Effects of Enflurane on Gill Withdrawal Behaviors and the Ability of Gill Motor Neurones to Elicit Gill Contractions in Aplysia

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We used the Aplysia gill withdrawal reflex model system in order to study how enflurane effected both gill withdrawal adaptive behaviors and the activity of single identified neurones which are involved with the mediation of the gill withdrawal response. We found that a continuous superfusion of enflurane (0.5 and 1.0%) solution over the abdominal ganglion (the CNS) resulted in an increase in the spontaneous gill respiratory movements; an increase in the spontaneous discharges in identified central motor neurones; and a depolarizing shift in the resting membrane potential of these neurones. Enflurane also significantly effected the ability of the gill motor neurones to elicit a gill contraction when the motor neurone was depolarized to produce action potentials by passing depolarizing current into the neurone. Although in most cases the ability of the motor neurone to elicit a gill withdrawal contraction was decreased, that in one third of the cases was increased. Enflurane may exert its actions by effecting the activity of CNS control neurones which exert both facilitatory and suppressive control over the peripheral nervous system in the gill as well as by having direct effects on the motor neurones. (Key words: enflurane, Aplysia gill contraction, gill motor neurones, CNS control neurones)

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Enflurane is a halogenated ether anesthetic which is widely used throughout the world. However, in addition to its general anesthetic properties, its administration can produce abnormal movements which consist of the twitching of individual muscle groups and in some cases tonic-clonic activity[1,2]. Despite its almost universal acceptance as an anesthetic agent, little is actually known as to how enflurane's effects are mediated by the central nervous system (CNS) and even less is known in regards to the neuronal mechanisms underlying its side effects. Therefore in an attempt to gain a better understanding of both the neuronal mechanisms of anesthesia and the CNS stimulating effects of enflu-

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rane we initiated a series of studies using the Aplysia siphon, mantle, gill and abdominal ganglion (AG) preparation.

The siphon, mantle, gill and AG preparation has been extremely used in neurobiological research to study both the neuronal basis of important homeostatic behaviors such as respiration[3] and adaptive behaviors of the gill and siphon[4,5]. In this *in vitro* preparation the gill and siphon exhibit rhythmic spontaneous contractions (spontaneous gill movements, SGMs) which are driven by a central pattern generator in the CNS and play a role in respiration in the intact animal[6]. In addition to those spontaneous respiratory movements, tactile stimulation of the gill or siphon elicits a gill withdrawal reflex (GWR)[7,8]. However, the GWR is not simply a graded contraction dependent on the applied stimulus strength (i.e. a reflex) but rather is a heterogeneous collection of at least four action patterns[9]. The GWR is mediated by the integrated activity of the central (CNS) and peripheral (PNS) nervous systems[10]. The central component of the GWR is mediated by a group of identified sensory, inter- and motor neurones located in the AG. These neurones are relatively large and can be recorded from whilst recording gill and siphon movements. In addition to these neurones there are central control neurones which exert both facilitatory and suppressive control over the PNS in the gill[11,12]. These control neurones have not as yet been unequivocally identified. But, their activity serves to either increase or decrease the amplitude of the GWR or the amplitude of a gill contraction elicited by the intracellular depolarization of an identified central gill motor neurone.

In this first study on the effects of enflurane in this preparation we examine the effects of enflurane perfusion over only the CNS.

Methods

Aplysia Californica (100–250g, Alacrity, Redondo Beach, CA) were used in these studies. They were maintained in a 1200*l* aquaria containing artificial sea water (ASW, Institute Ocean) at 15–17°C, pH 7.9. Animals were fed weekly, dried red sea weed (Atlantic Biologicals, St Andrews, New Brunswick, Canada). Only non-food satiated animals were used. Prior to dissection, animals were anesthetized by the injection of $MgCl_2$ (33% of body weight) into the hemocoel.

The semi-intact preparation (fig. 1) consisted of the siphon, mantle, gill and AG. The siphon, ctenidial and branchial nerves with which the AG innervates the mantle organs were left intact, all other nerves and connectives were severed. The preparation was pinned to a clear Sylgard (Dow-Corning)-coated base of a lucite chamber. The AG was further pinned dorsal side up on a Sylgard pedestal which was isolated from the rest of the preparation in a leak-proof chamber without interruption of its innervation of the mantle organs. Thus enflurane-containing ASW could be perfused over the AG without coming into contact with the gill or other peripheral structures.

A thread was attached to a single gill pinnule at one end and to a force-transducer (Narco-F60, Narco, Ontario, Canada) at the other end. The tension was adjusted to avoid stretching the pinnule. The transducer output was displayed on a storage oscilloscope and chart-recorder from which the measurements were made. The AG was transilluminated to aid impalement of identified central gill motor neurones. Single-barrel micropipettes (2M KCl) having a resistance of 10–20 MΩ were used. Identification of central gill motor neurones was based primarily on

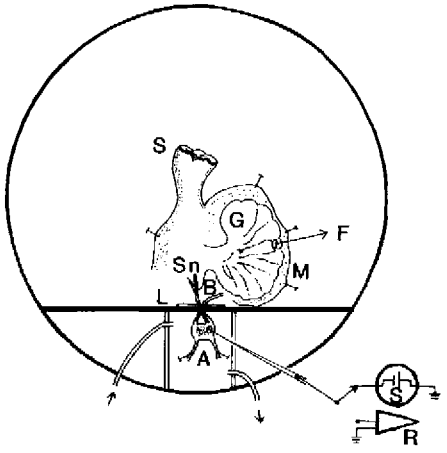

Fig. 1. The semi-intact preparation of Aplysia californica consisted of the siphon (S), mantle (M), gill (G) and abdominal ganglion (A). The siphon, ctenidial and branchial nerves (Sn, B) with which the abdominal ganglion innervates the mantle organs were left intact, all other nerves and connectives were severed. The preparation was pinned dorsal side down on a Sylgard (Dow-Corning) coated dish. The abdominal ganglion was further pinned dorsal side up on a Sylgard pedestal which was isolated from the rest of the preparation in a leak-proof chamber without interruption of its innervation of the mantle organs. The enflurane-containing artificial sea water (ASW) could be perfused over the abdominal ganglion without coming into contact with the gill or other peripheral tissues.

Standard electrophysiological techniques were used to record (R) from and to pass current (S) in central gill motor neurones. Depolarizing current pulses, 1–2 sec in duration were used to elicit gill withdrawal responses (GRs). Gill movements were measured using a force transducer (Narco F-60) connected to a single gill pinnule by fine surgical thread (F). Each preparation was first tested with ASW perfusion of the abdominal ganglion. Following the second control passing of current the perfusion was switched so that enflurane containing ASW was perfused over the abdominal ganglion for 20 min. A GR was then evoked. The perfusate was then switched back to ASW only and 20 min later the GR was again evoked. The preparations were superfused with ASW equilibrated with accurately monitored concentrations of enflurane vapor using a circuit specially designed to administer anesthetic at controlled concentrations and flow rates.

the type of gill movement elicited when depolarized[13] as well as their position in the ganglion and the pattern of spontaneous synaptic input to the cell[14]. The AG was bathed in a hypertonic sucrose/sea water solution[15] for 15 min prior to removal of the connective sheath. Desheathing was accomplished with a fine pair of scissors and forceps. Following desheathing, the sucrose solution was removed and the ganglion was washed 4 or 5 times in ASW.

A bridge circuit in the electrometer (Cell Explorer, Dagan, Minn) allowed for the simultaneous recording and passing current in the central gill motor neurones. Depolarizing current pulses, 1–2 sec in duration were used to elicit a gill withdrawal response (GR). Each preparation was first tested with ASW prefusion of the AG following a 1 hr rest. Two control GRs were elicited by intracellular depolarization of the motor neurone with an interstimulus interval (ISI) of 20 min. The experiment only proceeded if the elicted GRs were within 10% of each other. Approximately the same number of action potentials (APs) were elicited on each trial, a difference of 1 or 2 APs were acceptable. Following the second control depolarization, the perfusion was switched so that enflurane-containing ASW was perfused over the AG for 20 min. A GR was then elicited by depolarizing the central gill motor neurone to produce approximately the same number of APs as in the controls. The perfusate was then switched back to ASW and 20 min later a GR was then elicited.

In experiments where we monitored SGMs, stimuli were not applied to the

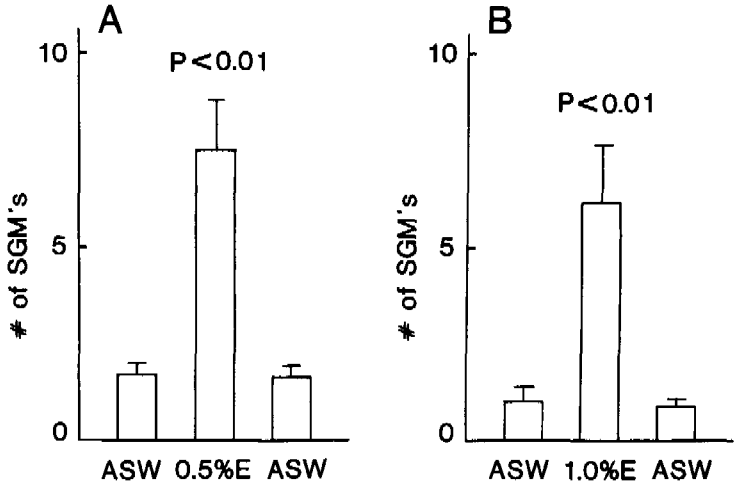

Fig. 2. The perfusion of enflurane (E)-ASW (artificial sea water) (0.5 and 1.0%) over the abdominal ganglion significantly increased in the number of spontaneous gill movements (SGMs). The measurements of the number of SGMs were made for 10 min.

A) ASW (pre): 1.69 ± 0.31/10 min, 0.5% E: 7.49 ± 1.37/10 min, ASW (post): 1.63 ± 0.30/10 min, n=30

B) ASW (pre): 1.0 ± 0.38/10 min, 1.0% E: 6.14 ± 1.48/10 min, ASW (post): 0.79 ± 0.24/10 min, n=14

preparation. The occurrence of SGMs were counted in the 10 min epoch before enflurane perfusion, during a 10 min perfusion of enflurane-ASW over the AG, and during the 10 min washout.

The preparations were superfused with ASW equilibrated with accurately monitored concentrations of enflurane (Dainabot, Osaka, Japan) vapor (volume for volume, in air) using a circuit specially designed to administer anesthetic at controlled concentration and flow rates. The rate of perfusion was 5–10 $ml \cdot min^{-1}$. Statistical significance was tested using a Student's t-test and two sets of data were assumed to be different at $P < 0.05$.

Results

Spontaneous gill movements (SGMs)

The superfusion of enflurane-ASW (either at 0.5 or 1.0%) over the abdominal ganglion had a significant but reversible effect on the rate of SGMs. Superfusion of enflurane (0.5%) resulted in an immediate and significant increase in the rate of SGM activity (n=30, $P < 0.01$) (fig. 2A). In addition, during the enflurane superfusion the most of the SGMs were large and saturated the transducer output. In a few preparations the gill remained contracted for periods of a few minutes. In other preparations where no SGMs were observed prior to enflurane superfusion, enflurane always induced the occurrence of SGMs. Following washout the preparations resumed their pre-enflurane like activity. However in some preparations washout was not immediate, enflurane effects persisted for up to 30 min. Results similar to these were also observed when a high concentration (1.0%) of enflurane – ASW was superfused over only the AG. In the preparations (n=14) this higher concentration of enflurane significantly increased the rate of SGMs ($P < 0.01$) (fig. 2B) while at the same

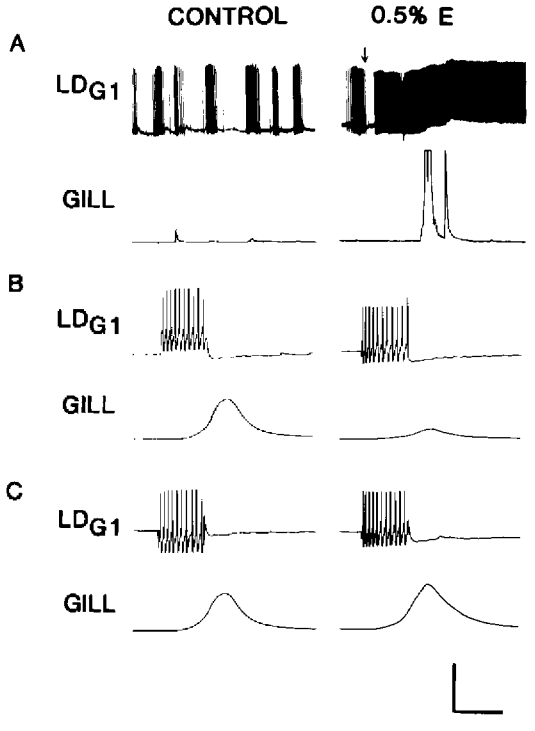

Fig. 3.

A) 0.5% enflurane (E) perfusion of the abdominal ganglion (arrow) resulted in an increase in the AP frequency of LDG_1, an increase both in the rate, amplitude and duration of the spontaneous gill movements and a depolarizing shift in the membrane potential. Scale: 50 mV; 100 sec

B) 0.5% enflurane brought abut a significant reduction in the amplitude of the gill contraction elicited by depolarization of the motor neurone. Scale: 50 mV; 1 sec

C) This example shows a significant increase in amplitide of the elicited gill contraction due to perfusion of enflurane. Scale: 50 mV; 1 sec

time producing the larger amplitude SGMs. As can be seen in data obtained from a single preparation (fig. 3A) the perfusion of enflurane – ASW over the AG not only effected the rate and amplitude of the SGMs but also effected the spiking activity of the central gill motor neurones. In this representative preparation 0.5% enflurane increased dramatically the frequency of APs in gill motor neurone LDG_1. In almost all preparations tested (n=60) 0.5% and 1.0% enflurane superfusion of the AG resulted in a remarkable increase in motor neurone activity. Accompanying the increase in firing frequency was a depolarizing shift in the membrane potential. This shift ranged between 5–14 mV and resulted in an increased firing rate of the neurone. Moreover, a change from a more regular firing pattern to one with more irregular burst was noted.

Effects on gill movement elicited by motor neurone depolarization

We determined whether enflurane would effect the ability of the gill motor neurones to elicit a gill withdrawal response. In these experiments we attempted to evoke the same number of APs on each trial. This was not always possible. We therefore only included data if there was only 1 or 2 APs difference. Nineteen experiments were performed which met this criterion. Of these in 13 cases, enflurane (0.5%) brought about a significant reduction in the amplitude of the gill contraction brought about by depolarization of the motor neurone (68.9 ± 6.3% of control; $P < 0.01$). An example of this is shown (fig. 3B) and the group data are presented in figure 4A. In the case of the other 6 preparations enflurane (0.5%) had the exact opposite effect, there was a significant increase in the amplitude of the elicited gill contraction (126.3 ± 8.5% of control; $P < 0.05$). An example of this is shown (fig. 3C) and the group data are presented in figure 4B. When a higher concentration of enflurane (1.0%) was used similar but more pronounced effects were observed (fig. 4). Thus we found two populations of preparations with the majority (13 out of 19) showing that enflurane suppressed the ability of the gill motor neurones to elicit gill contractions.

We tested the effect of an even

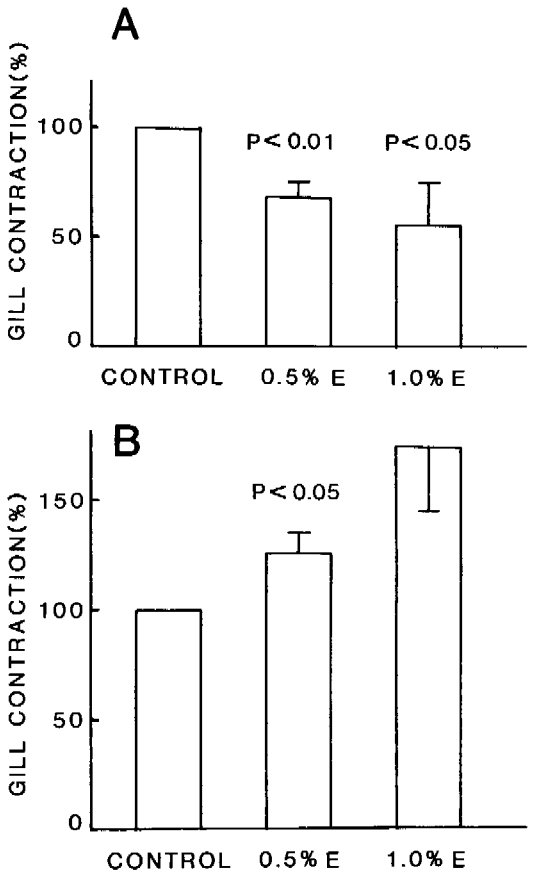

Fig. 4.

A) In 13 of 19 experiments, 0.5% enflurane brought about a significant reduction in the amplitude of the gill contraction brought by depolarization of the motor neurone (68.9 ± 6.3% of control, $P < 0.05$). In 5 of 7, 1.0% enflurane also brought about a significant reduction (56.6 ± 18.5% of control, $P < 0.05$).

B) In 6 cases of 19 experiments, 0.5% enflurane brought about a significant increase in the amplitude of the elicited gill contraction (126 ± 8.5% of control, $P < 0.05$). In 2 of 7, 1.0% enflurane also brought about an increase in the amplitude of the elicited gill contraction, however, this was not statistically significant (174.0 ± 41.0%, NS).

higher enflurance concentration (1.5%) and found that with this concentration depolarization of the neurone initiated more prolonged bursts of APs outlasting by at least 1 sec the applied current pulse. The gill contractions elicited were of much longer amplitude than control but because of the prolonged after-discharge of the neurones we could not really compare the amplitude of the contractions with that of controls.

Discussion

The data obtained in this study demonstrate that enflurane perfusion of the AG has significant effects on both neural activity and gill behaviors. As such, these data show that this Aplysia preparation may provide useful information regarding the mechanisms by which anesthetics effect both neural activity and behavior. Previous work using invertebrate model systems showed that halothane at low concentrations had excitatory effects on identified Lymnea neurones[16]. Moreover, in the same study, they showed paroxysmal depolarizing shifts in 4 groups of neurones when enflurane was used. Thus the results we have observed here agree in general with those of Winlows group. Our observations also agree with previous reports in humans and other mammals[1,2,17].

The ED_{50} of enflurane for Aplysia is unknown. Girdlestone et al.[18] reported an ED_{50} of 1.0% for enflurane in Lymnea stagnalis, another gastropod mollusc. Thus we estimate that we were at approximately one half to one of the ED_{50} in the marine mollusc Aplysia. In any case, perfusion of enflurane-ASW (0.5%) had significant and reversible effects on our preparation. In *in vitro* preparations SGMs occur at a frequency of 1–2 every 10 min, and their amplitude may vary considerably. The variance in the amplitude is probably due to the partial activation of the L25/R25 network which does not result in a high frequency burst of action potentials as previously decided[6,12,19].

Enflurane perfusion of the AG

brought about a significant change in the rate, amplitude and duration of SGMs. These changes in SGMs parameters were most likely due to changes in neural activity at the level of the Int-II network[20,21] and directly on the motor neurones. Further experiments examining the direct effects of enflurane on the electrophysiological properties of motor neurones will be necessary. As well, experiments will also have to be performed on the neural elements in the Int-II network to determine how enflurane alters this central pattern generator.

Enflurane perfusion on the AG had significant effects on the ability of gill motor neurones to elicit gill movements. Since enflurane did not come into contact with gill itself, this effect must be primarily due to enflurane effects on the CNS control neurones[11,12]. The activity of these control neurones can be affected by various endogenous neural peptides. The superfusion of arginine vasotocine (AVT) over the ganglion results in a suppression of a motor neurones ability to elicit a gill contraction when depolarized even though AVT has no measurable effect on the motor neurone itself[11]. The superfusion of another peptide small cardioactive peptide B (SCP_B) brings about the opposite effect. The ability of a gill motor neurone to elicit a gill contraction is enhanced even though SCP_B does not directly effect the motor neurone[11]. Thus it appears that these peptides affect the activity of the CNS control neurones which exert their effect in the gill periphery by either enhancing or suppressing the central motor neurones input to the muscle. Similarly, classical conditioning training affects the facilitatory control neurones which serve to enhance the motor neurones efficacy to elicit gill movements[24]. In most cases enflurane affecs the activity of control neurones which lead to a suppression of a motor neurones ability to elicit gill movement.

We do not understand why enflurane on some preparations had the opposite actions. Our first thought was that the animals might be in a different behavior state. However, we examined all our preparations closely and they appeared all to be in the same state. However, we cannot rule out the possibility that one group of animals was performing a behavior which led to a different behavioral state. Further studies will be performed in an attempt to clarify the differential effects of the anesthetic.

In summary, enflurane effects the neuronal activity of identified neurones which are involved in the mediation of a relevant behavior. This finding may also help us to begin to come to a better understanding of the mechanisms by which anesthetics affect neuronal activity and behavior.

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