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Enflurane is a potent inhibitor of high conductance Ca^{2+}-activated K^+ channels of *Chara australis*

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The volatile anaesthetic, enflurane, is commonly used in surgery for inducing the state of general anaesthesia. It is assumed, that general anaesthetics act on ion channels, but little is known of how they do so and what kinds of channels are sensitive. We found, that enflurane inhibits a large conductance Ca^{2+}-activated K^+ channel of the green alga, *Chara australis*. Effects occur at clinically relevant concentrations and are fully reversible. The actions of enflurane are distinct from those of charybdotoxin and tetraethylammonium, which are well known blockers of this channel type. Kinetic analysis of single-channel data demonstrates multiple effects of enflurane on the channel protein.

Enflurane; General anaesthetic; Ca^{2+}-activated K^+ channel; *Chara australis*

1. INTRODUCTION

There is general agreement that volatile anaesthetics act on ionic channels [1–3]. The efficiency of these compounds in inducing the anaesthetic state in animals is well predicted by the so-called Meyer–Overton rule, a rule that correlates the potency of general anaesthetics with their fat solubility [4,5]. Recently it was shown that Ca^{2+}-activated K^+-channels (K(Ca)) are (i) inhibited by clinical concentrations of volatile anaesthetics and (ii) that the efficiency of this inhibition follows the Meyer–Overton rule [6]. By these criteria these ion channels fulfill two requirements as a possible target of general anaesthetics in the central nervous system [7]. The effects of general anaesthetics on K(Ca) have previously been investigated but with contradictory results [6,8]. Since several types have been found, K(Ca) were classified as small (SK(Ca)), intermediate (IK(Ca)) and large conductance (maxi-K(Ca)) channels [9–11]. It seems possible that sensitivity to general anaesthetics is restricted to one of these classes.

K(Ca) of the green alga, *Chara australis*, have been studied intensively and were identified as maxi-K(Ca) [12–14]. These channels are ubiquitous in the animal and plant kingdom. Its presence in neurons has been documented (for review see [9–11]). A close evolutionary relationship within this class is indicated by characteristic features: maxi-K(Ca) exhibit a large unitary conductance of more than 100 pS in 150 mM KCl and a high selectivity for potassium vs. sodium ions. They are activated by micromolar cytosolic Ca^{2+} concentrations and show voltage dependence. Channel openings occur in bursts and complex schemes of activation have been proposed [12,13,15].

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2. MATERIALS AND METHODS

2.1. *Preparation and enflurane application*

Vacuolar droplets of about 10–100 μm in diameter were prepared as described elsewhere [14]. The surrounding membrane is derived from the tonoplast with the cytoplasmic face directed outward. The orientation of the membrane is still a matter of debate but we refer to investigations in which membrane identification was undertaken [14]. For enflurane application a small chamber was used to continously exchange the gas phase above the bathing solution. Enflurane was applied as a calibrated vapor (Dräger, Germany) at a flow rate of 1.1 l/s. To facilitate rapid diffusion to the membrane, the tip of the patch electrode was positioned just below the surface of the bathing solution.

2.2. *Electrophysiological measurements*

Electrodes were made from thick-walled borosilicate glass pulled to a resistance of about 20 MΩ. Measurements were carried out with 140 mM KCl, 1 mM $CaCl_2$ and 5 mM MOPS-KOH buffer, pH 7.2, on both sides of the membrane. Temperature was 25°C. The high Ca^{2+} concentration was choosen in order to activate the maxi-K(Ca) channels [6]. In the vesicle-attached configuration the cytoplasmatic surface of the channels faces the pipette solution. After excision of a membrane patch, the electrode tip was exposed to air for a few seconds. The membrane potential is given as the pipette potential with respect to the bathing solution, according to the usual conventions [16]. Currents were recorded with a patch clamp current-to-voltage converter (L/M EPC 7, List, Germany), digitized (VR100, Instrutech, USA), and stored on a video tape. For further analysis, data were played back, filtered at 2 kHz (−3dB corner frequency, 8-pole Besselt filter) and transferred to an IBM compatible computer (AT 386), using a 12 bit analog-digital converter (Labmaster DMA, Scientific Solutions, USA).

2.3. *Data analysis*

The kinetic analysis after enflurane application was started with single segments. When no further parameter change occurred, we concluded that a steady-state situation had been reached. All events observed prior to this time were rejected. The same method was used for analyzing the data after removing the anaesthetic.

Analysis was performed with pCLAMP and LPROC software (Axon Instruments, USA). The binning of events and the fitting procedure employed criteria described elsewhere [17–19]. Histograms were not corrected for missing events. Using square pulses with a rise time of 2 μs and a duration between 10 and 500 μs as a test input for the recording system and analysis software, the half-level threshold analysis detected events longer than 90 μs. Open-time distributions were fitted by maximum likelihood and a probability density function of the form: $f(t)=a/\tau^* \exp(-t/\tau)$. The fitting procedure was restricted to the area of the histogram showing at least 10 counts per bin. Three exponentials were necessary to fit the closed-time distribution in the range indicated in Fig. 3 [12]. The model was of the form: $f(t)=a_1/\tau_1^* \exp(-t/\tau_1) + a_2/\tau_2^* \exp(-t/\tau_2) + a_3/\tau_3^* \exp(-t/\tau_3)$. Events were binned on a logarithmic time scale using 10 bins per decade and indicated as counts per ms.

3. RESULTS AND DISCUSSION

We have tested the effects of enflurane on the activity of a maxi-K(Ca) in an excised membrane patch from *Chara*. A record from a typical experiment (n=32) is presented in Fig. 1a. Because of the high Ca^{2+} concentration in the bath, the channel was strongly activated at a membrane potential of −77 mV and exhibited long bursts of openings, separated by interburst segments lasting less than about 1 s. When enflurane was applied, after about 15 s, long gaps between bursts developed, however, occasional bursts of openings persisted. After enflurane had been removed, channel activity started to increase again.

In Fig. 1b–d histograms of the current amplitudes before, during and after enflurane treatment are shown. Enflurane strongly reduced the time the channel spent in the open state. The open probability was 0.57 before, and 0.09 in the presence of the anaesthetic. This effect was fully reversible.

There was also an effect on the current in the open state, but it was small compared with the changes in the open probability. Fitting the distributions in Fig. 1b–d with Gaussian functions and taking the difference of the mean values as the single-channel current, the calculated current was −11.0 pA before, −9.1 pA during and −11.4 pA after treatment.

Enflurane also affected closed-open transitions within bursts of openings. Before and after treatment, the channel remained open most of the time within a burst and only brief closings occurred (Fig, 2a). In the

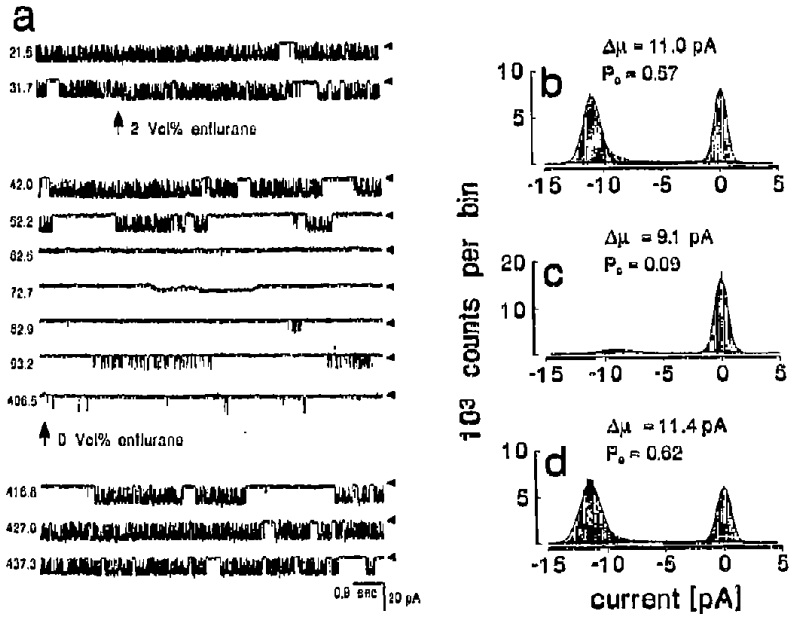

Fig. 1 (a) Effect of 2 vol.% enflurane on the steady-state activity of a single large conductance Ca^{2+}-activated K^+ channel (maxi-K(Ca)). Seal resistance was 12.8 GΩ. The membrane potential was clamped to −77 mV and a single channel was observed in the patch. Enflurane (2 vol.%) was applied and removed as indicated by the arrows. The numbers at the left of the current traces denote the time in seconds elapsed since the beginning the experiment. The arrowheads on the right indicate the baseline current. Data were digitized at 500 Hz and filtered at 200 Hz. Histograms of the current amplitude before (b) during (c) and after (d) enflurane application at a membrane potential of −77 mV are based on data of the steady-state situation. The value of 0 mV on the abscissa corresponds to the baseline. A sum of two Gaussians was fitted to the histograms. The difference between the mean values was taken as the single-channel current amplitude and is indicated in the figures as $\Delta\mu$. P_o denotes the open probability and was calculated from the fraction of data points associated with the channel in the open state. In the presence of enflurane the single-channel current is slightly decreased. Furthermore, the standard deviation of the open-state current was increased (0.7 pA before, 1.4 pA during and 0.9 pA after enflurane treatment), while the standard deviation of the closed-state current was unaffected (0.5 pA before, during and after treatment).

presence of enflurane, shorter openings were separated by longer closed times, as shown in Fig. 2b. The distributions of open and closed times are given in Fig. 3. The histograms of open times were fitted with single exponentials [12]. The time constant was 3.7 ms before treatment, but 0.8 ms in the presence of enflurane. When enflurane was removed, the time constant was again close to the first value. Furthermore, there was a difference in the closed time distributions. On a time scale between 0.5 and 100 ms enflurane reduced the number of channel closings shorter than 4 ms but increased those longer than 5 ms. This effect was also reversed after removing enflurane.

Within a range of ±80 mV the current–voltage relationship of the channel, under the conditions used here, is known to be almost linear [14]. In Fig. 4 the single-channel current in the presence of enflurane is plotted as a function of the membrane potential. It is evident that the shape of the curve also remains linear under the anaesthetic.

Because of the multiple effects of general anaesthetics on intact cells, they can act on ion channels directly, but can also affect second messenger systems, controlling channel activity [1,3]. Since our measurements were carried out on a cell-free system with controlled solutions on both sides of the membrane, the latter possibility is unlikely as an explanation for the results reported here.

From the finding that potassium fluxes across maxi-K(Ca) of *Chara* are inhibited in the presence of enflurane, it cannot be taken for granted that sensitivity to general anaesthetics is a common feature of maxi-K(Ca). However, there is clear evidence that K(Ca) in the central nervous system are also affected. Our results are in line with investigations on neuronal rat glioma C6 cells [6], in which halothane, enflurane, metoxyflurane and alcohols all reduced rubidium fluxes across K(Ca) as predicted by the Meyer–Overton rule. Although the type of K(Ca) in rat glioma C6 cells has not been identified, the channels were blocked by charybdotoxin, known as a blocker for maxi-K(Ca), but not by apamin, a blocker of SK(Ca) [9–11]. In contrast, K(Ca) of red blood cells are not sensitive to general anaesthetics [8]. In these cells the channels are of the SK(Ca) type [20]. Thus the difference in sensitivity of the two cells may be explained by the presence of different types of K(Ca). Recently maxi-K(Ca) have been identified in hippocampus neurons [21]. It was shown, that several volatile anaesthetics suppressed Ca^{2+}-activated K^+ currents and modified the spike pattern of the neurons [22].

Although there is increasing evidence that general anaesthetics do act on several ion channels in a clinical range of concentration, only for the nicotinic acetylcholine receptor (nAChR) have the molecular mechanisms been investigated so far [2]. Noise analysis of synaptic responses and single-channel studies revealed a shortening in the mean open time without changes in conductance [23,24]. Furthermore, desensitization of nACHR is enhanced in the presence of general anaesthetics.

The overall effects on maxi-K(Ca) of *Chara* are similarly complex, as has been shown here. Charybdotoxin and tetraethylammonium are well known blockers of maxi-K(Ca) [10]. Charybdotoxin is a slow dissociating drug and stays bound with a mean dwell time of several seconds, but does not alter the distributions of open and closed dwell times in the ms range [25]. In contrast, tetraethylammonium is a fast dissociating drug. In single channel studies, a dose-dependent reduction of single channel currents was found. Neither charybdotoxin

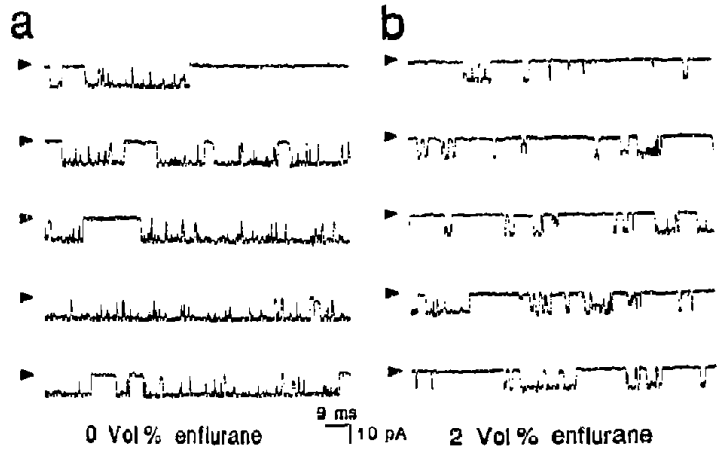

Fig. 2. Bursts of openings before and during enflurane treatment. Enflurane not only prolonged the periods during which the channel stayed closed, but also affected the open-closed transitions within the bursts. The membrane potential was −77 mV. Arrowheads indicate the baseline of the current traces. Records were digitized at 10 kHz and filtered at 2 kHz (−3 dB corner frequency). Comparing the traces in the absence (a) and presence (b) of enflurane, it is clear that during treatment short openings are separated by longer closed times. This effect is also documented in the open and closed time distributions of Fig. 4.

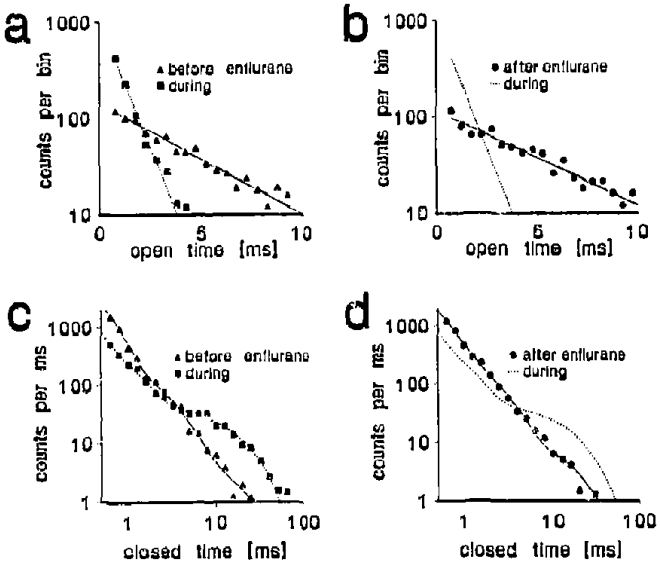

Fig. 3. Effects of enflurane on open and closed time distributions. In order to facilitate detection of the effects of enflurane treatment, histograms before, during and after the treatment contain 940 events in the case of open dwell times and 867 events in the case of closed dwell times. (a) Open time histograms before and during enflurane treatment. Direct comparison of the data shows, that the number of events shorter than 2 ms was greater in the presence of enflurane than before treatment. The distribution of open dwell times was approximated with a single exponential [8]. The solid line represents an exponential with a time constant of 3.7 ms, fitted to the distribution before enflurane treatment, and the broken line was fitted to the data in the presence of enflurane (time constant 0.8 ms). (b) Open-time histogram after removing enflurane (solid line). The fitted time constant was 4.4 ms. The dotted line is the same as in (a). (c) Closed-time distributions before (solid line) and during (dotted line) enflurane treatment within a range 0.5–100 ms on a double logarithmic plot. Time constants before treatment: τ_1 0.3 ms; τ_2 1.9 ms; τ_3 13.1 ms. During treatment: τ_1 0.1 ms; τ_2 0.6 ms; τ_3 13.3 ms. The histograms show that in the presence of enflurane the proportion of channel closings shorter than 5 ms is decreased whereas there were more events lasting longer than 5 ms. (d) Closed-time distribution after removing enflurane. Time constants are: τ_1 0.3 ms; τ_2 1.4 ms; τ_3 12.9 ms. The dotted line is the same as in (c).

nor tetraethylammonium mimic the actions of enflurane. Under enflurane the channel stays silent for periods of several seconds with a simultaneous change in the distribution of open and closed dwell times in the ms range (Figs. 1,2). Furthermore the single channel current is slightly reduced. We assume, that several binding sites on the channel protein or on the lipid–protein interface may be involved in producing these effects.

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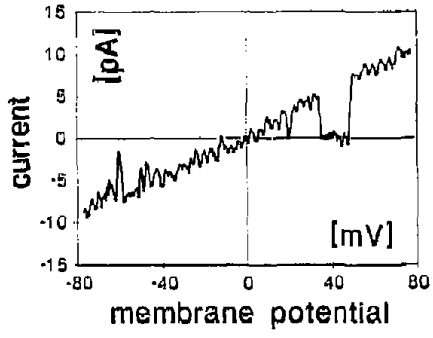

Fig. 4. Open-channel current–voltage relationship in the presence of enflurane, obtained with voltage ramps. The pipette potential was changed from +80 to −80 mV within 50 ms during a burst of openings. Ramps in the opposite direction were also tested, but hysteresis was not observed. The average leak currents from 5 records in the presence of enflurane were fitted with a 3-order polynomial and subtracted from current traces that were measured when channel openings occurred. Because of the small open probability in the presence of enflurane, only on rare occasions was a nearly complete current–voltage characteristic obtained from a single ramp. The current–voltage relationship of the channel within the tested voltage range is almost linear and reverses at about 0 mV. This value equals the Nernst potential for potassium ions. The short downward deflections at −60 and +40 mV indicate channel closings. Data were digitized at 10 kHz and filtered at 600 Hz.

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