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Steady-State Catecholamine Distribution in Chromaffin Granule Preparations: A Test of the Pump-Leak Hypothesis of General Anesthesia†

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ABSTRACT: The molecular mechanism of general anesthesia is not understood. Possible modes of action include binding at a protein site, such as a receptor or channel, or physical effects on membrane lipid properties. The pump-leak hypothesis suggests that anesthetics perturb the bilayer of synaptic vesicles, thereby increasing ionic permeability. This results in decay of proton gradients required for transport and accumulation of neurotransmitters. The subsequent loss of neurotransmitters from synaptic vesicles reduces the efficiency of synaptic transmission and results in the anesthetized state. We have determined the effects of general anesthetics on certain parameters of enzyme activity and membrane permeability relevant to the pump-leak hypothesis. We used chromaffin granules as a convenient model system and focused on clinically relevant anesthetic concentrations (ED_{50}), quantitative measurements of permeability changes, and the kinetics of gradient decay. General anesthetics at ED_{50} have little or no effect on the proton-transport ATPase activity, but do cause modest increments in proton permeability that change the catecholamine distribution in actively pumping chromaffin granule preparations. We found that pH gradients do not collapse entirely under these conditions and that only a fraction of total catecholamine is lost from the chromaffin granules. When total collapse is induced by other means, efflux of catecholamines occurs with a half-time near 30 min. These results suggest that if the pump-leak hypothesis is valid, then very small losses of catecholamines must be sufficient to induce anesthesia. We conclude that the weight of evidence favors other mechanisms, notably direct binding of anesthetics to sensitive proteins.

General anesthesia is induced by compounds as diverse as xenon, nitrous oxide, halothane, and normal alcohols up to 12 carbons in length. The effect involves partitioning of the anesthetic agent into membranes of neurons, followed by inhibition of axonal and synaptic transmission. The latter is considerably more sensitive to anesthetics (Richards, 1983), and synaptic junctions presumably represent the primary site of action.

Bangham and Mason (1980) first postulated that anesthetics inhibit synaptic transmission by causing release of catecholamines from synaptic vesicles into the nerve terminal cytosol. This "pump-leak hypothesis" is based on the observation that anesthetics increase ion permeability of lipid bilayers (Bangham et al., 1965; Johnson et al., 1973; Pang et al., 1979). Thus, when anesthetic compounds partition into synaptic

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vesicle membranes, the H^+/OH^- permeability would be expected to increase, causing the transmembrane pH gradient to decay. Accumulation and retention of catecholamine neurotransmitters in secretory vesicles is dependent upon a transmembrane proton potential (Johnson, 1988). It follows that any reduction in the pH gradient caused by an anesthetic-induced leak would cause catecholamines to diffuse from the vesicle interior into the cytosol. If a substantial leak occurred, a major fraction of the neurotransmitter might be expected to be lost from synaptic vesicles and the efficiency of synaptic transmission would be reduced, leading to the state of anesthesia.

Previous studies (Bangham & Mason, 1980; Barchfeld & Deamer, 1985, 1988) confirmed that general anesthetics increase the proton permeability of liposome bilayer membranes and cause release of accumulated dopamine from both liposomes and isolated synaptic vesicles. However, other evidence weighs against the pump-leak hypothesis. For instance, permeability increments at clinical levels of anesthetics are difficult to detect in liposome model systems. It might also be expected that synaptic vesicles would be less sensitive to permeabilizing effects of anesthetics than are liposomes because passive proton flux in biological membranes is 1–3 orders of magnitude greater than that of lipid bilayers (Deamer & Bramhall, 1986). Furthermore, the proton gradient across synaptic vesicle membranes is maintained by a proton-transport ATPase, which might accommodate modest increments in proton permeability by increasing the rate of active uptake. Finally, pH gradients with buffer capacities in the physiological range have half-times of decay measured in minutes, yet anesthetic effects on whole organisms can be observed in a minute or less. If the pump-leak mechanism is correct, significant decay of pH gradients and loss of neurotransmitters should occur in time intervals comparable to those of anesthetic action.

To provide a more quantitative basis for this discussion, we have investigated anesthetic effects on a relevant biological membrane. We chose to use chromaffin granules, the secretory vesicles of chromaffin cells in the adrenal medulla. These cells are derived from the same precursor cells as adrenergic neurons (Weston, 1970). Synaptic vesicles would be the model of choice, but in our hands the standard preparations are heterogeneous and unstable. In contrast to synaptic vesicles, chromaffin granules can be isolated in large quantities and are relatively stable.

The research reported here addressed the following questions:

(1) What is the intrinsic proton permeability of a model synaptic vesicle (the chromaffin granule)? The pump-leak hypothesis requires that the proton permeability must be relatively high as is true in other biological membranes (Deamer & Bramhall, 1986). One previous study inferred an extremely low proton permeability of the granule membrane (Johnson, 1988).

(2) Can ED_{50} levels of anesthetics cause measurable increments in proton permeability of the chromaffin granule membrane?

(3) What effect do general anesthetics have on the proton-transport ATPase of the membrane? It is important to know whether the ATPase can respond to an anesthetic-induced leak by increasing ATP hydrolysis rates. Alternatively, if anesthetics inhibit the enzyme, their effect on the pH gradient would be considerably enhanced.

(4) Is the induced proton leak sufficiently large to cause rapid loss of catecholamines from secretory vesicles? For the pump-leak mechanism to work, a physiologically significant quantity of catecholamine must diffuse into the neuron cytoplasm in the time it takes for anesthetics to induce anesthesia, typically 1 min or less.

Our results indicate that general anesthetics at clinical levels cause modest increments in proton and catecholamine efflux from chromaffin granules that can be correlated with known effects of general anesthetics in whole organisms. However, the small amount of catecholamine lost from the granules and the relatively slow rate of release are not consistent with the hypothesis that anesthesia is caused by a major loss of neurotransmitters from synaptic vesicles. The remaining alternatives are that minor leaks are sufficient or that anesthetics directly affect a protein site, as suggested by Franks and Lieb (1982, 1984, 1988).

Materials and Methods

Isolation of Chromaffin Granules. Bovine adrenal glands were acquired from a local slaughterhouse within 30 min after death. The tissue was immediately placed on ice and transported to our laboratory, requiring an additional 30 min of transit time. Chromaffin granules were isolated from the adrenal glands by the method of Carty et al. (1980), taking care to conduct all steps at 1–5 °C. The adrenal medullae were dissected, minced, and homogenized in 0.27 M sucrose and 10 mM Tris/maleate buffer at pH 7.0 by using a glass–Teflon Potter-Elvehjem homogenizer at 1000 rpm. The suspension was then centrifuged at 1250*g* for 5 min. The pellet was discarded, and the supernatant was recentrifuged at 8650*g* for 35 min. The pellet was gently rinsed with 1 mL of buffer to remove a thin film of contaminants, and the remainder was resuspended in 4 mL of buffer. This crude granule fraction was divided and layered onto two 35-mL columns of a 30% (v/v) Percoll, 0.27 M sucrose, and 10 mM Tris/maleate solution at pH 7.0 that had been pretreated by centrifugation at 20200*g* for 5 min. The chromaffin granules formed bands at the bottom of the column, which were removed by aspiration with a Pasteur pipet and washed by dilution (1:10 weight basis) in a 1 mM, pH 7.0, TES[1]/Tris buffer that contained 0.27 M sucrose. The washed suspension was centrifuged at 8650*g* for 15 min, and the purified chromaffin granule pellet was resuspended in the same buffer to a final granule protein content of approximately 20 mg mL^{-1} assayed according to the method of Lowry et al. (1951). This suspension was stored on ice and used up to 72 h after isolation. Alternatively, 0.1-mL aliquots of the granule stock were rapidly frozen in liquid nitrogen and stored at −80 °C prior to use.

Measurement of Proton Flux across the Chromaffin Granule Membrane. The external buffer used throughout this assay was 0.5 mM TES/Tris (pH 8.2) that contained 0.225 M sucrose and 0.015 M K_2SO_4. A relatively low buffer concentration was used so that proton uptake and release could be followed with a recording pH meter. Anesthetics were added to the reaction mixture from saturated buffer solutions that, upon partitioning, gave the desired membrane concentration in moles per liter. The membrane–buffer partition coefficients used to calculate the aqueous anesthetic concentrations required for a given membrane concentration are from Roth and Miller (1984). In a typical *n*-alcohol experiment, 2.46 mL of the external buffer (±alcohol) was added to a glass vessel held at 25 ± 1 °C by a circulating water bath. A combination pH electrode was inserted into the rapidly stirred

[1] Abbreviations: ACES, *N*-(carbamoylmethyl)-2-aminoethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; MES, 2-(*N*-morpholino)ethanesulfonic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; 9AA, 9-aminoacridine.

solution, and a stable reading was achieved within 1–2 min (recorded by a Fisher Acument recording pH meter). At that point, 40 μL of fresh chromaffin granule stock was added to the vessel to give a granule protein content of 0.25 mg/mL buffer. After 1.5 min, 0.5 μM valinomycin (final concentration) was added to the buffer to eliminate diffusion potentials generated by net proton efflux from the granule interior (pH 5.5) to the external medium (pH 8.0). Intrinsic proton flux across the granule membrane was estimated from a tangent to the line at the point of valinomycin addition. Changes in pH of the external buffer due to dissolution of CO_2 or leakage of electrolyte solution from the reference electrode were negligible over the 6-min course of each experiment.

Assays of proton flux in the presence of volatile anesthetics followed the same protocol except that the reaction vessels were sealed with zero air space and the temperature was held at 37 °C. For the purposes of this study, "clinical concentrations" of anesthetics were defined as ED_{50}, or the effective dose at which half of the test subjects are anesthetized. ED_{50} concentrations of anesthetics used in this study were taken from Roth and Miller (1984). The aqueous concentrations, expressed as millimolar, were ethanol, 190; butanol, 44; hexanol, 0.7; decanol, 0.013; diethyl ether, 24; halothane, 0.45; and chloroform, 0.89.

Measurement of Proton Flux in Liposomes Formed from Chromaffin Granule Lipids. Chromaffin granule membrane lipids were isolated from thawed granules by extraction into 2:1 (v/v) methanol/chloroform followed by separation by thin-layer chromatography (Kates, 1986). Liposomes were prepared according to the method of Barchfeld and Deamer (1988). Briefly, 6 mg of granule lipid was dried under argon and then dissolved in 1.0 mL of diethyl ether and 0.5 mL of pH 7.5 buffer containing 0.1 M K_2SO_4, 0.5 mM pyranine, and 1.25 mM each of ACES, MES, TES, and Tricine/KOH. The suspension was sonicated for 1 min, and the organic phase was removed by rotary evaporation under vacuum for 15 min. An additional 0.5 mL of buffer was added to the flask, and evaporation was continued for 45 min. The resultant vesicles were then sized by filtration through 0.2-μm pore diameter Nucleopore membranes. To remove the pH-sensitive pyranine probe from the external phase, the liposomes were passed over a Sephadex G-50 column. In a typical assay, 0.2 mL of liposomes was combined with 1.8 mL of buffer at room temperature in the presence of 0.5 μM valinomycin. The external pH was lowered to 7.00 by the addition of 1.0 M H^+ as H_2SO_4, and proton flux was measured by the initial change of entrapped pyranine fluorescence (Barchfeld & Deamer, 1988).

Measurement of ATPase Activities. ATPase activity was measured photometrically at 37 °C by coupled enzymatic oxidation of NADH (Bergmeyer, 1968). NADH (0.2 mM), phosphoenolpyruvate (1.25 mM), lactate dehydrogenase (10 IU), and pyruvate kinase (7.5 IU) were added to 0.96 mL of buffer composed of 0.195 M sucrose, 0.015 M K_2SO_4, 5 mM $MgCl_2$, and 10 mM each of ACES, MES, TES, and Tricine brought to pH 7.3 by addition of KOH. Frozen granules were thawed under lukewarm water and added to the buffer to give a final volume of 1 mL and a final concentration of 0.25 mg of granule protein mL^{-1}. After 2 min of equilibration in a temperature-controlled cuvette, 2 mM ATP (final concentration) was added. The rate of ATP hydrolysis was then measured by the decline in NADH absorbance at 340-nm wavelength vs time. The uncoupled treatments were measured in the same way except that the initial 2-min incubation was in the presence of 1 μM valinomycin and 1 μM CCCP. In the presence of those ionophores, the transmembrane proton motive force is completely released from chromaffin granules within 1 min. Proton ATPase activity under those conditions is not limited by proton back pressure.

Measurement of δΔpH across the Chromaffin Granule Membrane. We estimated the anesthetic-induced change in ΔpH across the chromaffin granule membrane (δΔpH) using 9-aminoacridine fluorescence. The practical and theoretical background for this assay is described by Deamer et al. (1972). Briefly, 10 μM 9AA was added to 2 mL of buffer composed of 5 mM $MgCl_2$, 0.1 M sucrose, 0.08 M KCl, and 0.05 M TES that was brought to pH 7.35 by addition of 0.02 M KOH (final K^+ concentration). ATP (2 mM) was added and fluorescence of the 9AA was measured at 390 nm (excitation) and 450 nm (emission) by using an Aminco-Bowman spectrofluorometer attached to a strip chart recorder. The temperature of the cuvette was maintained at 37 °C. Once a steady signal was established, chromaffin granules (0.3 mg of protein) were added from frozen stock that had been thawed under lukewarm water. Accumulation and quenching of 9AA was permitted to reach steady state, and then anesthetics were added to the cuvette. The anesthetic stock solutions were halothane (1 M solution in ethanol), butanol (glass distilled from Mallinckrodt analytical grade reagent), diethyl ether (glass distilled from Fisher analytical grade reagent), and 95% ethanol. The effect of anesthetic was measured as an increase in 9AA fluorescence (+ΔFU) compared to the control steady state. To transform ΔFU into the change in transmembrane ΔpH (δΔpH), we established a standard curve relating the two by titrating an identical granule suspension with 0.939 M H^+ as H_2SO_4. We assume that δΔpH due to acidification of the external phase gives the same change in 9AA quenching as δΔpH due to alkalinization of the internal phase upon addition of anesthetic.

Measurement of Catecholamine Uptake and Release from Chromaffin Granules. We measured catecholamine uptake and release by chromaffin granules using an on-line assay described previously (Hayflick et al., 1982; Johnson et al., 1982a) in which a polarizing electrode detects oxidizable substrates in the medium. In a typical experiment, 1 mL of fresh or thawed granule stock was passed over a Sephadex G-50 column that had been preequilibrated with 37 °C buffer containing 5 mM $MgCl_2$, 0.1 M sucrose, 0.08 M KCl, and 0.05 M TES that was brought to pH 7.35 by addition of 0.02 M KOH (final K^+ concentration). The granules were collected in 2.5 mL and immediately combined with 2.5 mL of the same buffer at 37 °C under nitrogen gas in an enclosed chamber. The final granule protein concentration was 3.4 mg mL^{-1} in 5-mL total volume. Total catecholamine concentration in the external volume was assayed at 2-min intervals by using a glassy carbon electrode (7-mm^2 surface area) at +0.5-V initial potential attached to a Princeton Applied Research Model 174A polarographic analyzer. Where specified, ATP was added to the reaction vessel as a 0.1 M ATP/TES stock at pH 7.0, and ammonium sulfate was added as a 1.0 M stock at pH 7.3. Halothane (Sigma) and hexanol (Kodak) were added from stock without further purification. Diethyl ether was added from a glass-distilled stock. We calibrated the external catecholamine concentration by addition of known amounts of dopamine. It is assumed that current at the glassy carbon electrode was due to oxidation of catecholamines alone and that ascorbate oxidation was insignificant. This is a reasonable assumption because catecholamines constitute 97% of the +0.5-V oxidizable substrate entrapped in chromaffin granules; ascorbate constitutes 3% (Johnson, 1988).

Results

The Intrinsic Proton Permeability of Chromaffin Granules

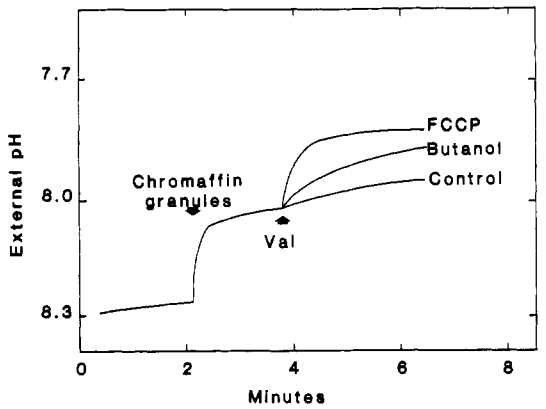

FIGURE 1: External pH vs time for a chromaffin granule suspension at 25 °C. Chromaffin granule addition was 0.25 mg of granule protein mL^{-1} in 2-mL total volume. The sharp decline in external pH upon granule addition is due to the pH 7.0 buffer in which they were stored. Valinomycin (0.5 μM final) was added to release the electrical potential generated by proton efflux. The abrupt decline in pH for the FCCP-treated vesicles in the presence of valinomycin demonstrates that counterion transport did not limit proton flux in the alcohol treatments. Addition of acidity by dissolution of CO_2 or by leakage from the KCl salt bridge was insignificant during the course of the experiment.

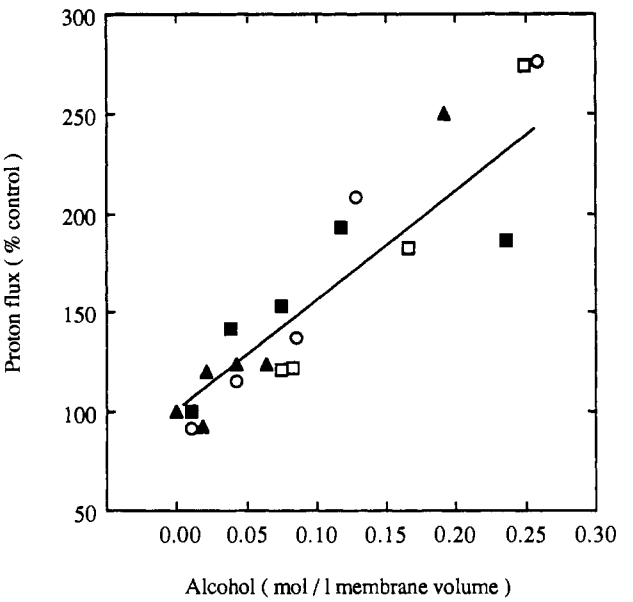

FIGURE 2: Effect of *n*-alcohols on proton flux across chromaffin granule membranes: (□) butanol; (○) hexanol; (■) octanol; (▲) decanol. Each point represents the mean of at least two replicates in which flux is expressed as percent of a control mean. A log transformation (performed to normalize residuals) gave a least-squares fit to the combined data $Y' = \log Y = 1.988 + 1.64X$, $R^2 = 0.75$. In linear form this is $Y = 97.3 + 549X$.

Is High. Figure 1 shows the rate of pH change of a 2-mL volume of dilute buffer in the presence of fresh chromaffin granules (0.5 mg of granule protein). Upon valinomycin addition, any diffusion potential due to proton efflux was eliminated, and the change in external pH could be used to calculate intrinsic proton permeability. For example, the total surface of the granules in Figure 1 was 750 cm^2, assuming 480 nmol of phospholipid per mg of granule protein and a membrane protein/phospholipid ratio of 0.5 on a weight basis (Johnson, 1988). Thus, proton flux for the control in Figure 1 was about 2×10^{-13} mol cm^{-2} s^{-1}, which yields a permeability coefficient of 2×10^{-5} cm s^{-1} at an internal pH of 5.5 and an external pH of 8.0. By comparison, K^+ permeability of erythrocytes is about 1×10^{-10} cm s^{-1}. This indicates that proton permeability of chromaffin granules is high and comparable to other biological membranes (Deamer & Bramhall, 1986).

Because a relatively high permeability is central to the pump-leak hypothesis, we ran an additional test using the pH-sensitive dye pyranine entrapped in liposomes formed from chromaffin granule lipids. Our experiments showed a net proton flux of about 6×10^{-14} mol cm^{-2} s^{-1}. Given an internal pH of 7.5 and an external pH of 7.0, the permeability coefficient was 6×10^{-4} cm s^{-1}. This is similar to the permeability of egg phosphatidylcholine liposomes and supports our conclusion that the granule membrane is relatively permeable to protons.

Clinical Levels of n-Alcohols and Volatile Anesthetics Increase Proton Flux across Chromaffin Granule Membranes. The pump-leak hypothesis requires that anesthetics increase the proton permeability of secretory vesicles. To test this, we used a pH electrode to measure proton efflux from chromaffin granules in the presence or absence of anesthetics. Typical results are shown in Figure 1, and a summary of our results for *n*-alcohols is shown in Figure 2. As expected, proton permeability of the granules in the presence of *n*-alcohols increases in a dose-dependent manner, with doubling of the rate at about 0.19 mol L^{-1} membrane volume.

It was important to determine the size of this effect at clinical levels of *n*-alcohols. A major deficiency of lipid domain theories of general anesthesia is that it is difficult to detect significant effects of the proposed mechanism at clinical anesthetic concentrations. Previous reports from our laboratory indicated that anesthetics can approximately double flux across liposome bilayers (Barchfeld & Deamer, 1985). However, relatively high anesthetic concentrations based on activities were used in those investigations. In the present study we applied lower concentrations related to ED_{50}. A typical membrane *n*-alcohol concentration equivalent to ED_{50} is 0.02 mol L^{-1} (Pringle et al., 1981), and the equation in the caption of Figure 2 predicts that the mean proton flux at this concentration is 105% of the control with upper and lower 95% confidence limits of 99.5% and 110%, respectively. Similar effects were measured for clinical concentrations of diethyl ether, chloroform, and halothane at 37 °C. The mean proton flux for these anesthetics was consistently 10% higher than that of controls, but variance in the data prevented us from showing a significant increase at the 5% confidence level. We conclude that *n*-alcohols, and most likely certain volatile anesthetics, cause at most 5–10% increments in the proton permeability of secretory vesicles at ED_{50}.

General Anesthetics Do Not Affect the Proton-Transport ATPase. Franks and Lieb (1984) proposed that the primary site of anesthetic action is a protein, rather than the lipid bilayer. If anesthetics both inhibited the proton-transport ATPase of secretory vesicle membranes and increased proton permeability, the resulting overall effect would be much greater than a permeability effect alone. Alternatively, if anesthetics did not inhibit the ATPase, but did increase proton permeability, one might expect to find increased ATPase rates. Johnson et al. (1982b) showed that proton ATPase activity of chromaffin ghosts increased by 40% per unit decrease in transmembrane ΔpH. We therefore determined the effect of several general anesthetics on ATPase activity. Positive controls were established by addition of the known protonophore CCCP.

Our results are summarized in Table I. At membrane concentrations 5 times ED_{50}, we were unable to demonstrate either uncoupling effects or inhibition of ATPase activity. However, we did observe doubling of ATPase activity in the

Table I: Effect of General Anesthetics on ATPase Activity of Chromaffin Granules at 37 °C[a]

anesthetic	aqueous concn (mM)	ATPase activity (% control)
butanol	59	107 ± 20
hexanol	6.9	101 ± 8
decanol	0.07	91 ± 10
diethyl ether	64	104 ± 20
halothane	3.6	103 ± 7

[a] Values represent the rate of ATP hydrolysis in the presence of anesthetic as a percentage of the rate in the absence of anesthetic. ATP hydrolysis was measured spectrophotometrically by enzymatically coupled NADH oxidation in the presence of 1 μM valinomycin and 1 μM CCCP. Aqueous concentrations shown correspond to membrane concentrations of 0.1 M. All values are the means of three replicates.

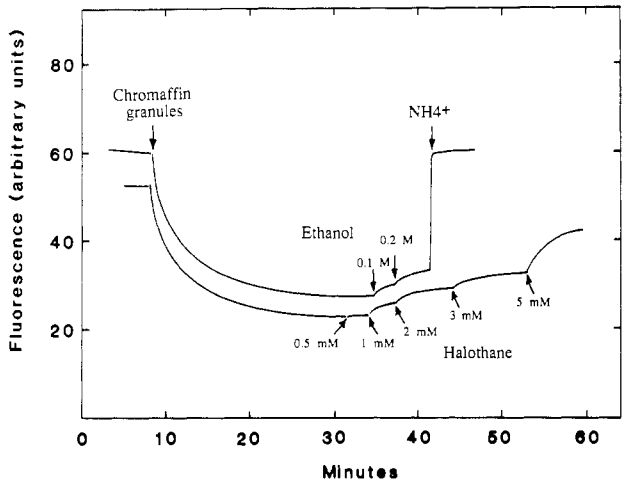

FIGURE 3: Effects of ethanol and halothane on 9AA quenching by chromaffin granules under physiological conditions. Chromaffin granules (0.25 mg of protein mL^{-1}) were added at time zero to 37 °C buffer containing 0.08 M KCl, 0.1 M sucrose, 0.05 M TES, 5 mM $MgCl_2$, and 2 mM ATP brought to pH 7.35 by the addition of KOH (0.02 M K^+ final concentration). Ethanol was added in 10-μL aliquots; halothane was added as microliter aliquots of a 1 M solution in ethanol. At the end of the experiment, the pH gradient was completely released by the addition of 5 mM $(NH_4)_2SO_4$ from a 1 M stock. The ethanol carrier contributed little to the fluorescence increase observed in the presence of halothane because at 5 mM halothane (10 μL of ethanol carrier) ΔFU = +20, while 10 μL of ethanol alone (0.1 M) gave ΔFU = +2. Addition of anesthetics in the absence of a pH gradient had no effect on 9AA fluorescence.

presence of CCCP. We conclude that the proton-transport ATPase is insensitive to general anesthetics and that any uncoupling effect due to an anesthetic-induced proton leak was too small to be detected.

Clinical Levels of Several General Anesthetics Diminish Transmembrane pH Gradients, but the Effect Is Small. It is possible that the increment in proton efflux that we measured could collapse the pH gradient across synaptic vesicle membranes. To address this question, we measured 9-aminoacridine quenching by chromaffin granules under physiological conditions (Figure 3). (As before, we assume that the chromaffin granule is a reasonable model for synaptic vesicles.) Addition of granules to a cuvette containing 9AA caused rapid quenching of the fluorescence signal, presumably due to permeation of the dye into the acidic granule interior. Additional acidification then occurred as protons were transported inward by the membrane ATPase, leading to further accumulation of 9AA and a steady decline in the fluorescence signal until a steady state was achieved (about 24 min). At steady state, we added ED_{50} levels of anesthetics either directly from stock or from 1.0 M solutions in ethanol. Subsequent

Table II: Change in Transmembrane ΔpH (δΔpH) of Chromaffin Granules Exposed to General Anesthetics As Measured by 9AA Quenching[a]

anesthetic	solution concn (mM)	δΔpH vs control	anesthetic	solution concn (mM)	δΔpH vs control
halothane	0.5[b]	0.00	diethyl ether	24.0[b]	−0.10
	1.0	−0.02		48.0	−0.21
	2.0	−0.12	ethanol	86.0	−0.02
butanol	44.0[b]	−0.02		172.0	−0.05
	88.0	−0.05			
	172.0	−0.15			

[a] All measurements were conducted at steady state following addition of 2 mM ATP to buffer at 37 °C. [b] ED_{50} concentration.

Table III: Effect of Clinical Levels of General Anesthetics on Catecholamine Concentrations in Suspensions of Chromaffin Granules under Physiological Conditions[a]

anesthetic	replicates	external catecholamine (% increase vs control)
	Thawed Granules	
diethyl ether	2	34 ± 15
hexanol	2	21 ± 12
halothane	1	24
	Fresh Granules	
diethyl ether	1	30
halothane	1	12

[a] Percent increase in external catecholamine refers to steady-state catecholamine concentration outside the granules following anesthetic addition compared to steady-state concentration prior to anesthetic addition in the same suspension. Typical experiments are shown in Figure 4.

aliquots of anesthetics were added to demonstrate dose dependence.

In most cases, addition of anesthetic at ED_{50} resulted in a measurable increase in the 9AA signal. From these values we calculated changes in transmembrane ΔpH (δΔpH), which are listed in Table II. ED_{50} diethyl ether elicited the largest response (δΔpH = −0.10), followed by ethanol (−0.05) and butanol (−0.02). Halothane had no measurable effect at ED_{50}, but at twice ED_{50} it caused a 0.02-unit decline in δΔpH. This evidence weighs against the original pump-leak hypothesis. Although clinical levels of anesthetics reduce the proton gradient across the chromaffin granule membrane, the gradient does not collapse and essentially all of the normal proton motive force is still available for catecholamine transport.

ED_{50} Levels of General Anesthetics Cause Measurable but Modest Losses of Catecholamines under Physiological Conditions. Having established that anesthetics at ED_{50} cause a small decrease in δΔpH across the chromaffin granule membrane, we next measured the amount of catecholamines lost from granules under conditions simulating physiological temperature, electrolyte concentration, and ATP concentration. Efflux of catecholamines was monitored with a glassy carbon electrode, and typical results are shown in Figure 4.

When fresh or thawed chromaffin granules were added to the reaction vessel at time zero, the electrode indicated a substantial leak of endogenous catecholamines as expected in the absence of an energy source (Figure 4A,B). Upon ATP addition, net catecholamine flux was rapidly reversed, presumably due to increased transmembrane ΔpH and ΔΨ generated by the proton ATPase. When steady state was established, we added anesthetics at levels approximating ED_{50}. Loss of catecholamine to the external medium began immediately in all cases and resulted in steady-state increases in external catecholamine from 12 to 34% above control (Table III). On average 50% of the change was achieved within 2–4

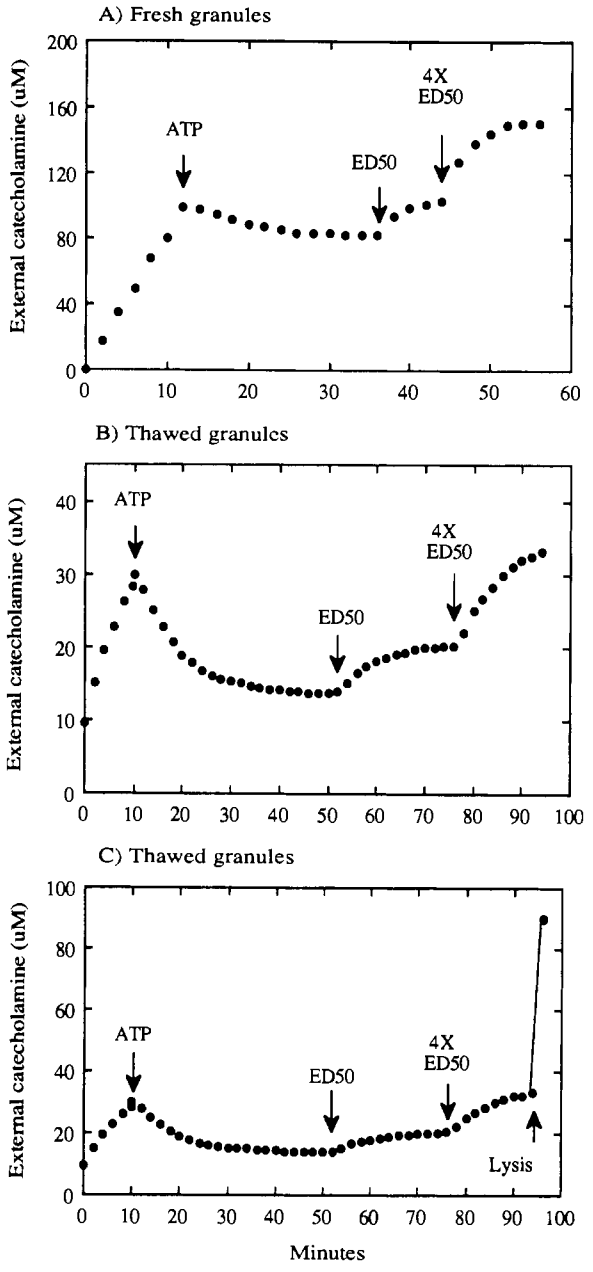

FIGURE 4: Effect of clinical levels of diethyl ether on external catecholamine concentration of chromaffin granule suspensions. Each data point represents measurement of total catecholamine in a 5-mL suspension by glassy carbon electrode (applied potential +0.5 V). At time zero, chromaffin granules (3.4 mg/mL protein) were added to buffer at 37 °C. After 10 min, 2 mM ATP was added as substrate for the membrane proton ATPase. ED_{50} represents addition of diethyl ether to 24 mM final solution concentration; $4 \times ED_{50}$ represents 96 mM diethyl ether. (A) Fresh granules; (B) granules that had been stored at −80 °C and thawed just prior to the experiment; (C) the same data set as in (B) except that it also includes a point for total entrapped catecholamine from a separate experiment in which granules were lysed in hypoosmotic buffer. Note the change in scale between the three figures. External catecholamine concentration was calibrated by addition of known concentrations of dopamine (data not shown).

min of addition; 20 min was required to achieve steady state. Addition of anesthetics had no effect on the measured current in the absence of catecholamine gradients.

The level of external catecholamine was always about 4-fold higher in assays using fresh granules (Figure 4A) compared to that in assays using thawed granules (Figure 4B). This difference was probably due to lysis of some of the granules caused by freezing and subsequent loss of catecholamines and other entrapped substrates. Nonetheless, the effect of anesthetics on catecholamine distribution across the granule membrane was proportionately the same whether or not the sample had been frozen. This necessarily follows from eq 1, which is given in the next section.

We conclude that clinical levels of general anesthetics release catecholamines from chromaffin granules as predicted by the pump-leak hypothesis. However, the quantity of catecholamine lost following anesthetic addition is only a small fraction (ca. 5%) of total entrapped catecholamine (Figure 4C). Furthermore, addition of 10 mM NH_4^+ as ammonium sulfate completely collapsed the transmembrane pH gradient, and yet the half-time for *total* catecholamine release was at least 30 min (data not shown). Thus, a major loss of catecholamine would be far too slow to be consistent with the rapid onset of anesthesia observed clinically.

Discussion

In this paper we have demonstrated that clinical levels of general anesthetics shift catecholamine distribution across the chromaffin granule membrane under physiological conditions at steady state. This phenomenon follows remarkably well from the predicted effect of membrane perturbants on the proton motive force available for catecholamine accumulation. That is, when anesthetics are inserted into the membrane at clinical levels, two effects occur simultaneously: (1) efflux increases by about 10% as shown in Figure 2; and (2) the resultant rise of pH_{in} causes proton ATPase activity to increase by 40% per unit of pH change (Johnson et al., 1982b). If these two functions are solved together, the steady-state internal pH should increase by 0.05 unit to pH 5.55. Our 9AA experiments indicated an anesthetic-induced decrease of transmembrane ΔpH between 0.02 and 0.10 unit (Table II), in reasonable agreement with the predicted outcome.

This change in transmembrane ΔpH necessarily leads to a change in catecholamine distribution across the granule membrane. Johnson (1988) presents evidence that chemiosmotically coupled transport distributes catecholamines across the chromaffin granule membrane according to

$$\log [C_T]_{in}/\log [C_T]_{out} = \Delta\Psi/Z + 2\Delta pH \qquad (1)$$

where $[C_T]_{in}$ and $[C_T]_{out}$ are total concentrations of a given catecholamine inside and outside the granule, $\Delta\Psi$ is the transmembrane electrical gradient (about +80 mV inside for chromaffin granules), Z = 59 mV under standard conditions, and ΔpH is the transmembrane pH gradient. Ordinarily, ΔpH is 1.8, leading to a value of about 135 000/1 for $[C_T]_{in}/[C_T]_{out}$. Given a modest increase of the steady-state internal pH to 5.55, the predicted ratio is reduced by 20% to about 107 000/1. Our measurements of external catecholamine concentration (Table III) showed a change of 12–34% above control for fresh and thawed granules in the presence of clinical concentrations of anesthetics.

We also note that the response we measured is kinetically feasible. That is, in the absence of anesthetics we have shown that the intrinsic proton permeability of chromaffin granules is substantial ($P = 2 \times 10^{-5}$ cm s^{-1}). At pH_{in} 5.5, this results in proton efflux of 2×10^{-13} mol cm^{-2} s^{-1}, which ordinarily is matched by inward pumping of protons by the membrane ATPase. Addition of anesthetics increases efflux by 10%, which means that the *net* efflux is initially 2×10^{-14} mol cm^{-2} s^{-1}. Johnson (1988) shows that the phospholipid content of chromaffin granules is 480 nmol (mg of granule protein)$^{-1}$ and the protein to phospholipid ratio in granule membranes is about 1 to 2 on a weight basis. If we assume that the surface-area-to-mass ratio is about the same for phospholipids and membrane proteins, then the total surface area would be 1500

cm^2 (mg granule of protein)$^{-1}$. This value multiplied by the buffering capacity of the granule interior [300 μmol of H^+ per pH unit per g of dry weight (Johnson et al., 1978) which is equal to 0.75 μmol of H^+ per pH unit per mg of granule protein] yields buffering capacity on a surface area basis equal to 5×10^{-10} mol of H^+ per pH unit per cm^2. Thus, at a net proton efflux of 2×10^{-14} mol cm^{-2} s^{-1}, pH shift from 5.50 to 5.55 would be achieved in 20 min. This is within a factor of 2 of the measured time to steady state in Figures 3 and 4. If, in contrast, proton permeability of the granule membrane were similar to K^+ permeability (e.g., $P = 1 \times 10^{-10}$ cm s^{-1} in erythrocytes), then a new steady state would not be achieved for many months.

Catecholamines also appear to be sufficiently permeable across biological membranes to allow for the steady-state shifts we measured. In an unpublished experiment, we found that the permeability of three neutral catecholamines across bilayers composed of the dominant phospholipid in chromaffin granules was relatively high, with P values ranging from 2×10^{-5} cm s^{-1} (dopamine) to 1×10^{-6} cm s^{-1} (epinephrine and norepinephrine). Similar values for epinephrine were measured by Bochain et al. (1981) and for dopamine by Bally et al. (1988). Furthermore, Johnson et al. (1982b) show that epinephrine accumulated into chromaffin granule ghosts is completely released within 10 min of ammonium addition. This could not be true if dopamine, norepinephrine, and epinephrine were relatively impermeable as reported for 5-hydroxytryptamine (Maron et al., 1983). An alternative explanation for the catecholamine release we observed follows from the work of Maron et al. (1983). In their view catecholamine efflux must be catalyzed by an amine transporter that is inactive at low intravesicular pH. If pH_{in} were increased (e.g., due to an anesthetic-induced proton leak), then net efflux could take place.

Previous studies have shown similar release of catecholamines from chromaffin granules in a sucrose medium (Sumikawa et al., 1980) and from intact tissue. For example, retention of dopamine in synaptosomes of rat caudate nuclei is significantly reduced (−8% control) by 0.17 M ethanol (Seeman & Lee, 1974), and a similar dose-dependent release of dopamine and serotonin occurs in rat brain cortical slices (Carmichael & Israel, 1975). In pheochromocytoma (PC-12) cells, high-affinity uptake of noradrenaline is reduced by clinical levels of several volatile anesthetics and by clinical levels of *n*-alcohols from ethanol to octanol (Tas et al., 1987a).

In vivo administration of halothane and ethanol is also known to increase circulating catecholamines. For example, patients given halothane/O_2 by face mask experience significant increases in blood plasma norepinephrine 15 min after induction of unconsciousness, which returns to normal 30–45 min later (Joyce et al., 1982). Because most of the catecholamine content of the body is in the adrenal medulla, the increased plasma content of catecholamines may result from an anesthetic effect on chromaffin granule membranes. However, leakage of catecholamines from chromaffin cells has not been observed directly. For instance, Pocock and Richards (1988) could not detect loss of catecholamine from halothane-treated chromaffin cells except at very high anesthetic concentrations (1–2 mM).

Critique of the Pump-Leak Hypothesis and Alternative Mechanisms for the Action of General Anesthetics. The results summarized above show that anesthetics qualitatively affect chromaffin granules as expected by the pump-leak hypothesis. However, quantitative and kinetic measurements reveal serious limitations to the hypothesis. For example, because the anesthetic-induced proton leak is small at clinical levels, the transmembrane pH gradient is diminished by about 0.05 pH unit, and only a small fraction of entrapped catecholamine is lost. In addition, when the pH gradient is caused to decay completely, loss of catecholamine is slow, with a half-time of 30 min.

If we assume that inhibition of synaptic transmission requires a collapse of the pH gradient followed by a major loss of neurotransmitter, then our results are not consistent with the pump-leak hypothesis. One may ask whether chromaffin granules are reasonable models of synaptic vesicles. For instance, proton pumping and internal buffering in synaptic vesicles may render them more susceptible to small increments in proton permeability. However, the results we obtained with chromaffin granules are similar to those obtained by Bangham and Mason (1980), who showed that benzyl alcohol at $6 \times ED_{50}$ caused only a modest release of labeled dopamine and that steady-state required 20 min to be reestablished. We must therefore consider alternative mechanisms.

First, it should be noted that a seemingly minor effect on permeability could be amplified in a relatively sensitive nervous function such as conscious behavior. Bangham (personal communication) has used the metaphor of a ship whose radio transmitter has been placed too near the water line, so that a small leak could inhibit communication. Although this bypasses the concern that measured permeability changes are small, it does not lead to a mechanism that could be tested at the biochemical or molecular level.

One possibility is that small amounts of catecholamines leaking from synaptic vesicles into the cytosol might inhibit nervous transmission. For instance, Christ and Nishi (1971) and Dun and Nishi (1974) showed that 10–1000 μM catecholamine produced ganglionic blockade in the rabbit cervical ganglion. These and similar observations led to the concept of inhibitory autoreceptors (Langer, 1977) reviewed recently by Starke (1987). Is it possible that catecholamine leakage produced by anesthetics inhibits synaptic transmission by binding to autoreceptors? There is some suggestive evidence. For instance, Prahdan et al. (1955) reported that epinephrine markedly potentiated urethan anesthesia in mice, and Mazel and Bush (1969) made similar observations with barbitol anesthesia. Feldberg and Sherwood (1954) implanted cannulas into the lateral ventricles of the cat brain and investigated the behavioral effects of a variety of pharmacological agents. It was found that intraventricular injections of 20–80 μg of adrenaline or noradrenaline (but not acetylcholine) produced an anesthetic state resembling that of pentabarbitone, which lasted for approximately an hour. Despite these intriguing results, possible effects of leaked catecholamines on inhibitory receptors appear unlikely for other reasons. For instance, if the receptors are on the cell surface, the catecholamines must cross the plasma membrane and accumulate to sufficiently high concentrations to bind to the receptors. Furthermore, anesthesia can be maintained over many hours, and it seems unlikely that leaking catecholamines could be maintained at the cell surface over such long periods of time.

Another possibility is that anesthetics interact directly with a protein site on neuronal membranes, thereby inhibiting nervous function. In support, clinical concentrations of several anesthetics have been shown to inhibit the function of certain enzymes and membrane proteins (Franks & Lieb, 1984, 1988; Tas et al., 1987b; Lechleiter & Gruener, 1984; Pocock & Richards, 1988). The protein hypothesis does not require anesthetics to interact with lipids. Instead, it is proposed that certain proteins have nonpolar binding sites that interact with

anesthetics according to the Meyer–Overton rule. In the absence of significant physical effects on lipid bilayers at ED_{50} concentrations of general anesthetics, the protein hypothesis is favored by the weight of evidence, at least in explaining anesthetic effects on the nervous system of higher organisms.

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Registry No. ATPase, 9000-83-3; H^+, 12408-02-5; ethanol, 64-17-5; butanol, 35296-72-1; hexanol, 25917-35-5; decanol, 36729-58-5; diethyl ether, 60-29-7; halothane, 151-67-7; chloroform, 67-66-3.

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