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The Enhancement of Proton/Hydroxyl Flow across Lipid Vesicles by Inhalation Anesthetics

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When *p*H gradients are created across model lipid membranes, there is a well-documented electrogenic flow of protons, which results in the development of a transmembrane potential. As a result, protons come to electrochemical equilibrium across lipid vesicles within tens of minutes. The ability of a series of inhalation anesthetics to enhance the conduction of protons across model membranes was examined. When clinically relevant concentrations of halothane, isoflurane, enflurane, or chloroform were equilibrated with sonicated lipid vesicles, significant increases in the proton conduction were found. However, for even high anesthetic concentrations (above 8 MAC) of ether and cyclopropane, no increases in proton conduction could be detected. These results rule out a common mechanism for general anesthesia involving enhanced proton conduction across membranes. (Key words: Anesthetics, gases: cyclopropane. Anesthetics, volatile: chloroform; enflurane; halothane; isoflurane. Membrane; potentials; proton permeability; spin labels. Theories of anesthesia.)

LIPID BILAYERS present a large energy barrier to the transmembrane movement of simple inorganic ions. As a result, the half-time for leakage of Na^+ and K^+ from model membranes, composed only of lipid, is on the order of weeks.[1,2] By comparison, the permeability of the membrane to protons or hydroxide ions (H^+/OH^- ions) is approximately 10^6 to 10^7 times larger than that for Na^+ or K^+. When *p*H gradients are established across lipid vesicles, membrane potentials develop that come to equilibrium with the *p*H gradient in tens of minutes. In recent years there has been considerable interest in this high permeability to H^+/OH^- ions. It is not clear what role (if any) this proton current has in biologic systems. It is not large enough to deplete proton gradients in membranes containing active proton pumps, but the leakage does contribute to the overall current of protons and is substantially greater than the background current for either Na^+ or K^+. The mechanism leading to this anomalously large passive transport of protons or hydroxide ions is currently under investigation.[3,4]

A number of agents are observed to enhance the passive transport of H^+/OH^- ions across membranes. The anesthetics chloroform or halothane, when added to lipid vesicle suspensions, increase the transmembrane current of H^+/OH^- ions.[5–8] Bangham and Mason suggested that this enhanced proton current might be the mode of action of general anesthetics.[5] By increasing H^+/OH^- flow across synaptic vesicles, anesthetics could deplete vesicle *p*H gradients. The storage of many neurotransmitters is driven by the transmembrane electrochemical potential of protons, and depleting the *p*H gradient would result in a loss of synaptic transmitter. Presently, the mechanism by which compounds such as halothane and chloroform increase the H^+/OH^- current is not understood. In addition, quantitative effects on the H^+/OH^- permeability for a range of general anesthetics are not available.

In the present article the effect of six general anesthetics on H^+/OH^- currents in lipid vesicles is measured. Two different approaches using spin resonance probes are taken to make this measurement. In the first approach the development of a transmembrane potential, $\Delta\psi$, which accompanies the establishment of a *p*H gradient, ΔpH, is measured. The H^+/OH^- current is then determined from the time dependence of $\Delta\psi$. In the second approach a time-dependent decay of ΔpH occurs if the buffer concentrations are low. This time-dependent decay of ΔpH is measured and then used to quantitate the H^+/OH^- current.[6] The phosphonium nitroxide, I, and alkylamine nitroxide, II, shown in figure 1, are used to quantitate $\Delta\psi$ or ΔpH, respectively. These probes are monitored using electron paramagnetic resonance (EPR) spectroscopy and are particularly well suited for the measurements made here.

The anesthetics tested could be divided clearly into two groups. One group enhanced the permeability of the membrane to H^+/OH^- ions at clinically significant concentrations, and another had little effect on the H^+/OH^- permeability even at high concentrations. This observation is discussed in terms of a model for general anesthesia involving H^+/OH^- flow.

Materials and Methods

The alkylphosphonium nitroxide (I) and the secondary amine label N-tempoyl-N-hexylamine (II) were synthesized as previously described.[9,10] Phosphatidylcholine (PC) from egg yolks was purified by column chromatography according to the procedure of Singleton *et al.*[11] and stored in chloroform under an argon atmosphere at −20° C. Halothane containing 0.01% thymol was obtained from Halocarbon Industries (Hackensack, New Jersey); enflurane and isoflurane from Anaquest (Madison, Wisconsin);

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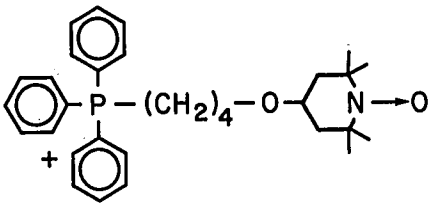
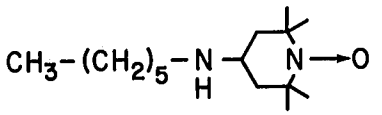


I



II

FIG. 1. Spin-labeled probes used in the present study. The phosphonium, I, is used to measure the transmembrane electrical potential, $\Delta\psi$, and the alkylamine, II, is used to measure the transmembrane pH gradient, ΔpH.

HPLC grade chloroform containing 0.075% ethanol from J. T. Baker (Phillipsburg, New Jersey); analytical grade anhydrous diethylether (free of peroxides) from Mallinckrodt (Paris, Kentucky); and cyclopropane from Aldrich Chemical Co. (Milwaukee, Wisconsin).

Formation of Lipid Vesicles

To prepare vesicles from egg PC, an aliquot of lipid in chloroform was dried under a stream of nitrogen and placed under vacuum (10^{-4} mmHg) for a minimum of 15 h. The lipid, which was free of any residual chloroform, was then dispersed in the appropriate buffer solution and sonicated as previously described.[12] This procedure for egg PC yields relatively uniform vesicles with a diameter of 300 ± 30 Å. The vesicles have an internal trapped volume of approximately 0.5 ml/g of phospholipid. All pH gradients were established across the vesicle membrane by diluting the vesicle suspension into the appropriate buffer solution. In some cases this was accomplished in the EPR cell using a pneumatically driven mixing system fitted to the EPR spectrometer. Unless otherwise noted, vesicles were sonicated in solutions containing a buffer of 2-(N-morpholino)-ethanesulfonic acid (MES) and diluted by a buffer of 3-{[tris-(hydroxymethyl)-methyl]amino} propanesulfonic acid (TAPS) to form the pH gradient. This produced vesicles with an internal pH of 6 and an external pH of 8. For the determination of electrogenic H^+/OH^- flow using the phosphonium, I, 100 mM buffer containing 125 mM Na_2SO_4 was used. For measurements of proton flux using probe II, 5 mM buffer containing 125 mM Na_2SO_4 was used.

Lipid concentrations of the final vesicle suspensions were determined by phosphate analysis using a modification of the procedure of Bartlett.[13]

Delivery of Anesthetics

Solutions containing egg PC vesicles and buffers were equilibrated with anesthetic using a system similar to that described by Young and Sigman.[14] In this system a carrier gas is passed through a sintered disk at the bottom of an anesthetic-filled vaporizer. The carrier gas, now saturated with anesthetic, is diluted with anesthetic-free gas to achieve the desired anesthetic concentration. This concentration is determined from the vapor pressure of the anesthetic, the ambient barometric pressure, and the flow rates of the carrier and diluent gases. Nitrogen was used in place of air to minimize lipid oxidation. Vesicle samples were equilibrated with anesthetic vapor for 15 min. This was judged to be adequate to achieve anesthetic equilibrium, and additional equilibration time did not change the H^+/OH^- permeability. The pH gradients were established as described above. In cases in which the solutions were transferred to a flat quartz EPR cell, anesthetic vapor, equal in concentration to that used during the equilibration step, was introduced into the dead volume of the EPR cell. The cell was sealed to avoid loss of anesthetic during the time course of the EPR measurements. Samples without anesthetic were equilibrated with 100% nitrogen.

EPR Measurements and Analysis of Spectra

EPR spectroscopy affords a number of advantages over other techniques, such as fluorescence spectroscopy, for these measurements. These EPR probes are highly quantitative, very sensitive, and not influenced by light scattering. The mechanisms by which they function are well understood, and they can be used at concentrations that are on the order of a few (or less) probe molecules per vesicle. The basis for the measurement is a $\Delta\psi$- or ΔpH-dependent partitioning of I or II, respectively. The partitioning of the probe, *i.e.*, the ratio of membrane bound to aqueous probe, is quantitated from its EPR spectrum. Hence, a measure of $\Delta\psi$ or ΔpH is obtained directly from the EPR spectrum.

Shown in figure 2 is an EPR spectrum of a phosphonium probe in solution without vesicles and a spectrum in the presence of vesicles. In the presence of vesicles the spectrum contains a contribution from both membrane-associated and aqueous probe populations. The spectrum associated with the membrane-bound probe is broad, and it makes a minimal contribution to the amplitude of the high-field resonance ($m_I = -1$). For this reason the amplitude of this signal provides a good measure of the population of aqueous probe. The partitioning of the probe, λ, or the ratio of membrane associated to aqueous probe is given by:

$$\lambda = \frac{A_f^\circ - A}{A - (\beta/\alpha)A_f^\circ} \quad (1)$$

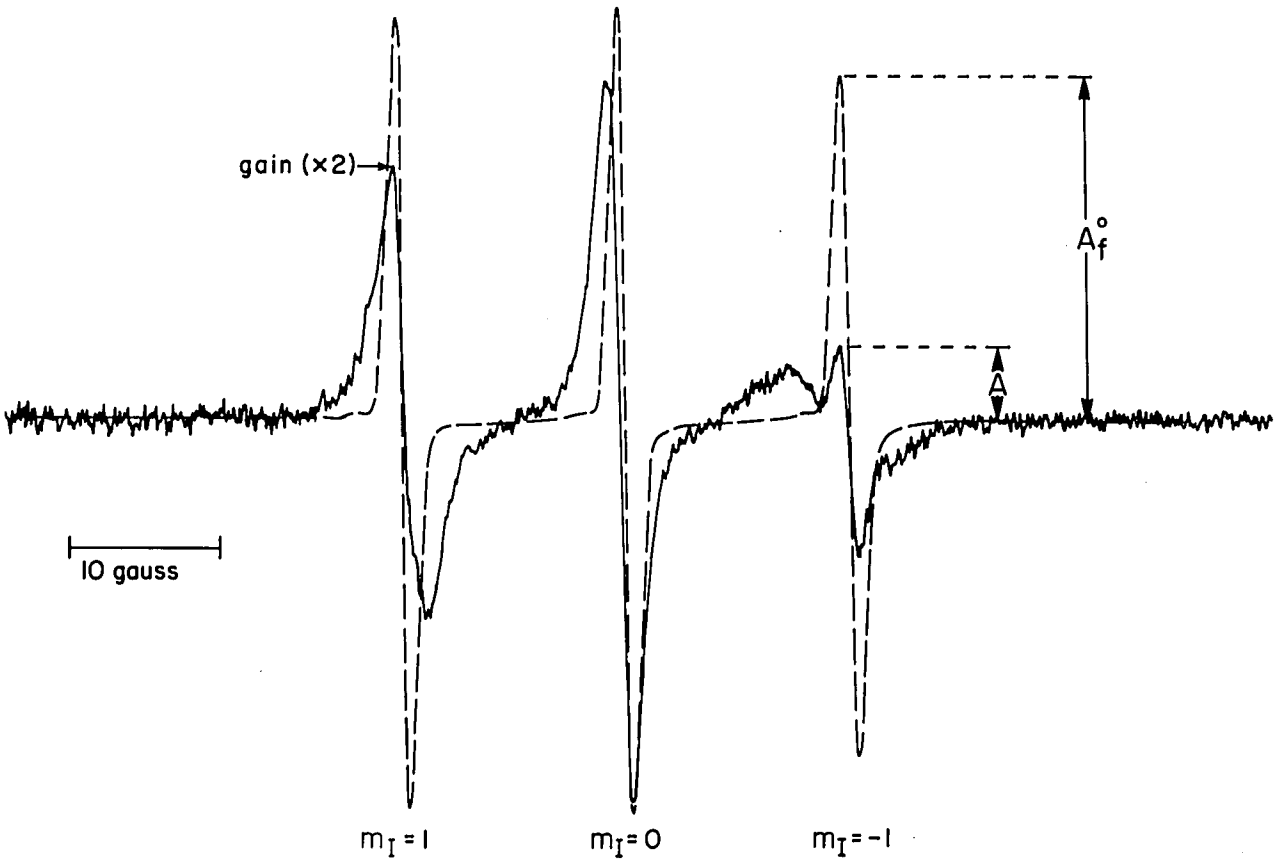


FIG. 2. An electron paramagnetic resonance (EPR) spectrum of the spin-labeled phosphonium I in the presence and absence of sonicated egg phosphatidylcholine vesicles. (——) and (– – –), respectively. The spin label is at a concentration of 20 μM and the vesicle suspension is at a concentration of approximately 20 mM lipid. In the presence of vesicles the spectrum is a sum of two components resulting from spin label in solution and spin label associated with the membrane. The phase partitioning of the probe is obtained from the amplitude of the high-field resonance, A, ($m_I = -1$), and the amplitude of this resonance in the absence of lipid A_f°.

Here, A is the amplitude of the high-field resonance of probes I or II (see fig. 2) and A_f° is the amplitude of the same quantity of probe in the absence of vesicles. The parameter β/α is a correction that takes into account the small contribution made by the bound probe to the amplitude of the high-field resonance, A. It is determined from the high-field resonance amplitude of totally free or totally bound probe and is approximately 0 for the probes used here. The time-dependent partitioning, $\lambda(t)$, can be obtained by setting the magnetic field of the EPR spectrometer to the high-field resonance and monitoring the amplitude, A, as a function of time.

As mentioned above, the partitioning, λ, is a function of $\Delta\psi$ or ΔpH for probes I or II, respectively. Probes I and II distribute across the vesicle as a function of the transmembrane potential or pH gradient. For example, if vesicles develop an inside negative transmembrane potential, the phosphonium probe I accumulates on the vesicle interior. Because of the large ratio of membrane surface to aqueous volume on the vesicle interior, the probe is entirely bound on the vesicle interior. By writing expressions for the chemical potential of the probe in each phase, the voltage (or pH) dependence of the probe partitioning (λ) can be determined. The physical basis for this dependence and the expressions for quantitating $\Delta\psi$ or ΔpH from λ are given in detail elsewhere.[10,15,16] All EPR measurements were made either on a Varian E-109 or a modified V-4500 spectrometer.

Determination of Proton Currents and Net Proton Permeability

Proton currents were determined in sonicated vesicles containing anesthetics using two different approaches described previously.[6] In the first approach an estimate of the H^+/OH^- current was made using the phosphonium probe I. When pH gradients are established under strongly buffered conditions a time-dependent trans-

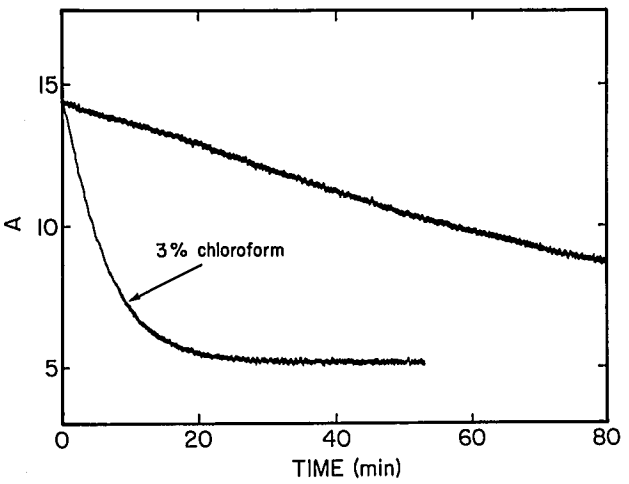


FIG. 3. High-field resonance ($m_I = -1$) amplitude of the spin-labeled phosphonium I following the creation of a pH gradient ($p\mathrm{H}_{in}$ = 6; $p\mathrm{H}_{out}$ = 8) under strongly buffered conditions in sonicated egg PC vesicles. The egg PC is at a concentration of approximately 30 mg/ml, the spin label is present at 30 μM, and 5 μM ϕ_4B^- is added to ensure that the phosphonium transmembrane migration is not rate-limiting. The decrease in the amplitude of this resonance results from the development of a transmembrane potential due to an electrogenic flux of H^+/OH^- ions. The time dependence is shown both with no additions and when the vesicles were equilibrated with a 3% chloroform vapor. The total signal intensity of the high-field resonance amplitude (A_f^o) with 30 μM spin probe is approximately 18.

membrane potential results from the current of H^+/OH^- ions. Again, this time-dependent potential is quantitated by measuring the phase partitioning of I. The initial H^+/OH^- current, i_o, is estimated from the initial slope of the time–voltage curve. The net H^+/OH^- permeability, P_{net}, is then readily calculated according to Equation 2[6]:

$$i_o = (\partial\Delta\psi/\partial t)_{\Delta\psi=0} \cdot c = FP_{net}([H^+]_{in} - [H^+]_{out}) \quad (2)$$

Here, we used a value of $\approx$0.9 μF/cm^2 for c, the membrane capacitance; $[H^+]_{in}$ and $[H^+]_{out}$ are values for the internal and external hydrogen ion concentrations, respectively; and F is Faraday's constant. These measurements were made in the presence of 0.5 to 2.0 μM tetraphenylborate, ϕ_4B^-. The presence of tetraphenylborate accelerates the transmembrane migration of probe I and ensures that its movement is not rate-limiting.[17]

In the second approach the alkylamine nitroxide II was used to measure time-dependent pH gradients. If pH gradients are established under weakly buffered conditions, the transmembrane flow of protons depletes the pH gradient, ΔpH. By measuring the change in the pH gradient, the net H^+/OH^- permeability can be calculated using Equation 3[6]:

$$P_{net} = \frac{B \cdot r_i^2 \cdot (\partial pH/\partial t)_{\Delta\psi=0}}{3r_o([H^+]_{in} - [H^+]_{out})} \quad (3)$$

Here, B is the buffer capacity and r_i and r_o are the internal and external vesicle radii, respectively. Again, the initial change in the pH gradient, $(\partial pH/\partial t)_{\Delta\psi=0}$, is determined from the time dependence of the partitioning of probe II as detailed previously.[6]

Results

Certain Inhalation Anesthetics Increase H^+/OH^- Currents

Several general anesthetics are observed to have a significant effect on the electrogenic H^+/OH^- flux at clinically relevant concentrations. Shown in figure 3 are tracings of the high-field resonance amplitude for the phosphonium I as a function of time following the creation of a pH gradient (inside acidic) in egg PC vesicles (the decay in the free signal amplitude of this label is due to formation of a transmembrane potential). In the presence or absence of anesthetic, protons come to electrochemical equilibrium, *i.e.*, the measured potential at equilibrium is in agreement with that expected from the magnitude of the proton gradient. The values estimated for P_{net} are similar to those obtained previously; from the data in figure 3 we estimate a value for P_{net} of $\approx 2 \times 10^{-7}$ (cm/s). When the vesicles are equilibrated with a 3% chloroform vapor [3.9 times the minimal alveolar concentration (MAC)], a tenfold increase in the H^+/OH^- current (and permeability) is measured. In almost all cases a membrane potential develops that is identical with that expected from a proton electrochemical equilibrium. This indicates that the membrane currents for the other ions present, such as Na^+ and $SO_4^=$, are not becoming large relative to the H^+/OH^- current. However, in the presence of isoflurane concentrations greater than approximately 2%, potentials do not come to equilibrium with the pH gradient and the vesicles depolarize within approximately 10 min. In this case the conductances of ions (other than H^+/OH^-) are becoming significant.

The H^+/OH^- permeability was also measured using the ΔpH sensitive probe II, under weakly buffered conditions, both in the presence and absence of anesthetics. The currents (and permeabilities) were identical within experimental error to those obtained using the phosphonium I. These results indicate that the phosphonium movement is not rate-limiting and that a significant electroneutral flow of protons does not occur in this system. If a significant electroneutral flow of protons had occurred, much higher proton permeabilities would be measured with probe II, and a stable potential in equilibrium with ΔpH would not have been observed using probe I.

Shown in figure 4 are plots of the fractional change in the electrogenic H^+/OH^- current *versus* the concentra-

tion (in units of the MAC for each anesthetic) for six different inhalation anesthetics. The ratio i'_o/i_o is the ratio of the current in the presence of anesthetic to the current in the absence of anesthetic. As seen in figure 4, inhalation anesthetics varied greatly in their effect on H^+/OH^- currents; for the anesthetics diethylether and cyclopropane, no significant effect on the value of the electrogenic H^+/OH^- current was observed.

Current Voltage Curve for H^+/OH^- Movement

In the absence of anesthetics, the H^+/OH^- flow is characterized by a highly linear current–voltage curve; this linearity is surprising and not expected for a simple diffusion process.[6] Simple diffusion processes are governed by the passage of the ion over an energy barrier that is centrally located in the membrane hydrocarbon. The currents for these processes can be described, to a first approximation, by an Erying rate analysis. In this case the rate for ion movement depends exponentially on the applied membrane potential so that the current increases in a superlinear fashion with applied voltage.[18,19] These current–voltage curves are of interest because they provide information about the shape and position of the energy barrier to ion transport. If the addition of anesthetics changed the shape of the energy barrier for H^+/OH^- movement (this could occur if the mechanism for H^+/OH^- permeation was dramatically different in the presence of chloroform), changes in the current–voltage curve might be observed. We examined the current–voltage curve for the H^+/OH^- current when vesicles were equilibrated with a 3% chloroform vapor. This curve is shown in figure 5. In the presence of this anesthetic (under conditions where the H^+/OH^- current is accelerated by approximately tenfold) the current–voltage curve remains linear.

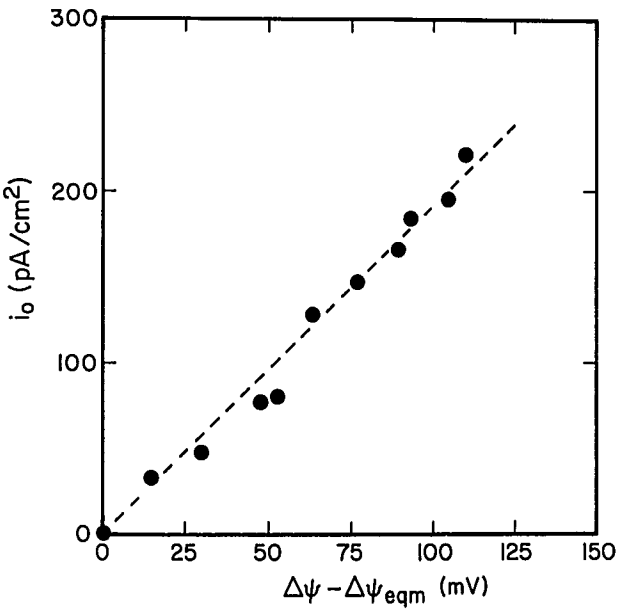


FIG. 5. A current–voltage curve obtained from the slope of the time–voltage curve for the data shown in figure 1. Here vesicles are equilibrated with a 3% chloroform vapor. The values for the currents are calculated from $\partial\Delta\psi/\partial t$ assuming a specific membrane capacitance of 0.9 $\mu F/cm^2$. The dashed line represents a constant integral membrane resistance of $5 \times 10^8\ \Omega\ cm^2$.

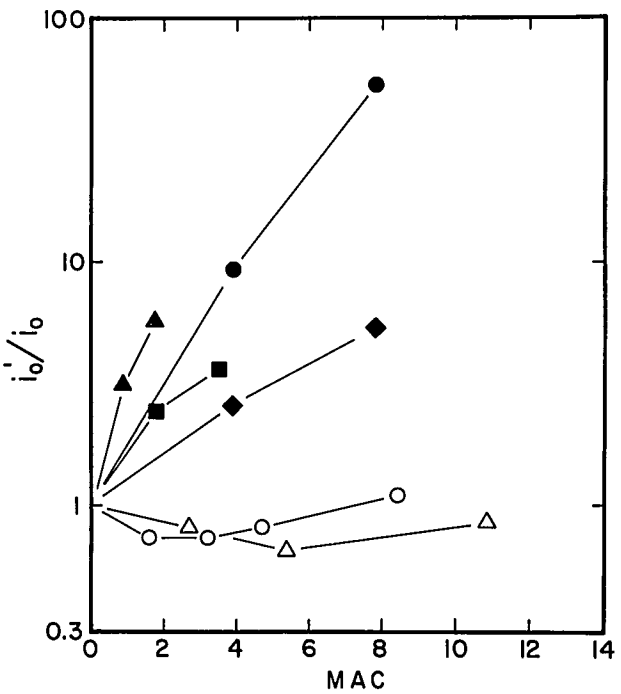


FIG. 4. Ratio of the H^+/OH^- current in the presence and absence of anesthetic when egg PC vesicles are equilibrated with (▲) isoflurane, (●) chloroform, (■) enflurane, (♦) halothane, (○) ether, or (△) cyclopropane. i'_o and i_o are the H^+/OH^- currents in the presence and absence of anesthetic, respectively. The MAC values for the six anesthetics shown were obtained from Firestone *et al.*[26] and are all for humans, except that for chloroform, which is for dog.

Discussion

The results obtained here clearly demonstrate that certain anesthetics increase the conductivity of H^+/OH^- ions across model membranes. These results were not unexpected and appear to be qualitatively consistent with previous studies in which proton permeability increases were promoted by inhalation anesthetics.[5–8] Here, dramatic quantitative differences between the action of inhalation anesthetics on the H^+/OH^- currents in lipid vesicles are found. The data shown in figure 4 clearly demonstrate that at equivalent MAC multiples, a range of inhalation anesthetics has dramatically different effects

on the H^+/OH^- permeability. The effects on H^+/OH^- currents are poorly related to the membrane/buffer partitioning of the anesthetics, and for diethylether and cyclopropane no significant effect on the H^+/OH^- current is observed even at high membrane concentrations.

Mechanisms of Anesthesia

The enhancement of the membrane proton permeability by general anesthetics has been suggested as a possible mechanism for general anesthesia.[5] Using the catecholamine granule as a model for neurotransmitter storage, Bangham and Mason[5] demonstrated that some general anesthetics increase the proton conductance across these granules. This leads to a reduction in the transmembrane *p*H gradient and a depletion of the neurotransmitter from the storage vesicle (in these systems the free energy driving the accumulation of neurotransmitter is obtained from the *p*H gradient).[20-23] The data shown here strongly argue against the enhancement of H^+/OH^- currents as a single mechanism for the action of general anesthetics. Cyclopropane and ether at concentrations that promote anesthesia do not measurably affect proton permeability.

These data do not exclude the possibility that enhancement of the H^+/OH^- current is responsible for other effects of these anesthetics. The mechanism proposed by Bangham and Mason may provide an explanation for other effects of anesthetics. For example, it is conceivable that this increase in H^+/OH^- conduction leads to a depletion of catecholamines from presynaptic storage vesicles and accounts, in part, for the circulatory depression seen with halothane, enflurane, isoflurane, and chloroform.[24]

Molecular Mechanisms for the Enhancement of H^+/OH^- Currents

The mechanisms by which H^+/OH^- ions are conducted across pure lipid bilayers are currently not understood. Previous work indicates that this flow is not the result of simple diffusion processes (*e.g.*, a permeation of H^+, H_3O^+, or OH^-) and is not the result of carriers.[3,4] Structures such as hydrogen-bonded networks of water have been proposed and appear to account for some of the available data.[4,25] It is not clear how anesthetics act to accelerate this transport. As shown in figure 5, the current–voltage curve for H^+/OH^- conduction remains linear in the presence of chloroform. This suggests that the mechanism behind this current flow has not changed. However, this linear current–voltage relationship does not allow a unique energy barrier to be defined,[6] and a new transport mechanism that also results in a linear curve is a possibility.

The enhancement in H^+/OH^- conductivity by these inhalation anesthetics does not appear to be simply correlated with the dielectric constant of the anesthetics. At equivalent membrane concentrations, chloroform and ether have dramatically different effects on the H^+/OH^- current: at concentrations in which chloroform results in a 50-fold increase in H^+/OH^- current, no effect by ether is detected. Their dielectric constants are 4.8 and 4.3, respectively, and are much greater than that of the membrane hydrocarbon. We could account for this behavior in terms of dielectric constant changes only if chloroform and ether were distributed very differently in the bilayer.

Halothane, chloroform, enflurane, and isoflurane all contain at least one hydrogen that is acidic. This molecular feature distinguishes them from anesthetics that do not enhance electrogenic H^+/OH^- fluxes (ether and cyclopropane). The role that this structural feature plays in the induction of H^+/OH^- currents is not known and is currently under investigation.

In conclusion, the effects of a range of inhalation anesthetics on the H^+/OH^- current in phospholipid vesicle systems were quantitated. Halothane, enflurane, isoflurane, and chloroform enhanced the H^+/OH^- current in a dose-related manner, whereas ether and cyclopropane did not. These results argue against increases in the H^+/OH^- current as a mechanism for general anesthesia, but they do not rule out the participation of H^+/OH^- permeability changes in other effects of inhalation anesthetics.

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