2% enflurane. In the same cell, the ACh D-response was also greatly reduced by the anaesthetic (Figure 1e–f). The amplitude of the spontaneous e.p.s.ps (Figure 1e) was also reduced but the frequency did not seem to be altered (Figure 1f).

Membrane resistance The membrane was estimated in 5 cells in which the membrane potential was held at − 60 mV. Current pulses of 250 ms duration and various amplitudes were injected into the cell. The resulting potential changes were recorded, and the membrane potentials at the end of the pulse were plotted as a function of current injected. Figure 2 depicts an example with an RB cell, indicating no change of the membrane resistance by 2% enflurane.

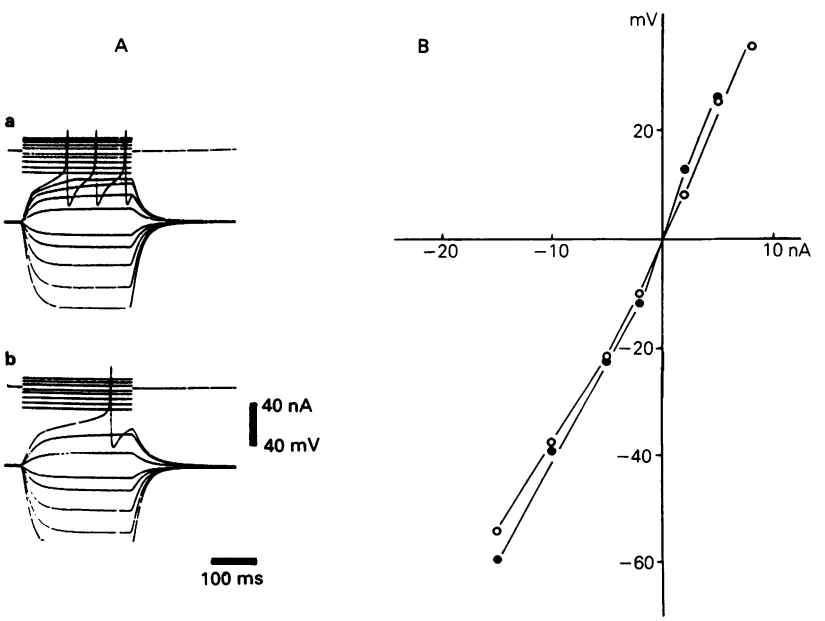

Figure 2 Effects of 2% enflurane on membrane resistance: (A) An RB cell was held at − 60 mV and 250 ms current pulses of various amplitudes were applied through the current passing microelectrode in control solution (a) and in 2% enflurane (b). (B) The voltage changes at the end of the pulse are plotted against the amount of the current passed: 2% enflurane (●) did not affect the membrane resistance; (○) control.

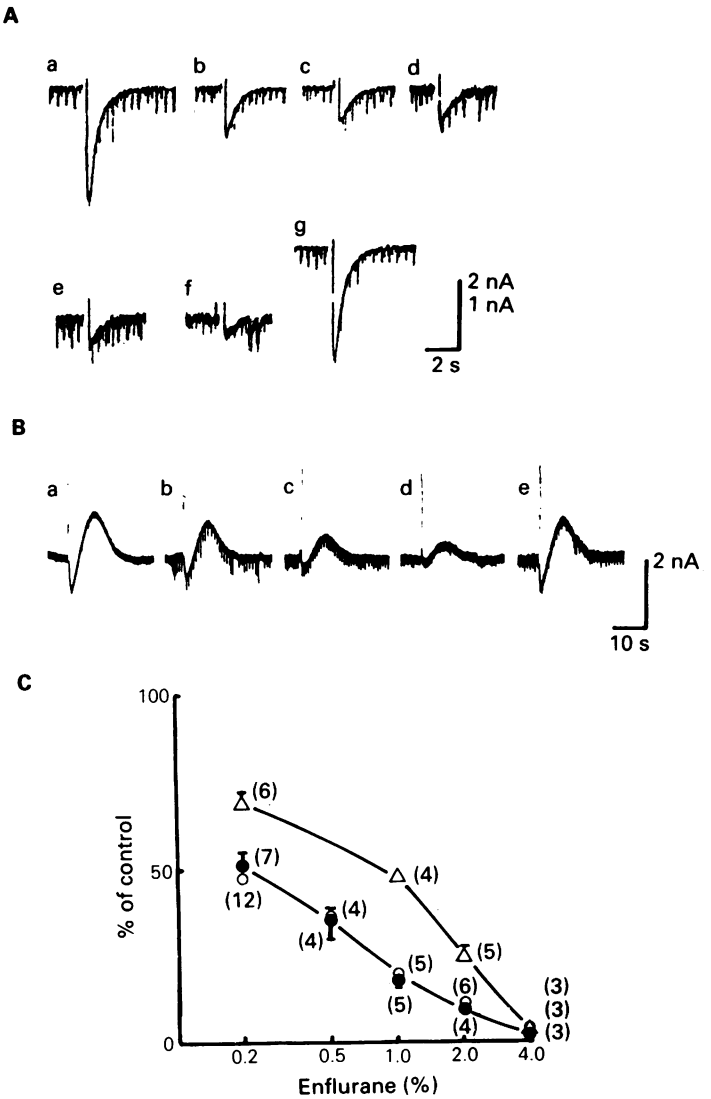

Figure 3 Effects of enflurane on the three types of acetylcholine (ACh)-response in voltage-clamp condition. (A) An RB cell was voltage-clamped at − 80 mV and ACh was applied iontophoretically every 2 min with 300 ms 400 nA current pulses to evoke the D-response. (a) Control, (b) 0.2%, (c) 0.5%, (d) 1%, (e) 2%, and (f) 4% enflurane, (g) wash in control solution for 15 min. The reversal potential was − 6 mV in control, which was not changed by enflurane. Current calibration: 2 nA for (a), (b), (c) and (g); 1 nA for (d), (e) and (f). (B) An identified neurone L-3 was voltage-clamped at − 60 mV. ACh was applied iontophoretically to evoke Cl- and K-responses every 2 min with 300 ms 400 nA current pulses. The reversal potential for Cl-response was − 58 mV at the beginning of the experiment and changed to − 45 mV after 40 min of ACh application in control solution. Thereafter it did not change throughout the experiment in the presence of the anaesthetic. The reversal potential for the K-response was − 80 mV and did not change. (a) Control, (b) 0.2%, (c) 1% (d) 2% enflurane, and (e) wash in control solution for 15 min. The K-response was less depressed than Cl-response by the agent. (C) Dose-response curve of the three ACh responses to enflurane. D-responses (●) were obtained with R-15s and RB cells voltage-clamped at − 80 mV. Cl-responses (○) were obtained with L-2-6 and pleural medial cells voltage-clamped at − 60 or − 80 mV. Most results were from those cells voltage-clamped at − 80 mV. K-responses (△) were obtained with L-2-6 and pleural medial cells voltage-clamped at − 50 or − 60 mV. The K-response was significantly less suppressed than the other two responses. Number of experiments is shown in parentheses. Bars indicate the s.e.mean when larger than the symbols.

This finding suggests that the depression of the e.p.s.p. and D-response shown in Figure 1 was not related to a decrease in the membrane resistance but rather to a reduction in the postsynaptic current. Thus, the voltage clamp experiment was carried out to elucidate the mode of action of enflurane on ACh-induced postsynaptic currents.

Voltage clamp experiments

The three types of acetylcholine response Figure 3A shows an experiment with an RB cell which was voltage-clamped at − 80 mV. ACh was applied iontophoretically every 2 min to evoke an inward current, which corresponded to the depolarizing response in Figure 1e. This inward current was reduced in a dose-dependent manner as the concentration of enflurane was increased from 0.2% to 4%, being practically abolished by 4% enflurane. In experiments with administration of a single concentration, the first ACh response evoked 1 min after perfusion with the anaesthetic was already reduced to some extent and a steady level of reduction was attained within several minutes. Drug action was completely reversed by washing in about 15 min (Figure 3A(g)). The reversal potential for the ACh current was between − 10 and 0 mV, and was not affected by enflurane ($n = 9$). Figure 3B shows ACh-responses of an identified neurone L-3, voltage-clamped at − 60 mV. ACh induced two phases of current response: the faster one was inward and had a reversal potential between − 60 and − 50 mV (Cl-response), and the slower one was outward with a reversal potential at about − 80 mV (K-response). The reversal potential for the chloride current shifted to a less negative potential between − 50 and − 40 mV in about 40 min of ACh application under control conditions. Thereafter it remained unchanged throughout the experiment. Enflurane did not alter the reversal potential of either current ($n = 7$ for Cl-response and $n = 9$ for K-response). Figure 3B shows that both responses were markedly depressed by enflurane, though the K-response was less affected. It was also observed that the first ACh responses were reduced 1 min after administration, even with a low concentration of enflurane (0.2%). The reduction reached a steady level within several minutes. Figure 3C illustrates the percentage depression of the three types of the ACh response in test solutions containing various concentrations of enflurane. The responses were significantly depressed by 0.2% enflurane and almost completely suppressed by a high concentration (4%). The K-response was significantly less sensitive to the anaesthetic than the other two at concentrations of 0.2%, 1% and 2% ($P < 0.05$).

Acetylcholine dose-response curve Dose-response curves for the effect of the ACh ejection current on the voltage-clamp current were obtained with the three types of the ACh response (Figure 4). Double reciprocal plots of the dose-response curves resulted in straight lines which intersect on the abscissa scale indicating that the depression was non-competitive for all three types of response.

Enflurane action on desensitization The effects on the desensitization process were assessed with four RB cells for 0.2% and three RB cells for 2% enflurane.

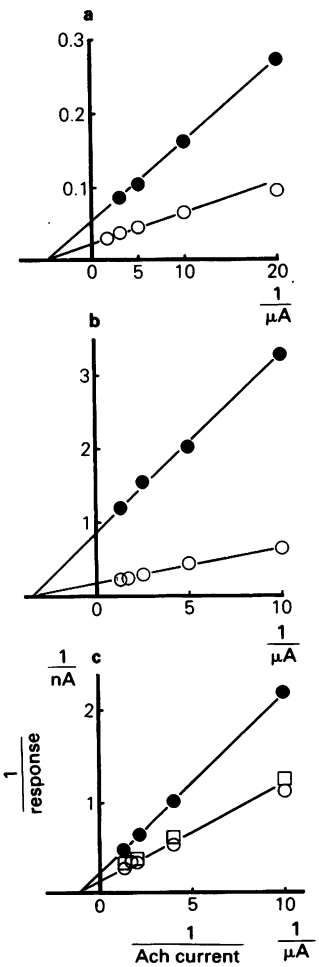

Figure 4 Effects of enflurane on acetylcholine (ACh) dose-response curves. ACh was applied iontophoretically for 200 or 300 ms with various amplitude current pulses. The dose-response curves are plotted as double reciprocals. (a) Effects of 0.5% enflurane (●) on D-response of an RB cell; (○) control. (b) Effects of 2% enflurane (●) on Cl-response of a left pleural medial cell (LPM); (○) control. (c) Effects of 0.2% enflurane (●) on K-response of an L-3; (○) control; (□) wash.

Figure 5a shows a rapid iontophoretic application of ACh at a frequency of 0.1 Hz, which developed desensitization in a single exponential mode (Figure 5b). The average time constant of the desensitization process was 22 ± 3 s ($n = 4$). This value agrees with those found in snail neurones by Bregestovksi *et al.* (1979). Enflurane 0.2% did not affect this. With 2% enflurane, the ACh current became too small to estimate the time course but no evidence was obtained to indicate a faster development of desensitization.

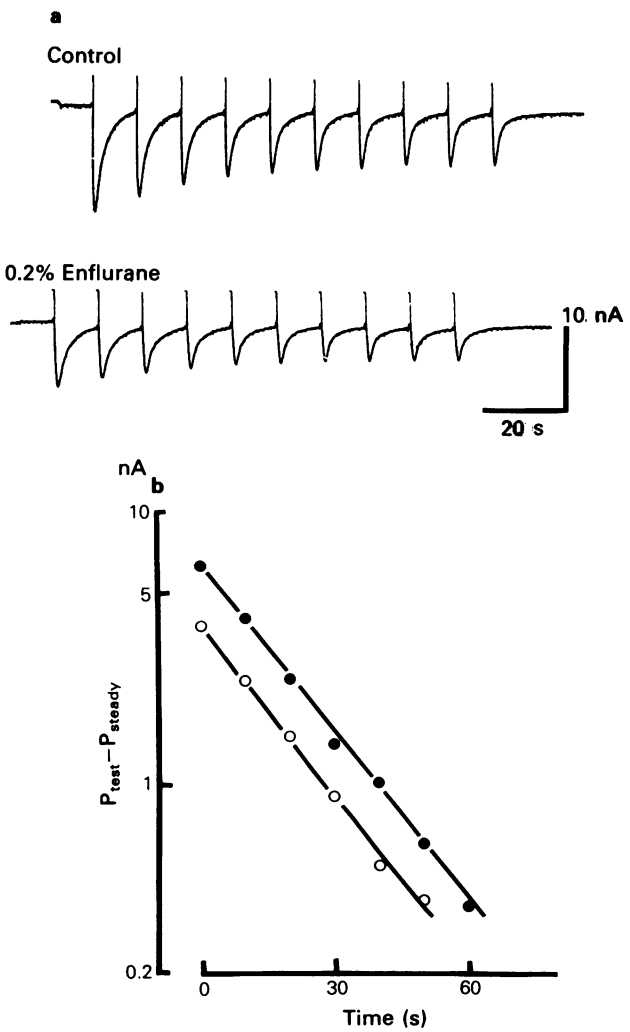

Figure 5 Enflurane action on the time course of desensitization of D-response: (a) acetylcholine (ACh) was applied iontophoretically with 200 ms 400 nA current pulses every 10 s to an RB cell voltage-clamped at − 80 mV. Enflurane 0.2% decreased the response current to about 50%. (b) The difference between the peak of each current (P_{test}) and that of the tenth response current (P_{steady}) is plotted against time. Desensitization developed exponentially and the time constant, which was 21 s in the control solution (●) was not affected by enflurane. (○).

Postsynaptic currents Effects of enflurane on the excitatory postsynaptic current (e.p.s.c.) and inhibitory postsynaptic current (i.p.s.c; Cl-component) were examined with R-15 and L-2-6, respectively. E.p.s.cs were evoked on R-15 voltage-clamped at − 80 mV by stimulation of the right pleuroabdominal connective every 5 s. The amplitude of the e.p.s.c. was reduced by enflurane, in a dose-dependent manner (Figure 6), and to a similar extent to the iontophoretically evoked D-response. The decay of the e.p.s.c. followed a single exponential, and, when plotted semilogarithmically, the rate of decay was seen to increase with higher concentrations of enflurane (Figure 6B). The time constant of decay was significantly decreased to 65 ± 4% ($n = 5$) of the control value by 2% enflurane. The decrease was not significant in the presence of 0.2% of the agent ($n = 5$). The reversal potential for the e.p.s.c. was not affected by the anaesthetic. In some experiments with identified neurones of L-2-6, spontaneous i.p.s.cs were recorded continously, as was the case in buccal ganglia of *Aplysia* reported by Adams *et al.* (1982). Seven cells were voltage-clamped at various membrane potentials and inward i.p.s.cs of Cl-component were monitored and photographed. Figure 7 illustrates an example with an L-2. The i.p.s.c. was suppressed by enflurane in a concentration-dependent manner to an extent similar to that of the ACh Cl-response of an identical cell. The time constant of decay plotted against the membrane potential in Figure 7B shows that 2% enflurane enhanced the decay of the i.p.s.c, to 67 ± 4% ($n = 7$) of the control. A low concentration of the agent (0.2%) did not alter the decay significantly. The voltage dependency of the time constant was not affected in the voltage range explored. The reversal potential for the i.p.s.c. was not changed (Figure 7C).

Discussion

Action potential and membrane resistance

Alteration in the rate of rise of membrane action potential reflects a change in the fast inward Na current in *Aplysia* neurones (Adams & Gage, 1979; Farquharson & Jahan-Parwar, 1984). Therefore, the stability of the action potential in the presence of up to 4% enflurane suggests that the anaesthetic does not alter this inward Na current. However with very high concentrations of volatile anaesthetics a decrease in the sodium current was noted in the crayfish giant

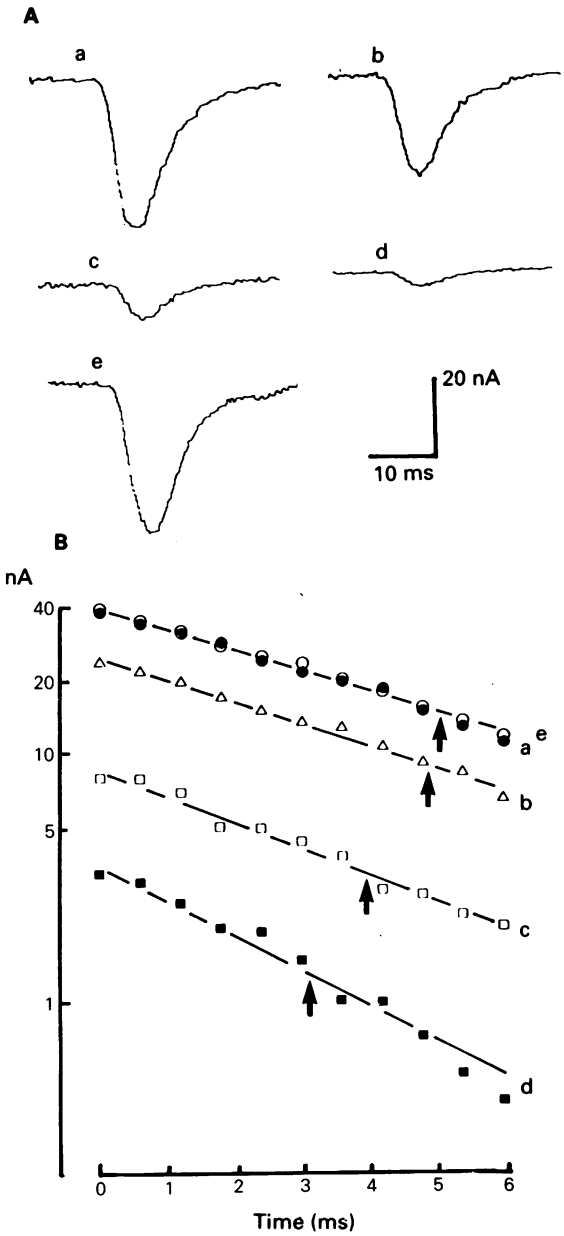

Figure 6 Effects of enflurane on e.p.s.c. of R-15 cell: e.p.s.cs were elicited on an R-15 voltage-clamped at − 80 mV by stimulation of the right pleuroabdominal connective with 0.3 ms duration voltage pulse every 5 s. (A) Ten e.p.s.cs were averaged in each solution: (a) control; (b) 0.2%, (c) 1%; (d) 2% enflurane and (e) wash in control solution for 15 min. (B) The decay phase of e.p.s.cs plotted semilogarithmically, showing a single exponential mode of decay. The time constant (shown by arrows) was shortened by higher concentrations of the anaesthetic.

axon (100 mM diethyl ether, 30 mM halothane; Bean *et al.*, 1981) and squid giant axon (100 mM diethyl ether, 3 mM methoxyflurane, 5–7 mM chloroform and 4–5 mM halothane; Haydon & Urban, 1983).

Enflurane 2% did not affect the membrane resistance. This is consistent with Barker's finding (1975) that 1 mM chloroform did not alter the membrane resistance of molluscan neurones. Since the enflurane containing air was bubbled into the reservoir solution

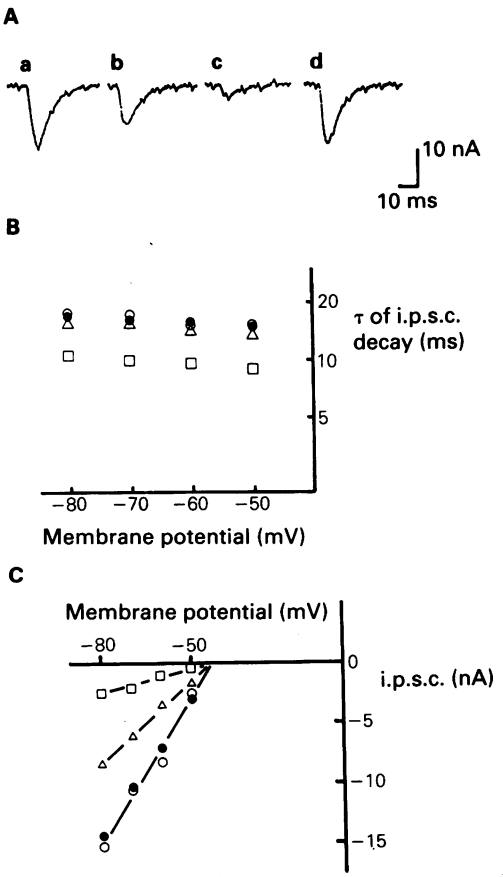

Figure 7 Effects of enflurane on spontaneous i.p.s.cs (Cl-response). (A) Spontaneous i.p.s.cs were obtained on an L-2 voltage-clamped at − 80 mV. The i.p.s.cs were recorded continuously at frequencies of 0.5–1 Hz. (a) control; (b) 0.2%, (c) 2% enflurane; and (d) wash in control solution for 15 min. (B) The holding potential was changed between − 50 and − 80 mV. The time constant of i.p.s.c. decay was measured at each membrane potential and plotted against the potential. Each point is the average of 5–10 i.p.s.cs: (●) control; (△) 0.2% enflurane (□) 2.0% enflurane; (○) wash. (C) The amplitudes of the i.p.s.c are plotted against the membrane potential. Enflurane reduced the amplitude but did not alter the reversal potential for the i.p.s.c. Each point is the average of 5–10 i.p.s.cs.

at about 20°C in the present experiments, 1% of the anaesthetic is calculated to result in a concentration of 0.58 mM (Halsey, 1980). Consequently, 0.2% and 4% enflurane are 0.12 and 2.32 mM concentrations, respectively. The clinical concentration with inhalation of MAC (Minimal Alveolar Concentration) of the anaesthetic (1.68%) is calculated to be 0.53 mM in water phase at 37°C. Although no data are available, to our knowledge, for *Aplysia* and enflurane, extrapolation of data of Cherkin & Catchpool (1964) predicts that ED_{50} of halothane to anaesthetize gold fish at 37°C is about 14 mmHg or 057 mM. This is about 2.5 times the concentration at MAC of halothane in man, which indicates that goldfish is less susceptible to this volatile anaesthetic. Since it is known that the anaesthetic potency increases at lower temperatures (Cherkin & Catchpool, 1964; Franks & Lieb, 1982), the enflurane concentrations used in the present experiments may not differ greatly from that needed to induce anaesthesia in *Aplysia*.

Depression of both excitatory and inhibitory responses

Barker (1975) reported that central depressants including 1–2 mM chloroform suppressed the depolarizing Na-K dependent response to ACh without altering the Cl-dependent response in molluscan neurones, thereby indicating a selective depression of the excitatory transmission by these agents. Judge & Norman (1982) showed that general anaesthetics depressed the Cl-dependent inhibitory response to ACh in *Helix* neurones. In the present study, enflurane depressed both excitatory and inhibitory ACh-responses in a dose-dependent manner and the K-dependent response was less sensitive to the anaesthetic. These findings are in agreement with those of Cote & Wilson (1980), who studied the effects of barbiturates on the current responses evoked on *Aplysia* neurones by ACh and γ-aminobutyric acid.

In the present experiments with enflurane, the iontophoretically evoked ACh-responses were reduced to almost the same extent as the low frequency e.p.s.c. or i.p.s.c. This finding suggests a minimal effect of this agent on the presynaptic mechanism affecting the transmitter release. This is consistent with the findings of Kennedy & Galindo (1975) that enflurane had a minor effect on transmitter output at the neuromuscular junction of rat phrenic nerve-diaphragm preparation. Richards *et al.* (1975) also suggested that ether and methoxyflurane did not significantly affect the process of transmitter synthesis and mobilization in the guinea-pig olfactory cortex. On the other hand, barbiturates were reported to reduce the amount of transmitter released from presynaptic nerve terminals in cat spinal cord (Weakly, 1969).

Interaction of enflurane with postsynaptic channel mechanism

In the present experiments, double reciprocal plots for the three types of ACh-response revealed a non-competitive inhibition of all three responses by enflurane. Non-competitive inhibition of the ACh response by chloroform (1 mM) was also reported by Barker (1975) with cell 11 of land snails. Enflurane did not promote desensitization, excluding the possibility of quicker desensitization in the reduction of the neurally and iontophoretically evoked postsynaptic currents. The reduction in the first ACh response evoked iontophoretically 1 min after administration of enflurane indicates that the closed channels became blocked. The gradual increment in the depression during several applications of ACh may suggest a kind of open state blockade of the channel. It was reported that the time constant of decay of neurally evoked postsynaptic currents corresponds to the mean open time of the channel (Magleby & Stevens, 1972a,b). Anaesthetics were reported to facilitate the decay of endplate and postsynaptic currents (Gage & Hamill, 1975; 1976; Adams *et al.*, 1982). Increase in the decay rate of e.p.s.c. and i.p.s.c. in the present experiments may be accounted for by a faster relaxation of the open state to the closed state, and/or by a blockade of the open state by enflurane.

Lechleiter & Gruener (1984a,b) reported a shortening of the burst duration of the ACh-induced channel current by halothane in patch clamp experiments with *Xenopus* skeletal muscle cells, and they attributed the effect to the disordering action of the anaesthetic on the membrane lipids. Rosenberg *et al.* (1977) reported a biphasic action of halothane, low concentrations ordering and high concentrations fluidizing synaptic plasma membranes of rat brain tissue and artificial phospholipid membranes. Recent work showed that clinically relevant concentrations, changes in lipid bilayer structure and fluidity were virtually undetectable (Franks & Lieb, 1978; 1982; Turner & Oldfield, 1979; Lieb *et al.*, 1982). Franks & Lieb (1982) pointed out that warming of only a few tenths of a degree produces similar fluidity increases in cholesterol–containing bilayers but does not induce anaesthesia in animals. These considerations suggest that enflurane produces some conformational change of the receptor-channel complex, and that blockade of both closed and open states of the channel plays at least some role in reducton of the postsynaptic current.

In conclusion, enflurane depresses cholinergic transmission in *Aplysia* ganglion cells at concentrations similar to those used in mammalian anaesthesia.

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