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Actions of general anaesthetics and arachidonic acid pathway inhibitors on K^+ currents activated by volatile anaesthetics and FMRFamide in molluscan neurones

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1 K^+ currents activated by volatile general anaesthetics ($I_{K(An)}$) and by the neuropeptide FMRFamide ($I_{K(FMRFa)}$) were studied under voltage clamp in isolated identified neurones from the pond snail *Lymnaea stagnalis*.

2 $I_{K(An)}$ was activated by all the volatile anaesthetics studied. The maximal responses varied from agent to agent, with halothane ≈ sevoflurane > isoflurane > enflurane ≈ chloroform.

3 $I_{K(An)}$ was inhibited rather than activated by the *n*-alcohols from hexanol to dodecanol and by the 6- and 8-carbon cycloalcohols. The *n*-alcohols exhibited a cutoff effect, with dodecanol being unable to half-inhibit $I_{K(An)}$.

4 Unlike $I_{K(An)}$ which did not desensitize at reasonable halothane concentrations, $I_{K(FMRFa)}$ desensitized at most FMRFamide concentrations studied. This desensitization could be substantially removed by halothane. Nonetheless, both $I_{K(An)}$ and $I_{K(FMRFa)}$ had similar sensitivities to the potassium channel blockers tetraethylammonium and 4-aminopyridine, consistent with both currents flowing through the same channels. Responses to low concentrations of halothane and FMRFamide showed synergy.

5 The phospholipase A_2 inhibitor aristolochic acid inhibited $I_{K(An)}$, consistent with a role for arachidonic acid (AA). The lipoxygenase and cyclooxygenase inhibitor nordihydroguaiaretic acid blocked $I_{K(FMRFa)}$ but did not affect $I_{K(An)}$. $I_{K(An)}$ and $I_{K(FMRFa)}$ were little affected by the cyclooxygenase inhibitor indomethacin. These findings suggest that neither lipoxygenase nor cyclooxygenase pathways of AA metabolism are involved in the anaesthetic activation of $I_{K(An)}$.

6 Inhibitors of a third, cytochrome P450-mediated, pathway of AA metabolism (clotrimazole and econazole) potently blocked $I_{K(An)}$, suggesting possible roles for certain cytochrome P450 isoforms in the activation of $I_{K(An)}$.

Keywords: General anaesthesia; volatile anaesthetics; alcohols; arachidonic acid; cytochrome P450; FMRFamide; potassium channels

Introduction

About a decade ago, we found a K^+ current that is activated by volatile general anaesthetics in certain identified neurones of the pond snail *Lymnaea stagnalis* (Franks & Lieb, 1988). Perfusion of these neurones with volatile anaesthetics such as halothane and isoflurane opened K^+ channels, causing hyperpolarizations and decreases in membrane resistance which in turn tended to inhibit neuronal electrical activity. The targets to which volatile agents bind to open these channels have yet to be determined, although they are known to be protein rather than lipid in nature. This conclusion follows from the results of experiments with the optical isomers of the volatile anaesthetic isoflurane, which show stereoselectivity for activating the K^+ current (Franks & Lieb, 1991a) but not for partitioning into lipid bilayers (Franks & Lieb, 1991a; Dickinson *et al*., 1994). In this paper, we describe new results on the concentration dependence of five volatile and eight alcohol general anaesthetics for activating and inhibiting, respectively, this K^+ current, which we call $I_{K(An)}$ (Franks & Lieb, 1988).

This reversible, volatile anaesthetic-activated current is not appreciably voltage-gated and does not desensitize at pharmacological levels of volatile anaesthetic agonist. $I_{K(An)}$ further differs from a number of other K^+ currents (Hille, 1992) in that it is an outwardly rectifying current whose activity persists in both the absence of intracellular free Ca^{2+} and in the presence of moderate concentrations of 4-AP (4-aminopyridine) and extracellular TEA^+ (tetraethylammonium ion) (Franks & Lieb, 1988, 1991b). Taking into account the properties of $I_{K(An)}$, we have designed conditions under which $I_{K(An)}$ is effectively isolated from other possible currents. In preliminary experiments carried out using this selective protocol, we found that the molluscan neuropeptide FMRFamide activated a K^+ current, which we shall here call $I_{K(FMRFa)}$. This was presumptive evidence that $I_{K(An)}$ and $I_{K(FMRFa)}$ might share some common pathways of activation and perhaps even the same ion channels. Indeed, it has recently been shown at the single-channel level that halothane and FMRFamide probably open the same K^+ channels (the so-called S channels) in *Aplysia* neurones (Winegar *et al*., 1996). The mechanism of FMRFamide activation of $I_{K(FMRFa)}$ has been much studied in *Aplysia* neurones (Piomelli *et al*., 1987; Volterra & Siegelbaum, 1988; Belardetti *et al*., 1989; Buttner *et al*., 1989; Critz *et al*., 1991), and also investigated in *Helisoma* neurones (Bahls *et al*., 1992), and a central role for metabolites of arachidonic acid (AA = 5,8,11,14-eicosatetraenoic acid) has been demonstrated.

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In particular, it has been shown convincingly that lipoxygenase, but not cyclooxygenase, pathways of AA metabolism are involved; a possible role for cytochrome P450 has also been proposed (Belardetti *et al*., 1989) but is controversial (Piomelli, 1994).

Given the importance of AA metabolites in the activation of $I_{K(FMRFa)}$ in related molluscan species and the apparent similarities between $I_{K(An)}$ and $I_{K(FMRFa)}$, we have looked at and compared the actions on these two currents of inhibitors of the three major pathways (cyclooxygenase, lipoxygenase and cytochrome P450) of AA metabolism (Needleman *et al*., 1986; Piomelli, 1994) in volatile anaesthetic-sensitive neurones of *Lymnaea*. These results, together with those of experiments on the interactions between responses to both classes of general anaesthetic and FMRFamide, are presented here.

Methods

Dissection and isolation of single neurones

Lymnaea stagnalis snails (between 25 and 35 mm in length) were obtained from Blades Biological (Cowden, U.K.) and maintained in an aquarium at 18°C on a diet of lettuce. Before the arrival of a new batch of snails, the aquarium was cleaned and half-filled with fresh tap water (~40 l), which was then aerated for at least 24 h before adding the snails and continuously thereafter. The central nervous system was excised and the loose connective tissue around the right parietal ganglion removed. The tough endoneurium was removed after softening in pronase (type E, ~5 mg ml^{-1}, Sigma Chemical Co. Ltd., Poole, U.K.) for ~20 min. The right parietal ganglion was dissected away and held by its nerve roots in a small bath (volume ≈50 μl) using light suction. The neurones used in the experiments reported here were the three largest neurones (cell bodies 80 to 100 μm in diameter) visible on the surface of the right parietal ganglion, located in a lobe-like protrusion in the ventral region of the ganglion. A single neurone was isolated by first impaling it with two electrodes and then slowly moving the rest of the ganglion away from the neurone. This left the isolated neurone suspended in the bath, which was perfused at a rate of 0.5–2.0 ml min^{-1} with the same solution used for the dissection. The composition of this dissection solution was (mM): NaCl 50, KCl 2.5, $CaCl_2$ 4, $MgCl_2$ 4, HEPES 10, glucose 5; titrated to pH 7.4 with NaOH.

Electrophysiology

Currents in isolated neurones were measured with a two-electrode voltage-clamp amplifier (Axoclamp 2A, Axon Instruments, Foster City, CA, U.S.A.). The current records were filtered (10 Hz, −3 dB) by an 8-pole Bessél filter (Model 902, Frequency Devices, Haverhill, MA, U.S.A.) before being digitised (20 Hz) and stored on a computer. Electrodes, fabricated from 1-mm (o.d.) filamented borosilicate glass capillary tubes (GC100F, Clark Electromedical Instruments, Reading, U.K.) and filled with electrode solution (2.4 M K-acetate + 0.1 M KCl), had resistances between 15 and 45 MΩ.

Isolated neurones were continuously perfused (at 0.5–2.0 ml min^{-1}) with either control or test solutions, delivered by gravity feed from one of ten glass reservoirs. Reservoirs containing volatile anaesthetics were sealed with rigid plastic floats, and all tubing and valves were made of PTFE. With these precautions, losses of volatile agents from the perfusion system were found to be negligible when measured by gas chromatography (Hall *et al*., 1994). Acetate saline, the standard extracellular control solution used for electrophysiological recordings, had the following composition (mM): TRIS-acetate 55, TEA-acetate 5, K-acetate 2.5, Mg-acetate 4, Co-acetate 4, glucose 5; titrated to pH 7.4 with TRIS-OH. Co-acetate and glucose were always added on the day of the experiment. This saline was designed to pharmacologically isolate $I_{K(An)}$ from other currents found in these neurones. TEA was used to minimise contributions from other K^+ currents, and TRIS, Co^{2+} and acetate were used in place of Na^+, Ca^{2+} and Cl^-, respectively, to minimize currents due to movements of these latter ions. The efficacy of this saline is apparent from Figure 1, which shows quasi-steady-state current-voltage relationships for a typical neurone in acetate saline, both with and without halothane. It is clear that the background current is small in the range of membrane voltage from −100 to −40 mV, and the halothane-activated current $I_{K(An)}$ can easily be isolated. Except when such current-voltage relationships were being measured, neurones were routinely clamped at −40 mV. All experiments were performed at room temperature.

Anaesthetics

The sources of the anaesthetics were as follows: isoflurane, enflurane, sevoflurane (Abbott Laboratories Ltd., Queenborough, U.K.); halothane (ICI Ltd., Macclesfield, U.K.); chloroform, ethanol, *n*-butanol, *n*-hexanol, *n*-heptanol, *n*-octanol, *n*-nonanol, *n*-decanol, *n*-dodecanol (BDH Chemicals Ltd., Poole, U.K.); *n*-tetradecanol (Aldrich Chemical Co. Ltd., Gillingham, U.K.), cyclohexanol, cyclooctanol, (Lancaster Synthesis Ltd., Morecambe, U.K.); *n*-undecanol (Sigma Chemical Co. Ltd., Poole, U.K.). The volatile anaesthetics were made up as fractions of saturated solutions in acetate saline at room temperature. The concentrations of the saturated solutions were taken to be: isoflurane, 15.3 mM (Franks & Lieb, 1991a); enflurane, 11.9 mM (Seto *et al*., 1992); sevoflurane, 11.8 mM (Watts *et al*., 1992); halothane, 17.5 mM (Raventós, 1956); chloroform, 66.6 mM (Firestone *et al*., 1986). The lower *n*-alcohols (carbon numbers ⩽C8) and the cycloalcohols were added directly or were diluted from stock solutions in acetate saline. Solutions of the higher *n*-alcohols were prepared using concentrated ethanolic solutions;

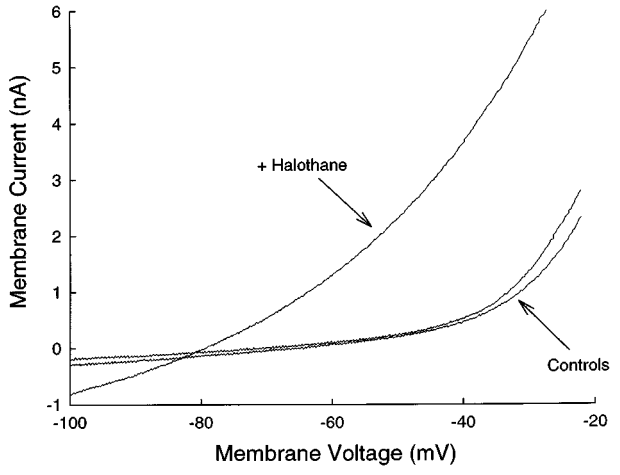

Figure 1 Current-voltage relationships for a typical isolated anaesthetic-sensitive neurone bathed in acetate saline, in the presence and absence of 1.4 mM halothane. The control curves were obtained before and after application of the anaesthetic. The membrane potential was ramped at 3 mV s^{-1} in the depolarizing direction.

the final concentration of ethanol in acetate saline was always 17 mM, and an identical concentration of ethanol was present in the relevant control solutions.

Other chemicals

Where not specified otherwise, all chemicals mentioned both here and above were obtained from Sigma Chemical Co. Ltd. (Poole, U.K.). Solutions containing FMRFamide (Phe-Met-Arg-Phe-amide) and aristolochic acid (sodium salt, Cascade Biochem Ltd., Reading, U.K.) were diluted from stock solutions in acetate saline. Solutions containing clotrimazole, econazole, NDGA (nordihydroguaiaretic acid), and indomethacin were prepared using concentrated ethanolic solutions; the final ethanol concentration in acetate saline was always 17 mM, and an identical concentration of ethanol was present in the relevant control solutions.

Analysis of concentration-response data

Concentration-response data for currents activated by volatile general anaesthetics or FMRFamide were fitted to an activation Hill equation of the form

$$E = \frac{E_{\max}}{1 + \left(\frac{EC_{50}}{A}\right)^{n_H}} \quad (1)$$

where E is the agonist-induced current measured at its peak, E_{max} is the maximal agonist-induced current, A is the agonist concentration, n_H is the Hill coefficient and EC_{50} is the agonist concentration for a half-maximal effect.

Inhibitory concentration-response data were fitted to an inhibitory Hill equation of the form

$$y = \frac{100}{1 + \left(\frac{I}{IC_{50}}\right)^{n_H}} \quad (2)$$

where y is the percentage of the control peak current remaining in the presence of an inhibitor at a concentration I, n_H is the Hill coefficient and IC_{50} is the inhibitor concentration for 50% inhibition.

Numerical fits to these Hill equations were obtained using the method of unweighted least squares (Marquardt-Levenberg algorithm, SigmaPlot®, Jandel Scientific, San Rafael, CA, U.S.A.) applied to all of the individual (rather than the mean) responses.

Values throughout the paper are given as means ± s.e.means.

Results

Activation of $I_{K(An)}$ by volatile general anaesthetics

All of the volatile general anaesthetics tested (halothane, isoflurane, enflurane, sevoflurane and chloroform) activated $I_{K(An)}$. Halothane was studied in the most detail. The halothane concentration-peak response data are plotted, together with typical current traces from a single neurone, in Figure 2. The responses did not desensitize except at extremely high (~5 mM) halothane concentrations, where a 'bounce-back' effect was noted upon removal of halothane but not studied further.

Concentration-response data for all volatile general anaesthetics studied are given in Figure 2, where the currents are normalized to those activated by 1.4 mM halothane. The data were fitted to Eqn. 1, and the EC_{50} concentrations, maximal currents (E_{max}) and Hill coefficients (n_H) are listed in Table 1. Perhaps the most interesting feature of these results is that quite different maximal currents were elicited by the different volatile agents, with halothane and sevoflurane

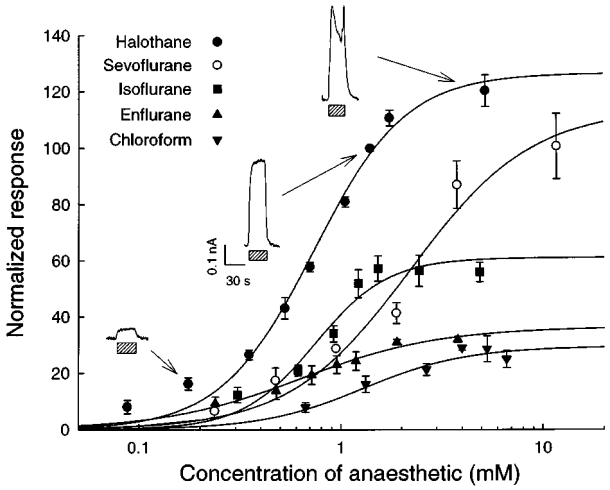

Figure 2 Concentration-response curves for activation of $I_{K(An)}$ by five volatile anaesthetics. The data points give the peak currents (means ± s.e.means for at least four cells), normalized to 100 for the current produced by 1.4 mM halothane. The curves are fits to Eqn. 1, with the Hill parameters listed in Table 1. The insets are typical current traces (all to the same scale) in response to halothane at the concentrations to which the arrows point.

Table 1 EC_{50} concentrations, maximal currents and Hill coefficients for activation of $I_{K(An)}$ by volatile general anaesthetics, together with EC_{50} concentrations for general anaesthesia in the *Lymnaea stagnalis* snail and in man

	Hill equation parameters for activation of $I_{K(An)}$				*General anaesthesia EC_{50} (mM) for*	
Anaesthetic	*EC_{50} (mM)*	*E_{max}*	*Hill coefficient*	*d.f.*	Lymnaea stagnalis	*Man*
Halothane	0.73 ± 0.04	127 ± 4	1.89 ± 0.15	60	0.6	0.21
Isoflurane	0.74 ± 0.07	61 ± 4	2.39 ± 0.47	40	0.7	0.31
Enflurane	0.63 ± 0.18	36 ± 6	1.27 ± 0.38	25	0.6	0.52
Sevoflurane	2.22 ± 0.53	114 ± 14	1.45 ± 0.35	18	–	0.30
Chloroform	1.21 ± 0.31	29 ± 4	1.70 ± 0.75	28	–	0.79

Hill equation parameters for activation of $I_{K(An)}$ are given as means ± s.e.means for *d.f.* degrees of freedom, obtained from fits of the data to Eqn.1. The maximal currents (E_{max}) are normalized to 100 for the current elicted by the standard test concentration (1.4 mM) of halothane. The criterion for 'general anaesthesia' in *Lymnaea stagnalis* was loss of the whole-animal withdrawal reflex in response to gentle mechanical stimulation at 20°C; EC_{50} data (Girdlestone *et al.*, 1989) expressed as partial pressures were converted to concentrations in water (Franks & Lieb, 1993) using estimates of water/gas partition coefficients at 20°C obtained or calculated from Allott *et al.* (1973) and Franks & Lieb (1993). EC_{50} concentrations (MAC) for general anaesthesia in man (criterion: lack of purposeful response to a surgical incision) were calculated for water and are taken from Franks & Lieb (1993), except for sevoflurane, which is for mammalian physiological saline (Franks & Lieb, 1996).

being much more effective than enflurane and chloroform, and isoflurane lying in between. Hill coefficients, on the other hand, were more similar from agent to agent and tended to be greater than unity, consistent with more than one anaesthetic molecule being involved in the activation of $I_{K(An)}$.

Where EC_{50} data for volatile agents are available for *Lymnaea* 'general anaesthesia' (i.e, for halothane, isoflurane and enflurane), the EC_{50} concentrations are close to those for activating $I_{K(An)}$ (see Table 1). However, no whole-animal snail data are available for sevoflurane and chloroform. For comparison, human aqueous MAC concentrations are also given in Table 1, and these are generally much lower than EC_{50} concentrations for activating $I_{K(An)}$.

Inhibition of $I_{K(An)}$ by medium- and long-chain n-alcohols and cycloalcohols

The *n*-alcohols hexanol, heptanol, octanol, nonanol, decanol, undecanol, and dodecanol, plus the 6- and 8-carbon cycloalcohols, behaved quite unexpectedly. Unlike the volatile general anaesthetics, these alcohols did not activate $I_{K(An)}$ on their own but instead reversibly inhibited the activation of $I_{K(An)}$ by halothane. Typical traces showing inhibitions of the response to 1.4 mM halothane by 5 mM *n*-hexanol, 150 μM *n*-nonanol and 16 μM *n*-dodecanol are given in Figure 3, and concentration-response data for the *n*-alcohols from hexanol to undecanol are plotted in Figure 4 together with fits to Eqn. 2. The IC_{50} concentrations and Hill coefficients are listed in Table 2 for the *n*-alcohols and cycloalcohols. Notice that IC_{50} concentrations for the *n*-alcohols decrease (i.e., potencies increase) with increasing chain-length up to decanol, after which they level out. In addition, the Hill coefficients (n_H) are greater than unity for the medium-chain *n*-alcohols but not significantly different from unity for the long-chain *n*-alcohols nonanol, decanol and undecanol (see inset to Figure 4). This behaviour of IC_{50} and n_H is consistent with the *n*-alcohols binding to a site of circumscribed volume capable of binding two or more molecules of the smaller alcohols but only one molecule of the larger alcohols, and it forms the basis of a cutoff effect (see Discussion).

Inhibition of $I_{K(An)}$ by *n*-hexanol was studied in more detail and found to depend on the concentration of halothane used to activate $I_{K(An)}$. This is shown in Figure 5, where hexanol concentration-response curves are plotted for hexanol inhibition of $I_{K(An)}$ activated by three different concentrations of halothane. It can be seen that hexanol inhibition increases with increasing concentrations of halothane, in a manner suggestive of halothane increasing the affinity of the hexanol-binding sites.

The behaviour of the short-chain alcohol *n*-butanol was found to occupy an intermediate position between that of the volatile general anaesthetics and that of the larger alcohols, in that it both activated $I_{K(An)}$ on its own and inhibited $I_{K(An)}$ activated by halothane. However (possibly because of this dual effect), butanol effects were very variable, and they were therefore not studied in detail.

Activation of a potassium current by FMRFamide

Under conditions designed to isolate $I_{K(An)}$, the neuropeptide FMRFamide activates a K^+ current ($V_{rev} \approx -80$ mV) in a concentration-dependent manner (Figure 6). Unlike $I_{K(An)}$, the FMRFamide response desensitized even at moderate agonist concentrations. Nonetheless, both currents were insensitive to inhibition by the channel-blocking agents TEA^+ and 4-AP. Inhibition of both currents by a very high concentration of TEA^+ is shown in Figure 7. 4-AP (3 mM) had no significant inhibitory effect on either the 50 μM FMRFamide-activated response (10 ± 9% inhibition, $n = 5$ cells) or on the 1.4 mM halothane-activated current (25 ± 4% *potentiation*, $n = 5$ cells). Taken together, these results suggest that, although both

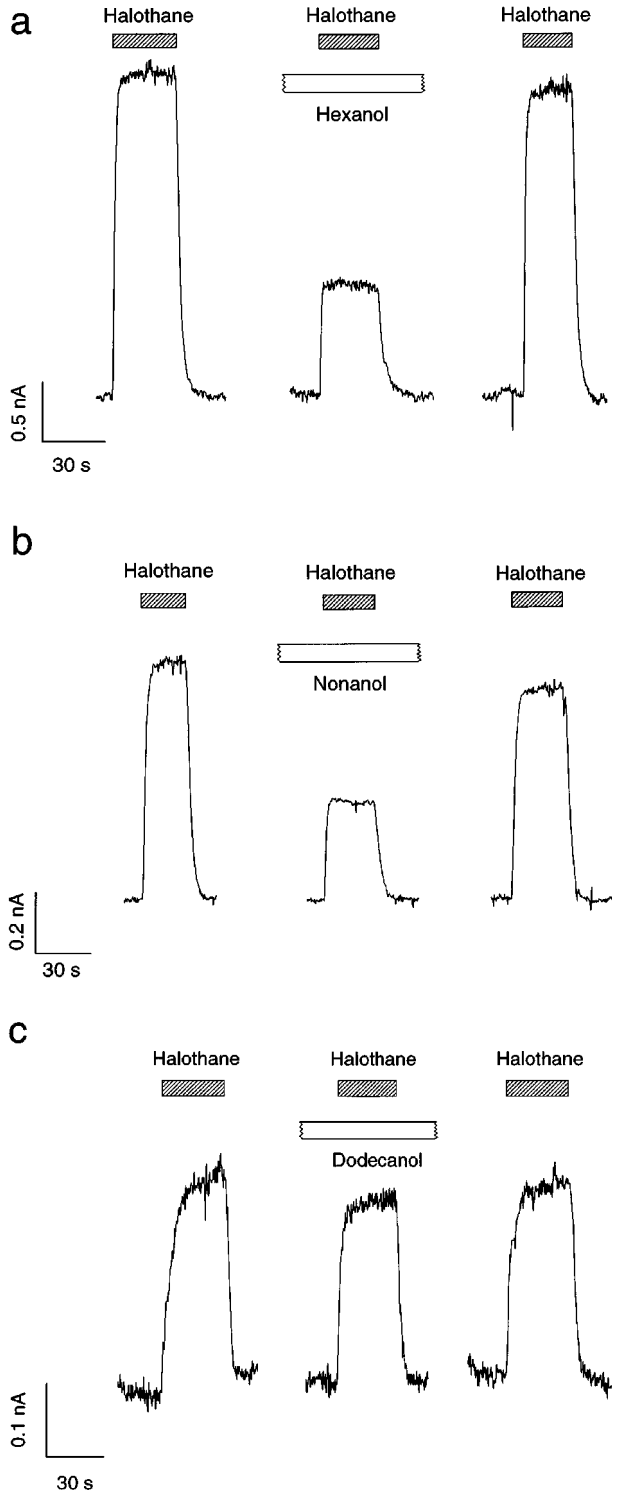

Figure 3 Typical current traces showing *n*-alcohol inhibition of $I_{K(An)}$ by (a) 5 mM hexanol, (b) 150 μM nonanol and (c) 16 μM dodecanol. Note that this nearly saturated concentration of dodecanol (74% saturated) gave only small inhibitions (17 ± 4% for 10 cells). Cells were pre-exposed to the *n*-alcohols for at least 2 min. The halothane concentration was 1.4 mM.

agents act at different primary sites, the K^+ channels ultimately opened by halothane and FMRFamide may be similar or identical.

In this regard, it is interesting that *n*-hexanol inhibits responses to both volatile anaesthetics and FMRFamide with comparable potencies. For four cells in each of which responses were obtained to 1.4 mM halothane, 1.2 mM isoflurane and 10 μM FMRFamide, the inhibitions (mean $\pm$ s.e.mean) by 5 mM hexanol were found to be $46 \pm 8\%$, $45 \pm 13\%$ and $61 \pm 5\%$, respectively.

Interactions between responses to halothane and FMRFamide

The effects of halothane and FMRFamide were not simply additive. First, the presence of halothane had the unexpected effect of substantially removing the desensitization of the response to FMRFamide ($n = 14$ cells). Halothane did not have to be preapplied for this effect to be observed. This can be seen from the currents in Figure 8 in response to 75 μM FMRFamide and 1.4 mM halothane applied both separately and together. In addition, for lower concentrations of

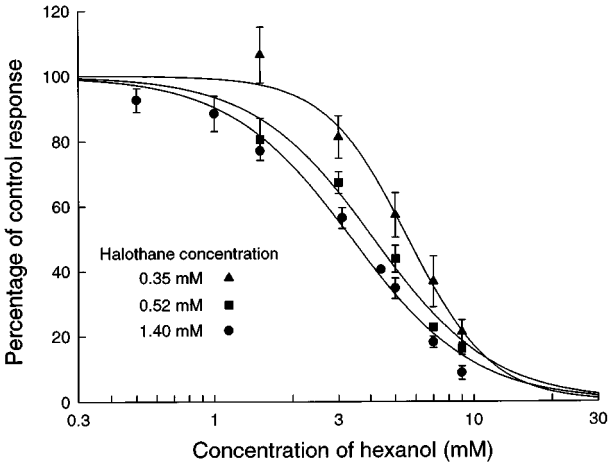

Figure 5 Hexanol concentration-response curves for *n*-hexanol inhibition of $I_{K(An)}$ activated by three different concentrations of halothane. Each data point is the mean $\pm$ s.e.mean for at least four cells. The curves are fits to Eqn. 2. The Hill parameters for 0.35, 0.52 and 1.40 mM halothane are respectively $IC_{50} = 5.61 \pm 0.36$, 4.12 ± 0.20 and 3.38 ± 0.14 mM hexanol and $n_H = 2.71 \pm 0.51$, 1.88 ± 0.20 and 1.84 ± 0.17, with 33, 24 and 28 degrees of freedom.

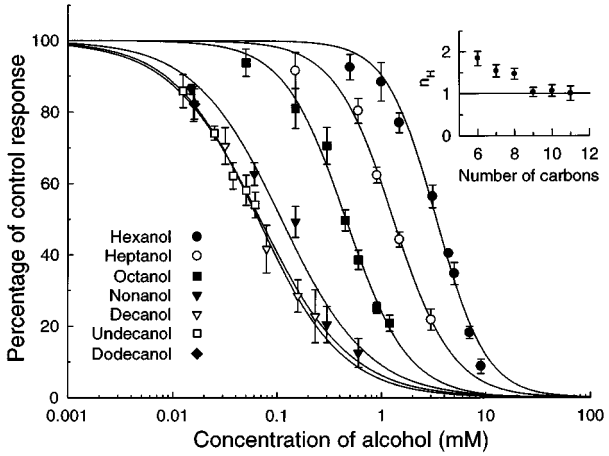

Figure 4 Concentration-response curves for inhibition of $I_{K(An)}$ by the medium-and long-chain *n*-alcohols. Each data point gives the percentage of the 1.4 mM halothane-activated current remaining in the presence of *n*-alcohol (mean $\pm$ s.e.mean for at least three cells). The curves are fits to Eqn. 2, with the Hill parameters listed in Table 2. The inset shows the Hill coefficients (n_H) as a function of chain length.

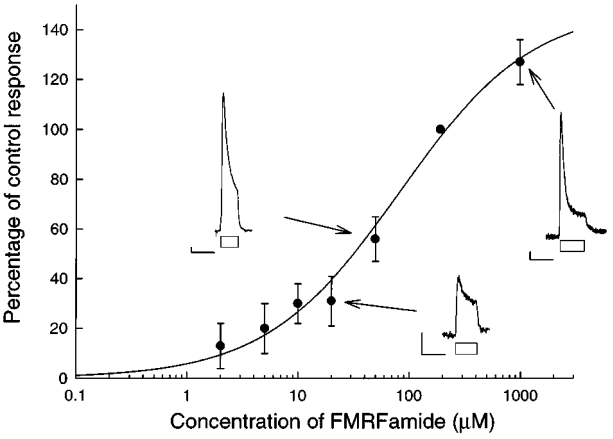

Figure 6 FMRFamide concentration-response curve. The data points give the peak currents (means $\pm$ s.e.means for at least four cells), normalized to 100 for the current produced by 200 μM FMRFamide. The curve is the fit of 55 individual responses to Eqn. 1. The Hill parameters are $EC_{50} = 81 \pm 43$ μM, $n_H = 0.73 \pm 0.15$ and $E_{max} = 150 \pm 30$. The insets are typical current traces in response to FMRFamide at the concentrations to which the arrows point. The scale bars for all three insets correspond to 50 pA and 30 s.

Table 2 IC_{50} concentrations and Hill coefficients for inhibition of $I_{K(An)}$ by alcohols, together with EC_{50} concentrations for general anaesthesia in tadpoles

	Hill equation parameters for inhibition of $I_{K(An)}$			*Anaesthesia*
Anaesthetic	*IC_{50} (mM)*	*Hill coefficient*	*d.f.*	*EC_{50} mM*
n-Hexanol	3.38 ± 0.14	1.84 ± 0.17	28	0.57
n-Heptanol	1.31 ± 0.07	1.54 ± 0.15	13	0.23
n-Octanol	0.46 ± 0.02	1.47 ± 0.13	32	0.057
n-Nonanol	0.11 ± 0.01	1.04 ± 0.11	24	0.037
n-Decanol	0.066 ± 0.007	1.07 ± 0.14	22	0.013
n-Undecanol	0.069 ± 0.008	1.01 ± 0.17	17	0.0081
n-Dodecanol	0.075 ± 0.002	(1.00)	9	0.0047
Cyclohexanol	1.73 ± 0.13	1.20 ± 0.11	42	5.0
Cyclooctanol	2.10 ± 0.33	1.44 ± 0.41	18	0.39

Hill equation parameters for inhibition of 1.4 mM halothane-activated $I_{K(An)}$ are given as mean $\pm$ s.e.means for *d.f.* degrees of freedom, obtained from fits of the data to Eqn. 2. The IC_{50} for *n*-dodecanol was calculated assuming a Hill coefficient of unity. EC_{50} concentrations for tadpole general anaesthesia are taken from Alifimoff *et al.* (1989) for the *n*-alcohols and from Curry *et al.* (1991) for the cycloalcohols.

FMRFamide (10–20 μM) and halothane (0.35 mM) where less FMRFamide desensitization occurred, synergism was observed: the current in the presence of both FMRFamide and halothane was greater than the sum of the currents due to each agent applied separately. This manifested itself as a larger net response to FMRFamide in the presence than in the absence of halothane. The degree of this potentiation varied considerably from cell to cell (range: 20–800%, mean ± s.e.mean: 145 ± 80%, $n = 10$ cells).

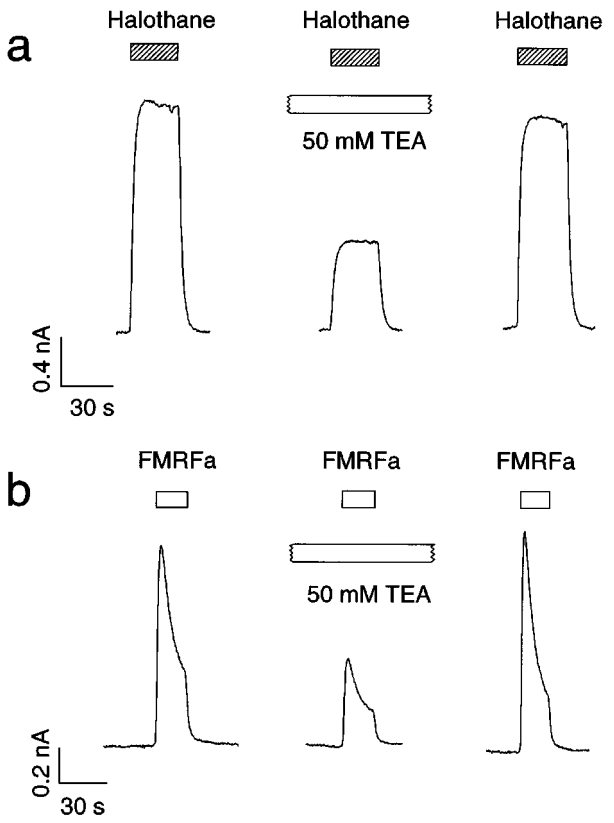

Figure 7 Similarity of inhibitions by a high concentration of TEA^+ of currents activated by (a) 1.4 mM halothane and (b) 50 μM FMRFamide. For the middle traces, the TEA^+ concentration was increased to 50 mM from its normal value of 5 mM (the TRIS concentration was correspondingly decreased from 55 to 10 mM), and cells were pre-exposed to this high TEA^+ solution for at least 1 min. Typical current traces are shown. The average inhibition of halothane-activated currents was 43 ± 4% ($n = 6$ cells), and the corresponding block of FMRFamide-activated currents was 59 ± 6% ($n = 6$ cells).

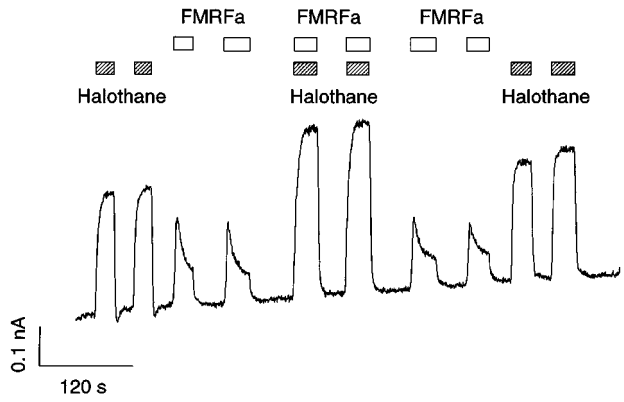

Figure 8 Interaction between responses to FMRFamide and halothane. Responses are shown to 75 μM FMRFamide and 1.4 mM halothane, applied separately and together. The record is a continuous current trace from a single neurone.

Effects of AA pathway inhibitors on currents activated by halothane and FMRFamide

In *Aplysia* and *Helisoma* neurones, activation of a K^+ current by FMRFamide is mediated by lipoxygenase metabolites of arachidonic acid (see Introduction). AA (arachidonic acid) can be directly released from membrane lipids by phospholipase A_2, and we found that aristolochic acid (10 μM), an inhibitor of this phospholipase (Denson *et al*., 1996), inhibited 1.4 mM halothane-activated $I_{K(An)}$ by 40 ± 6% ($n = 5$ cells). This suggested a possible role of AA metabolites in the generation or modulation of $I_{K(An)}$. To pursue this possibility, we looked at and compared the effects on $I_{K(An)}$ and $I_{K(FMRFa)}$ of inhibitors of the three major pathways that have been described for the metabolism of arachidonic acid: the cyclooxygenase, lipoxygenase and cytochrome P450 pathways.

NDGA (nordihydroguaiaretic acid) is both a lipoxygenase and cyclooxygenase pathway inhibitor (Piomelli & Greengard, 1990). While 10 μM NDGA very effectively inhibited FMRFamide-activated currents (76 ± 8% inhibition, $n = 3$ cells; see Figure 9a), the same concentration of NDGA had almost no effect on halothane-activated currents (3.0 ± 1.2% inhibition, $n = 4$ cells; see Figure 9b). Indomethacin, on the other hand, is a much better inhibitor of the cyclooxygenase than of the lipoxygenase pathway, and 10 μM indomethacin, a concentration 20 fold greater than the IC_{50} for inhibiting prostaglandin synthesis in *Aplysia* ganglia (Piomelli *et al*., 1987), had only a small effect on FMRFamide-activated currents (23 ± 8% inhibition, $n = 4$ cells, $P < 0.07$) and no significant effect on halothane-activated currents (2 ± 8% inhibition, $n = 5$ cells). These results are consistent with responses to FMRFamide being mediated mainly by lipoxygenase (rather than cyclooxygenase) metabolites of arachidonic acid, and with responses to halothane being mediated by neither lipoxygenase nor cyclooxygenase metabolites of arachidonic acid.

Clotrimazole and econazole are imidazole anti-fungal agents which inhibit cytochrome P450 pathways (Alvarez *et al*., 1992). Figure 10a shows that 1 μM clotrimazole reversibly inhibits $I_{K(An)}$ (by about 50%) as well as a basal outward current. This latter current appeared to be related to $I_{K(An)}$, since the clotrimazole-inhibited basal current reversed at the same potential as $I_{K(An)}$, and its magnitude correlated with that of $I_{K(An)}$ (data not shown). The slow time course of clotrimazole inhibition compared to that for halothane activation is striking and may indicate that halothane acts directly on the $I_{K(An)}$ channel; alternatively, it may simply reflect a much lower cellular permeability of clotrimazole and a subsequent slow access to intracellular sites. Figure 10b gives the clotrimazole concentration-response data for $I_{K(An)}$, fit to Eqn. 2 with $IC_{50} = 1.2 \pm 0.1$ μM clotrimazole and $n_H = 0.93 \pm 0.09$. Similarly to clotrimazole, 1 μM econazole blocked 51 ± 5% ($n = 4$ cells) of the 1.4 mM halothane-activated $I_{K(An)}$. Inhibition of $I_{K(An)}$ was independent of voltage, in the range from about −75 to −25 mV, for both clotrimazole ($n = 5$ cells) and econazole ($n = 2$ cells). A few experiments studied the inhibition of the 10 μM FMRFamide response by 1 μM clotrimazole, which was found to be 47 ± 8% ($n = 3$ cells), comparable to the inhibition found for $I_{K(An)}$ (see Figure 10b).

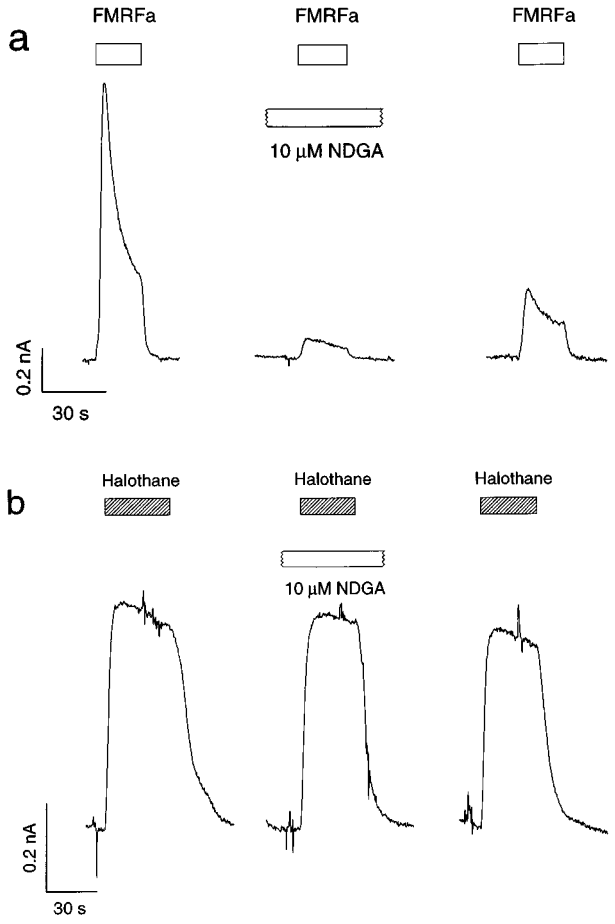

Figure 9 Inhibition of (a) the FMRFamide-activated response and (b) lack of inhibition of the halothane-activated response by the cyclooxygenase and lipoxygenase inhibitor NDGA. Concentrations were 10 μM NDGA, 50 μM FMRFamide and 1.4 mM halothane. Typical current traces are shown for two cells, pre-exposed to NDGA for 5 min (FMRFamide response) and 3 min (halothane response). NDGA inhibition of the FMRFamide-activated current reversed only partially after a 12 min washout. The average inhibition of the FMRFamide-activated currents was $76 \pm 8\%$ ($n = 3$ cells), but the corresponding inhibition of the halothane-activated currents was only $3.0 \pm 1.2\%$ ($n = 4$ cells).

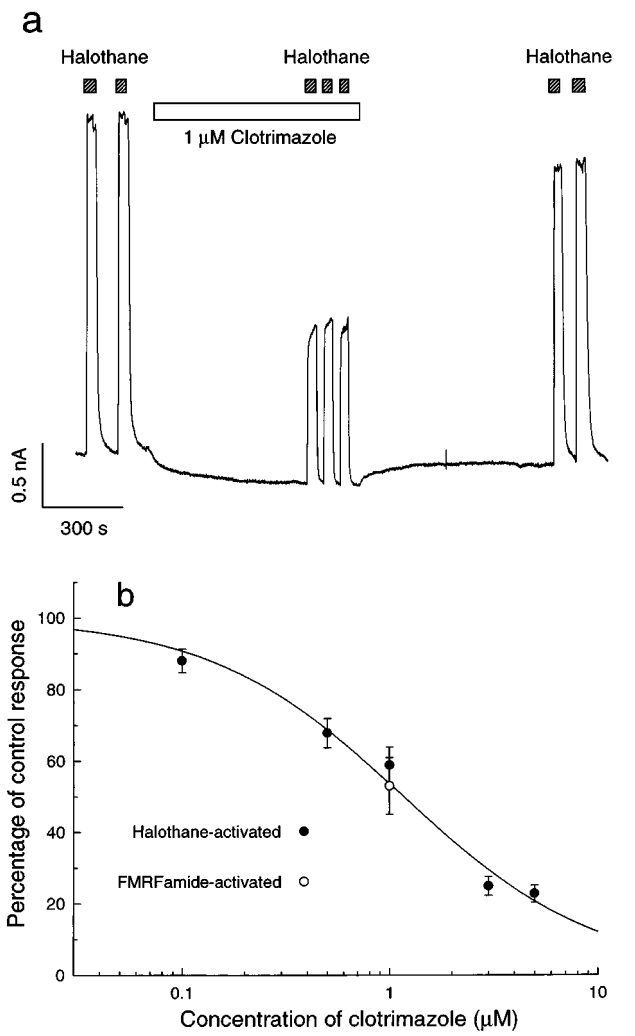

Figure 10 Inhibition of $I_{K(An)}$ by clotrimazole. (a) Clotrimazole (1 μM) blocks about half of the current activated by 1.4 mM halothane. The trace is a continuous record from a single neurone. (b) Clotrimazole concentration-response data for inhibition of 1.4 mM halothane-activated currents. Each filled-in circle is the mean $\pm$ s.e.mean for at least three cells. Cells were preincubated with clotrimazole for 10 min. The curve is the fit (of 23 individual data points) to Eqn. 2. The Hill parameters are $IC_{50} = 1.2 \pm 0.1$ μM clotrimazole and $n_H = 0.93 \pm 0.09$ (21 degrees of freedom). For comparison, a single open circle shows the effect of 1 μM clotrimazole on currents activated by 10 μM FMRFamide (mean $\pm$ s.e.mean for $n = 3$ cells).

Discussion

The volatile general anaesthetics: activation of $I_{K(An)}$

In the present study we have confirmed that $I_{K(An)}$, a molluscan anaesthetic-activated K^+ current, is reversibly switched on by a variety of volatile general anaesthetics (Franks & Lieb, 1988), but, surprisingly, with efficacies that vary by more than 4 fold, in the order halothane $\approx$ sevoflurane > isoflurane > enflurane $\approx$ chloroform (see Figure 2 and Table 1). For those volatile agents (halothane, isoflurane and enflurane) for which *Lymnaea* EC_{50} concentrations for 'general anaesthesia' have been determined, these concentrations compare closely with EC_{50} concentrations for activating $I_{K(An)}$ (see Table 1).

The n*-alcohols and cycloalcohols: inhibition of $I_{K(An)}$ and the cutoff effect*

In contrast to the volatile general anaesthetics, the *n*-alcohols larger than pentanol and two cycloalcohols (cyclohexanol and cyclooctanol) do not activate $I_{K(An)}$ on their own but instead inhibit halothane-activated $I_{K(An)}$. (*n*-Butanol both activates and inhibits $I_{K(An)}$, thus behaving in this respect both as a volatile agent and a larger *n*-alcohol.) Alcohol concentration-response experiments for inhibition of $I_{K(An)}$ yielded IC_{50} concentrations which were generally considerably higher than EC_{50} concentrations for tadpole general anaesthesia (see Table 2). (Alcohol data for molluscan general anaesthesia are not available). Thus both qualitative and quantitative arguments can be made against an important role for $I_{K(An)}$ in the general anaesthesia produced by these alcohols.

IC_{50} concentrations for *n*-alcohol inhibition of halothane-activated $I_{K(An)}$ decrease with increasing chain-length from hexanol to decanol, thereafter remaining constant from decanol to dodecanol (see Table 2 and Figure 4). This forms the basis of the well-known cutoff effect (Franks & Lieb, 1985; Alifimoff *et al*., 1989). At the molecular level, the levelling off of IC_{50} after nonanol (Figure 11) is consistent with the *n*-alcohols binding to a circumscribed water-facing

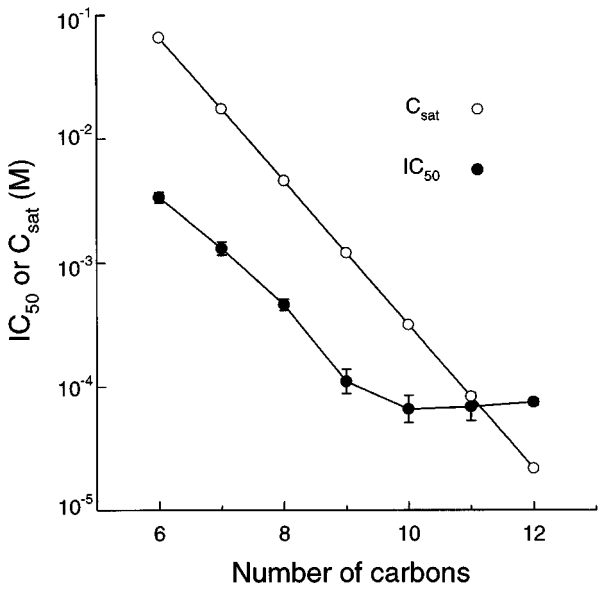

Figure 11 The cutoff effect for inhibition of $I_{K(An)}$ by the homologous series of *n*-alcohols. IC_{50} concentrations from Table 2 are plotted on a semilogarithmic scale against the number of carbon atoms in the *n*-alcohols. Values for the aqueous solubilities (C_{sat}), together with the straight line, are from Bell (1973) and sources referenced therein.

hydrophobic protein pocket with a volume roughly equal to that of *n*-decanol. Larger *n*-alcohols would then fill the pocket and extend into aqueous solution, gaining no additional free energy of binding (Franks & Lieb, 1985). Further evidence for this view comes from a consideration of the Hill coefficients n_H (see Table 2 and inset to Figure 4). These are greater than unity for the smaller *n*-alcohols studied (hexanol, heptanol and octanol) but not significantly different from unity for the larger *n*-alcohols (nonanol, decanol and undecanol), consistent with binding of only one molecule of the larger but more than one molecule of the smaller alcohols.

Do the alcohols inhibit $I_{K(An)}$ by binding to the same sites as do the volatile general anaesthetics, which activate $I_{K(An)}$? Just as the volatile agents differ in their efficacies while presumably binding to the same sites, it could be that the alcohols bind nonproductively (with efficacies of zero) and competitively inhibit binding of volatile anaesthetics. However, Figure 5 shows that the inhibitory *n*-hexanol concentration-response curves are dose-dependently shifted to the left by increasing concentrations of halothane, suggesting that halothane and hexanol mutually enhance the binding of each other, possibly by an allosteric mechanism. It thus appears that alcohols inhibit $I_{K(An)}$ by binding to different sites than those to which volatile general anaesthetics bind to activate $I_{K(An)}$.

Effects of inhalational and alcohol general anaesthetics on FMRFamide-activated K^+ currents

FMRFamide, an endogenous peptide present in *Lymnaea*, activates a K^+ current $I_{K(FMRFa)}$ in a concentration-dependent manner (Figure 6). This current exhibits similarities to $I_{K(An)}$ in its sensitivities to TEA^+ (Figure 7) and 4-AP, suggesting that the two K^+ currents reflect activation of the same or very similar K^+ channels. This interpretation is supported by the results of a recent study by Winegar *et al.* (1996), who found that in *Aplysia* neurones both halothane and FMRFamide can activate a K^+ channel having almost the same single-channel conductance and outwardly rectifying properties as a serotonin-inhibited and FMRFamide-activated K^+ channel (the S-channel) previously described in *Aplysia* (Siegelbaum *et al.*, 1982; Belardetti *et al.*, 1987). Unlike $I_{K(An)}$, however, the *Lymnaea* FMRFamide peptide-activated current exhibits desensitization even at quite low agonist concentrations.

Interestingly, this desensitization of the FMRFamide response is abolished by halothane (see Figure 8), perhaps due to an effect on the FMRFamide receptor or its associated G-proteins (Volterra & Siegelbaum, 1988). In addition, at low levels of FMRFamide, the FMRFamide response is potentiated by low levels of halothane. This synergism between FMRFamide and halothane responses is consistent with these two activators acting at different sites, perhaps on different molecules. Finally, *n*-hexanol, which inhibits $I_{K(An)}$, was found to inhibit FMRFamide responses with the same potency, suggesting common or similar inhibitory sites for alcohols acting on the $I_{K(An)}$ and FMRFamide pathways.

A role for metabolites of AA in the activation of $I_{K(An)}$ and $I_{K(FMRFa)}$?

A good case has been made for the positive involvement of lipoxygenase, but not cyclooxygenase, metabolites of arachidonic acid in the FMRFamide activation of a K^+ channel in *Aplysia* (Piomelli *et al.*, 1987) and *Helisoma* (Bahls *et al.*, 1992) neurones. Our *Lymnaea* results with the phospholipase A_2 inhibitor aristolochic acid, the combined lipoxygenase and cyclooxygenase inhibitor NDGA (Figure 9), and the cyclooxygenase inhibitor indomethacin support this view for the FMRFamide-activated K^+ current $I_{K(FMRFa)}$ but not for $I_{K(An)}$, which is insensitive to both lipoxygenase and cyclooxygenase inhibitors.

Nonetheless, inhibition of $I_{K(An)}$ by the phospholipase A_2 inhibitor aristolochic acid suggests a role for arachidonic acid metabolites in the activation of $I_{K(An)}$. This led us to look at inhibitors of a third pathway of arachidonic acid metabolism: the cytochrome P450-dependent monooxygenase pathway. The cytochrome P450s are products of a vast gene superfamily; cDNAs for over 300 cytochrome P450s have been cloned and sequenced (Estabrook, 1996).

We found potent block ($IC_{50} \approx 1$ μM) of $I_{K(An)}$ by two P450 inhibitors, clotrimazole and econazole. Clotrimazole can block at least 75% of $I_{K(An)}$ and also blocks part of a basal current present in the absence of volatile anaesthetics (Figure 10). The size of the clotrimazole-sensitive basal current was found to correlate with that of $I_{K(An)}$, consistent with volatile anaesthetics potentiating an endogenous current already partially activated in the absence of anaesthetics. Clotrimazole and econazole have also been found to potently inhibit Ca^{2+} activated K^+ channels in human red cells, where cytochrome P450 has not been detected, and it has been suggested that they might act on closely related hemeproteins (Alvarez *et al.*, 1992). Alternatively, clotrimazole and econazole may act directly on K^+ channels. However, we found no voltage dependency for the block of $I_{K(An)}$ by either clotrimazole or econazole (both weak bases), as might be expected if they were open-channel blockers. Nonetheless, it should be borne in mind that these inhibitors are not necessarily specific for cytochrome P450.

Clotrimazole was also found to inhibit, with equal potency, the FMRFamide response, reminiscent of the finding of Belardetti *et al.* (1989) that the related P450 inhibitor proadifen inhibits the activities of *Aplysia* S-channels activated

by FMRFamide, AA and 12-HPETE (12-hydroperoxyeicosatetraenoic acid), a lipoxygenase metabolite of AA. Furthermore, in cell-free patches hematin increased S-channel opening in the presence of 12-HPETE. These investigators concluded that a heme-containing enzyme, possibly cytochrome P450, is required to transform the lipoxygenase AA metabolite 12-HPETE into a form which directly activates the S-channel. Thus cytochrome P450s or related hemeproteins appear to play a role in the activation of $I_{K(An)}$ and $I_{K(FMRFa)}$.

Molecular basis of activation and inhibition of $I_{K(An)}$ by general anaesthetics

How can one account for our observation that volatile anaesthetics activate but large alcohol general anaesthetics inhibit $I_{K(An)}$? The simplest explanation is that both classes of general anaesthetics bind directly to separate excitatory and inhibitory sites on the $I_{K(An)}$ channel protein, with the inhalational agents being most potent at the excitatory sites and the alcohols being most potent at the inhibitory sites. Such an explanation has been suggested for the observed (Jenkins *et al*., 1996) potentiation and inhibition of the 5-HT_3 receptor in mouse neuroblastoma cells by volatile agents and alcohols, respectively. The 'bounce-back' effect observed at very high concentrations of halothane (see Figure 2) might be explained by binding of volatile anaesthetics to inhibitory sites, and this in turn might account for the different apparent efficacies of the inhalation agents for activating $I_{K(An)}$ (see Table 1).

On the other hand, we found that $I_{K(An)}$ is potently inhibited by agents that inhibit cytochrome P450 pathways. This suggests that general anaesthetics may be acting at excitatory and inhibitory sites on certain cytochrome P450s or related hemeproteins that produce metabolites (of an endogenous substrate such as AA) that activate $I_{K(An)}$. A consistent story emerges if volatile anaesthetics enhance but large alcohols inhibit activities of the relevant cytochrome P450s (in addition to direct effects on the K^+ channels). Interestingly, it has been found that the cytochrome P450-mediated aniline hydroxylase activity of rat liver microsomes is stimulated by a number of volatile anaesthetics (Van Dyke & Rikans, 1970; LaBella & Queen, 1995) but inhibited by *n*-alcohols (LaBella & Queen, 1995).

Implications for mechanisms of general anaesthesia

The channels underlying $I_{K(An)}$ are closely related if not identical to the so-called 'background' (Siegelbaum *et al*., 1982) or 'baseline' (Winegar *et al*., 1996) K^+ channels found in *Aplysia* neurones. They also have properties in common with members of a recently discovered family of two-pore-domain 'leak' K^+ channels (Goldstein *et al*., 1996). A recent study (Gray *et al*., 1998) has shown that one of these two-pore-domain K^+ channels is opened by volatile agents, while the mRNAs of two others have been found to be abundantly expressed in mouse brain (Fink *et al*., 1996; Lesage *et al*., 1997). Thus volatile anaesthetic-activated currents analogous to $I_{K(An)}$ may turn out to be widespread in nature.

Anaesthetic activation of background K^+ currents analogous to $I_{K(An)}$ would tend to reduce neuronal activity both by hyperpolarizing neurones and by increasing their membrane conductances. Such behaviour need not be limited to neurones. For example, metabolites of AA, and cytochrome P450 monooxygenases in particular, are prime candidates for the elusive endothelium-dependent hyperpolarizing factor (EDHF), which is released from endothelium in response to shear stress or ligands acting on endothelial receptors (Mombouli & Vanhoutte, 1997), and it is tempting to suppose that anaesthetic activation of K^+ currents such as $I_{K(An)}$ might contribute to the peripheral vasodilation and subsequent hypotension that often accompanies the general anaesthesia produced with volatile agents.

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