INCREASES IN INTERNAL Ca^{2+} AND DECREASES IN INTERNAL H^+ ARE INDUCED BY GENERAL ANESTHETICS IN SQUID AXONS

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ABSTRACT Squid axons were injected with arsenazo III and treated with sea water containing compounds usually classified as general anesthetics, (pentanol-decanol and a variety of hydrocarbons and their derivatives). Such treatment led to an increase in absorbance by arsenazo III at wavelengths sensitive to $[Ca]_i$. The effect was independent of the presence or absence of Ca^{++} in sea water and it was not modified by substances that release Ca from internal stores. The effect was easily reversible. In axons injected with phenol red or impaled with a glass electrode sensitive to H^+, a similar treatment led to an alkalinization that was also readily reversible. Both Ca release and the change to an alkaline pH had identical time courses. The dose required for action by all of the chemical agents studied could be predicted from a knowledge of their fractional saturation in sea water, i.e. from their thermodynamic activity. For compounds with 8–10 carbon atoms, Ca-release effects can occur at concentration less than those necessary to block either conduction or Na/Ca exchange. A special chemical agent was octylamine, which induced a marked rise in pH_i and in addition its nonionic form produced the typical Ca release associated with general anesthetics.

INTRODUCTION

The effects of substances classified as general anesthetics on excitable cells are not fully understood. Detailed studies by Haydon's group (Haydon et al., 1980; Haydon and Urban, 1983 *a*, *b*, *c*) have related the block of nerve impulse propagation to alterations of several Hodgkin-Huxley parameters. They suggested that the way in which nonpolar molecules, such as hydrocarbons act is consistent with their adsorption into the interior of a lipophilic region, so causing its expansion in a direction normal to the membrane surface. Polar molecules, such as alcohols, would have their polar ends localized at the interfaces. Other studies have been conducted to explore the effects of general anesthetics on cell Ca content, since Ca ions are known to be involved in the regulation of many processes including voltage-dependent and neurotransmitter-induced ion fluxes and transmitter release from nerve endings. Sensitization of nerve terminals or muscle by volatile anesthetics has been related to increased Ca^{2+} binding, as observed in erythrocyte ghost membranes (Seeman et al., 1971). However, it was also found that all anesthetics, regardless of charge or molecular geometry, deplete the red cell membrane to the same level of residual Ca, except at low concentrations of straight chain alcohols (Low et al., 1979). Ethanol produced a dose-dependent depletion of brain Ca levels (Ross, 1976) and it also appeared to augment calcium-mediated mechanisms, both pre- and post-synaptically, by possibly increasing intracellular Ca concentration (Carlen et al., 1982) or by changing the affinity of the proteins (Baker and Shapira, 1980). Such enhancements can also account for the uncoupling produced in cardiac cells by general anesthetics (White et al., 1984; Wojtczak, 1984). Furthermore, Ca transport systems are also modulated by anesthetics. The Ca uptake and the ATPase activity of the sarcoplasmic reticulum from skeletal muscle was increased by diethyl ether (Salama and Scarpa, 1980), while the Na-dependent Ca fluxes in brain synaptic vesicles would be stimulated by methanol and low doses of ethanol or inhibited by large doses of ethanol and other short-chain aliphatic alcohols (Michaelis and Michaelis, 1983). Finally, a larger decrease in the Ca than in the Na and K conductances was observed in *Aplysia* neurons treated with ethanol (Camacho-Nasi and Treistman, 1985), while ethanol caused an immediate increase in the Ca permeability of the plasma membrane of resealed human red blood cell ghosts (Yingst et al., 1985).

In a recent study on the influence of chemical agents on the level of ionized Ca in squid axons it was found that octyl alcohol appeared to inhibit Ca buffering in axoplasm (Requena et al., 1985). The present study was undertaken to extend such observations. We find that general anesthetics, such as C_5 to C_{10} alcohols, some alkanes and chloroform, applied in Ca-free sea water, increase the internal free Ca level and simultaneously induced alkalinization of

axoplasm. These effects are independent of the external Na and Ca concentrations and can be attributed to an exchange of Ca^{2+} for H^+ on some sites inside the nerve fiber. Preliminary results have been published (Vassort et al., 1985).

METHODS

Dye Spectrophotometry and Ion Sensitive Electrodes

The measurement of Ca_i with arsenazo III and H_i with phenol red as well as the use of ion specific electrodes for the measurement of Na_i and H_i were as described by Mullins et al. (1983). Glass electrodes for Na and H measurement were made from the same batches of glass (NAS 11-18 0150; Corning Glass Works, Medfields, MA). The results with arsenazo III were recorded as 685–660 and 675–685 nm difference spectra and the isosbestic wavelength 570 nm was used as a control; with phenol red we used 504–525 or 481–525 nm difference spectra and 481 nm for the isosbestic wavelength. The results section shows the difference spectra 685–660 nm for arsenazo III and 504–525 nm for phenol red. The excitability of the axon was checked every second using external stimulating and recording electrodes while transmembrane potential was recorded by the reference electrode inserted axially.

Experimental Materials

The experiments were done using living animals of the species of *Loligo pealei* collected at the Marine Biological Laboratory, Woods Hole, MA during May–June, 1984. After killing the squid, an axon was rapidly dissected and cleaned so that it was ready for an injection of dye generally within 1 h of the death of the animal.

Microinjection

Stock solutions of arsenazo III, phenol red or the inhibitor orthovanadate, were made up in 330 mM K TES buffer, pH 7.3. Small volumes, 15–20 μl, of these were microinjected over a length of 15–20 mm. The usual final concentrations within the axoplasm were 500 μM for arsenazo, 300 μM for phenol red, and 1 mM for orthovanadate.

Solutions

Sea water had the following composition (in millimolars) Na, 455; K, 10; Mg, 55; Ca, 3; Tes (pH 7.8), 10; Cl, 571; EDTA, 0.1 (to protect against heavy metal contamination). For solutions with 112 mM Ca, Mg was omitted and NaCl reduced so that the solution was isosmotic with 1,000 mosml/L. High K solutions and Na-free solutions were made by replacing all Na with either K or Li, respectively. Cyanide sea water had 2 mM NaCN at pH 7.8 added to normal sea water. All solutions were adjusted to 1,000 ± 10 mosmol/L and pH 7.8. Temperature was controlled at 13°C except when another temperature is given.

Hydrocarbon Derivatives

The chemical reagents used for this study (alcohols, paraffins) were of analytical grade and were obtained from Eastman Kodak. For the relatively insoluble compounds such as octanol, decanol, octane, hexane, Br-hexane, solutions of these substances were prepared in sea water by adding sufficient of the chemical reagent so that a saturated solution was obtained. Dilutions of this solution were then used to obtain the desired final concentrations. For the more water soluble compounds (pentanol, octylamine, hexanol, and chloroform) an appropriate amount of the reagent was added to sea water to produce the desired final concentration. Special care was taken to deliver sea water solutions of substances at micromolar concentrations to the experimental cell with fast flow and all glass tubing so that any loss of such insoluble compound was minimized. While the common name octanol of octyl alcohol is used in the text, this should be understood to mean 1-octanol and all substituents on paraffins were in the 1 position. The paraffins themselves were always the straight chain substances. We have checked that these hydrocarbons derivatives in the dose employed do not alter the absorbance spectrum of arsenazo III and phenol red by adding hexanol to a cuvette containing the dye and measuring absorbance with a spectrophotometer.

Sensitivity of pH Measurements

An important part of this study involved measuring the changes in internal H^+ when various chemical reagents were applied. These changes were of the order of 0.02 pH unit, a value that is difficult to measure in axoplasm with high precision when pH_i is ~7. The use of the pH-sensitive dye phenol red expands the sensitivity of this measurement by a factor of ten so that changes in H_i are much more readily followed. An example of this can be noted in Fig. 8. An additional advantage of phenol red is that it measures a mean hydrogen ion concentration so that changes in pH_i produced by the local binding of Ca in the periphery of the axoplasm are readily seen. We recognize that our Ca indicator, arsenazo III is sensitive to changes in ambient pH and that such changes can be confused with changes in Ca_i, but it is to be noted that with the pH changes being confined to the range of 0.01–0.03 the error in arsenazo reading is quite negligible.

RESULTS

Alcohols Increase Internal Free Ca

Among the substances classified as general blocking agents (anesthetics), alcohols with a chain of 5–10 carbons were initially selected because of their ease of use and because they block electrical activity at concentrations that do not alter the osmotic pressure of the artifical sea water (ASW) significantly. Fig. 1 shows the effects of hexanol (8 mM), pentanol (18 mM) and octanol (0.54 mM) applied successively on a given axon and the effects of decanol (0.04 mM) on another axon. At the concentrations used here the alcohols induced similar increases in resting $[Ca]_i$, as shown by the increased absorbance of the arsenazo III signal. The longer the carbon chain of the alcohols the longer the time to reach steady state: <5 min with pentanol, up to 20 min or more with decanol. Furthermore, the shorter chain length alcohols (pentanol and hexanol) besides blocking action potential propagation, induced a

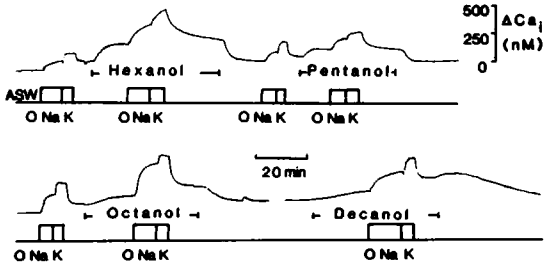

FIGURE 1 An axon injected with arsenazo III was treated for short periods of time to Na-free ASW (0 Na) and to high K (K) ASW in the absence or in the presence of hexanol (8 mM), pentanol (18 mM), and octanol (540 μM). Same protocol on another axon in the presence of decanol (40 μM). Notice the increase in resting absorbance when the alcohols were added.

5–7 mV depolarization. Action potentials were still recorded with the longer chain alcohols and the membrane potential was not affected. The effects were reversible with a time course of recovery similar to the on effect, except for decanol. In this case, as with more potent general anesthetics (see below) there was a significant further increase in absorbance on switching back to ASW. As a general protocol we have applied to the axon a Na-free (Li-substituted) and a high K (no Na) ASW; this gives some insights to the internal Ca buffering capacity on switching back to ASW. The increases in free Ca induced by these two solutions were very small at the beginning of the experiment. Hexanol increased these two signals, although the rate of increase in Ca_i was less when induced by the high K solution (in the presence of hexanol). The increases in free Ca induced by the Na-free solution in the presence of pentanol and octanol were larger than in the absence of alcohols, but the effects of the alcohols on the further increases induced by the depolarization were rather inhibitory. On switching back from the high K to the normal ASW the presence of alcohols reduced the rate of recovery to the steady Ca level when compared with the same solution changes applied just before in the absence of alcohols. The concentration-$[Ca]_i$ curves for pentanol, hexanol, or octanol are shown for three different axons (Fig. 2). In all three cases, increasing the alcohol concentration increased, nearly linearly, the resting absorbance. Notice that in pentanol the same concentrations were much more potent when applied at the end of the experiment (to an axon loaded with Na as judged by the size of the increase in $[Ca]_i$ induced by a Na-free solution). There was no large changes in the $[Ca]_i$ induced both by Na-free or high K solutions; in fact, there was a decrease at the higher concentrations of pentanol and hexanol used. The details of responses of axons to alcohols are given in Table I.

Due to variations in the magnitude of the effects induced by a given concentration of one alcohol or the other on different axons at different times along one experiment, the effects of hexanol on the resting absorbance were checked several times on an axon with different Na and Ca loads (Fig. 3). Although the first 8 mM hexanol application was preceded by switching to ASW + 0.8 mM hexanol soon after the axon was equilibrated in the set up, clearly the response was larger and faster than the second one elicited by 8 mM hexanol applied 100 min later. However, a third application soon after the second one had recovered gave rise to the same increase in resting absorbance that had just occurred. Then, the axon was stimulated for a period of 10 min at 100/s in Ca-rich Na containing solution, and was thus Na and Ca loaded. A further application of hexanol-containing solution induced a larger increase in resting absorbance than previously; a final application of hexanol after a period of further Ca-loading by high K depolarization did not show a significant increase in the response. This experiment started in 0Na ASW so that after the internal Na electrode had equilibrated, it showed a slow but consistent decrease in $[Na]_i$ from 22 to 12 mM. The variation in the amplitude of the three first responses suggests that the response to hexanol was sensitive to the Na-load of the axon. This was confirmed by analyzing the responses of axons bathed in normal Na, 3 Ca, ASW, and then treated with ASW plus hexanol; after stimulation in normal ASW, the response increased, but decreased after the axons were stimulated in Na-free ASW.

In conclusion, a given concentration of hexanol elicits increases in the resting absorbance that are larger with both Na and the Ca loading of the axon; these situations are known to reduce the Ca buffering capacity. Thus, we are prevented from making a more quantitative analysis.

The increase in the internal free Ca elicited by adding alcohols to ASW might have several origins including alterations in transmembrane Ca movements. Fig. 4 shows results of an experiment in which an axon was first treated with octanol (0.54 mM) in normal 3 Ca ASW. An increase in resting absorbance was observed. The same increase was again observed when reapplying octanol at the same concentration but after the axon has been washed for 2 h in nominally Ca-free ASW (no Ca chelator added). The lower part of the figure shows the effect of octanol on another axon in 3 Ca and in high (104) Ca ASW. Notice that the increase in resting absorbance was not greater than the sum of the increase induced by octanol and by the high Ca solution separately. Similar results were obtained with hexanol and pentanol. This suggests that alterations in transmembrane Ca movements are not the primary cause for the increase in internal Ca, and indicates that alcohols release Ca ions from internal stores.

Ca uptake by organelles in axoplasm is well established (Brinley, 1978). In an attempt to distinguish between possible impairment of Ca uptake by smooth endoplasmic reticulum or mitochondria and alteration of Ca binding by internal stores we have attempted to inhibit these organelles.

Since caffeine has been reported to increase light emis-

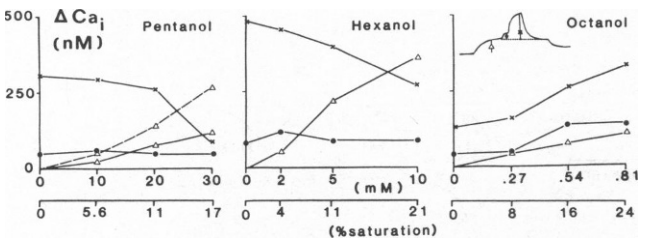

FIGURE 2 Concentration-dependent effects of pentanol, hexanol and octanol on the resting absorbance and on the magnitude of the Ca increase induced by Na-free (Li substituted) or high K ASW in three axons. Dashed line shows the effect of pentanol on the same axon after Na-loading. *Insert* on right relates symbols to: application of alcohol in sea water, in Na-free solution, and X to depolarization with 450 mM K sea water.

TABLE I

	mM	ASW → 0 Na ASW				0 Na ASW → high K				High K → ASW			
		dCa/dt nM s^{-1}	ΔCa_i nM	R 1,000 s^{-1}	%	dCa/dt nM s^{-1}	ΔCa_i nM	R 1,000 s^{-1}	%	dCa/dt nM s^{-1}	ΔCa_i nM	R 1,000 s^{-1}	%
Axon 050484A													
ASW	—	0.42	60	7.0	—	4.17	75	55.6	—	− 4.58	−100	45.8	—
ASW	—	0.33	40	8.2	—	5.17	100	51.7	—	− 4.17	−105	39.7	—
Octanol	0.27	1.17	70	16.7	217	4.17	120	34.7	65	− 3.67	−135	27.2	64
Octanol	0.54	1.50	160	9.4	121	7.50	120	62.5	117	− 4.5	−240	18.7	44
Octanol	0.81	1.58	160	9.9	128	3.75	225	16.7	31	− 5.17	−450	11.5	27
Axon 050484A													
ASW	—	1.04	100	10.4	—	4.17	120	34.7	—	− 3.75	−120	31.2	—
Pentanol	18	1.00	80	12.5	82	1.58	65	24.3	72	− 1.83	− 85	21.5	72
ASW	—	1.42	70	20.3	—	4.00	120	33.3	—	− 4.00	−140	28.6	—
Axon 050584A													
ASW	—	2.08	165	12.6	—	6.67	200	33.3	—	− 5.83	−300	19.4	—
Octanol	0.54	3.33	285	11.7	92	3.92	225	17.4	50	− 8.33	−650	12.8	66
Pentanol	18	1.00	100	10.0	79	1.50	90	16.7	50	− 2.33	−275	8.5	44
Hexanol	8	1.00	150	6.7	53	1.08	200	54	162	− 2.00	−450	4.4	23
Axon 050684A													
ASW	—	0.47	70	6.7	—	2.58	65	39.7	—	− 1.88	− 65	28.9	—
Hexanol	8	0.92	160	5.7	85	1.00	130	7.7	19	− 1.42	−270	5.3	18
ASW	—	0.83	70	11.9	—	2.25	85	26.5	—	− 2.25	−125	18.0	—
Pentanol	18	1.04	90	11.6	98	1.25	60	20.8	79	− 2.25	−150	15.0	83
Axon 050784A													
ASW	—	0.58	60	9.7	—	5.83	290	20.1	—	− 7.08	−340	20.8	—
Pentanol	10	1.08	65	16.6	148	6.08	275	22.1	123	− 4.58	−315	14.5	90
Pentanol	20	0.92	70	13.1	115	3.17	210	15.1	84	− 4.17	−260	16.0	99
Pentanol	30	0.21	50	4.2	37	0.5	50	10	56	− 0.25	− 50	5.0	31
ASW	—	1.42	110	12.9	—	5.83	375	15.5	—	− 5.42	−475	11.4	—
Axon 050884A													
ASW	—	2.83	105	26.9	—	7.50	405	18.5	—	−10	−605	16.5	—
Hexanol	2	2.08	155	13.4	50	6.67	350	19.1	103	− 7.08	−500	14.2	79
Hexanol	5	1.42	125	11.4	37	7.08	320	22.1	120	− 5	−500	10.0	56
Hexanol	10	0.5	—	—	—	0.5	—	—	—	− 0.33	—	—	—
Axon 051584A													
ASW	—	0.63	115	5.5	—	2.33	125	18.6	—	− 2.33	−180	12.9	—
ASW	—	0.58	115	5.1	—	2.00	150	13.3	—	− 2.75	−225	12.2	—
Hexanol	5	0.92	160	5.7	107	2.33	100	23.3	145	− 1.67	−185	9.0	72

The effects of pentanol, hexanol, and octanol on the rates of rise and recovery and on the amplitude of the increase in internal Ca were estimated from the variation in absorbance of arsenazo III when the axons were submitted to Na-free (0 Na) ASW, then to 450 K (high K) ASW and on return to ASW. To allow for comparison the ratios (R) = d Ca_i/dt over Ca_i are given as well as their relative variations induced by the alcohols in a percentage of the control values in ASW.

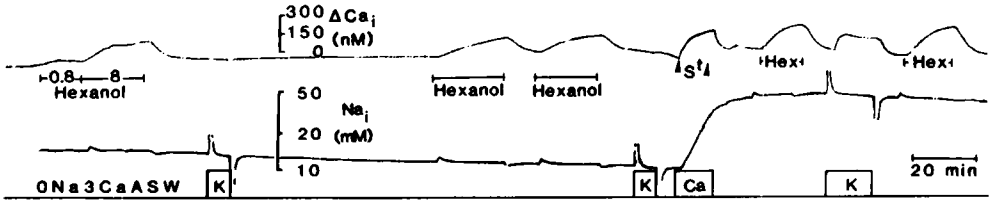

FIGURE 3 Continuous tracings of arsenazo III signal (upper line) and Na-sensitive electrode (lower line) on an axon treated several times with 8 mM hexanol. Successive application induced about the same increase in resting absorbance at the same internal Na_i. Larger increases occurred when internal Na and Ca were increased as a result of a period of 10 min of stimulation at 100 s^{-1}. The Na^+ and reference electrodes sense significantly K treatment, which results in the discontinuities in the Na electrode trace.

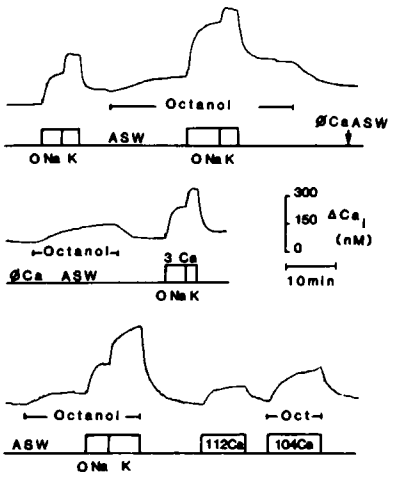

FIGURE 4 Octanol (16% saturated) induced the same increase in resting absorbance in an axon bathed with normal 3 Ca ASW or after 2 h in 0 Ca ASW. In another axon, the increase in arsenazo III signal in the presence of ASW with high Ca and octanol (16% saturated) was about the sum of the increases elicited by high Ca ASW and octanol alone.

sion of aequorin by releasing Ca from intracellular stores in mammalian neurones (Neering and Mc Burney, 1984) we looked at its effect on an axon injected with arsenazo III. Caffeine appeared to decrease resting absorbance (see Fig. 5 in the presence of vanadate). However, we have noted that caffeine interacts with arsenazo III and alters its spectrum in a cuvette (Vassort, unpublished results). It has been recently brought to our attention that arsenazo III has a one-to-one interaction with caffeine (Best and Abramcheck, 1985). Ryanodine, another substance known to alter sarcoplasmic reticulum did not change the resting absorbance at 10^{-6} M nor did it change the effects of hexanol which appeared equally potent in increasing internal Ca (Fig. 5). Mitochondria are active in Ca sequestration only when the level of ambient free Ca is high. In a fresh axon cyanide does to change resting Ca. Fig. 5 confirms that on an axon that had been treated for 15 min with 2 mM cyanide resting absorbance did not change. The figure also shows that resting absorbance was increased to about the same extent as in other experiments when alcohols were added. All active transport mechanisms are expected to be highly impaired by orthovanadate. Three axons were injected with vanadate (6 mM, final concentration) together with arsenazo III. Although the signals in response to usual procedures (Na-free or high K solutions) were smaller due to a leakage of arsenaso III (or to an interaction of vanadate with the dye), hexanol (8 mM) always induced an increase in resting absorbance (Fig. 5). These results suggest that alterations of smooth endoplasmic reticulum and mitochondrial transport by alcohols are unlikely to be responsible for the increase in internal free Ca.

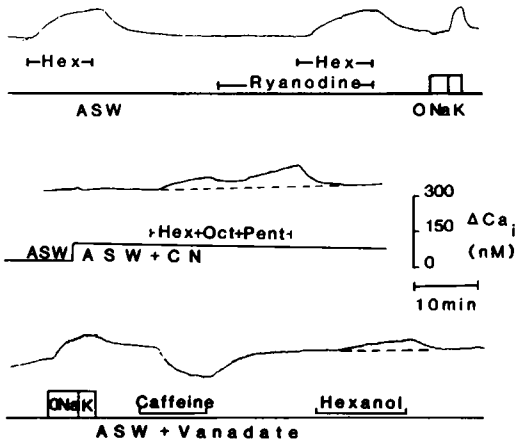

FIGURE 5 No change in resting absorbance was observed on an axon injected with arsenazo III and bathed in ryanodine ASW (10^{-6} M). Ryanodine also did not alter the response to 6 mM hexanol (*upper part*). Different alcohols, hexanol (6 mM), octanol (10% saturation), and pentanol (30 mM) still elicited increases in resting absorbance on an axon injected with arsenazo III in CN (2 mM) ASW (*middle part*). In an axon injected with vanadate (6 mM) together with arsenazo III 1 h before the beginning of the tracing, hexanol (6 mM) induced a significant increase in resting absorbance. The apparent decrease observed with (10 mM) caffeine application is the result of a direct interaction of arsenazo III with caffeine (*lower part*).

The above experiments suggest that the increases in internal Ca induced by alcohols do not result from (*a*) a larger Ca entry (Fig. 4); (*b*) nor from an alteration in the active Ca uptake by internal organelles (Fig. 5). This increase might thus be due to a decrease in the internal buffering as is shown by the experiment illustrated in Fig. 6. An axon was first soaked in a high K solution and then in a Na-free solution before switching back to ASW. In Na-free solution the decrease in the arsenazo II signal reflects mostly the rate of internal Ca buffering, while on return to ASW it reflects an additional component owing to the rate of Ca extrusion by the Na-Ca exchange mechanism (for more discussion see Mullins et al., 1983). The same two test solutions applied in the presence of hexanol (10 mM) revealed that both means of reducing free Ca were slowed down; the rate constant decreases

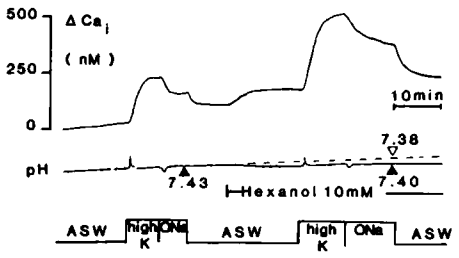

FIGURE 6 In an axon injected with arsenazo III, hexanol induced an increase in resting absorbance and reduced the rate of recovery to basal absorbance on switching from high-K to Na-free solution and from Na-free to normal ASW solution; thus indicating a decrease in internal Ca uptake or binding and a decrease in Na-dependent Ca efflux. Hexanol also induced an alkalinization superimposed on the slowly developing acidification as shown by the H^+-sensitive microelectrode.

(initial rate of fall/amplitude) in absorbance when switching to ONa and then to ASW were decreased five- and fourfold in the presence of hexanol. This was also observed in three other experiments (see also Table I).

Not only was the Na-dependent Ca efflux reduced in the presence of alcohols but also the Na-dependent Ca influx induced either by Na-free solution or by high K solutions. Table I lists the initial rate of rise (dCa_i/dt) and the amplitude (Ca_i) of such Ca influx activation in the absence (ASW) or presence of different alcohols. Although amplitudes could be increased up to threefold there was not a consistent increase in the rate of rise or even some decrease. Thus, one has to use the ratio initial rate of rise/amplitude as an approximation of the rate constant or the percentage of variation induced by alcohols compared to the ASW conditions; this shows generally a significant reduction during alcohol exposures except in the presence of octanol. Table I also reports the initial rate of fall, amplitude, and rate constant when switching back from high K to normal ASW. Rate constants were markedly reduced (up to fourfold) as expected from the result of decrease buffering capacity and reduced Na-dependent Ca efflux.

Alcohols Decrease Internal Free H

Whether an axon is injected with phenol red for spectrophotometric measurement of H^+, or has a glass electrode inserted, it is a common observation that there is a slow (0.1–0.6 nM H^+/min) acidification of the fiber, presumably because artificial sea water has no bicarbonate and this has been shown to be essential for pH regulate ion (Boron and Russell, 1983).

In experiments where a pH electrode was inserted for measurement of axoplasmic pH it was noted that the addition of alcohols to ASW prevented or even reversed the slowly developing acidification. An example of this effect is shown in Fig. 6. Here, the fiber had an initial drift of pH toward the more acid side of ~0.1 nM H^+/min and this was reduced to zero upon the application of hexanol. Similar observations were obtained during pentanol and octanol with an alkalinization of the order of 0.01 to 0.03 pH unit.

To further elucidate this point, axons were injected with phenol red. Two examples taken from six experiments are illustrated. In Fig. 7 the pH electrode signal and the phenol red dual wavelength difference signal are compared. On adding hexanol to ASW the drift was transiently reversed, indicating an internal alkalinization. The on and off effects occurred in ~10 min, as did the increase and recovery of free Ca under similar conditions. At the maximal effect the internal free H had decreased by only 1 nM. Thus, it is not surprising that the variation on the pH electrode trace is barely visible since, in this acidic axon (pH 7.13), a 1-nM change is an increase of <0.006 pH unit.

The following experiment was performed in 0 Na 0 Ca ASW, a solution which together with the lack of bicarbonate should block transmembrane control of internal pH by the H-Na-Cl-HCO_3 exchanger. These conditions did not prevent the increase in internal free Ca upon the addition of alcohols (see Fig. 4). The effects of pentanol (30 mM) and of hexanol at two concentrations (0.8 and 8 mM) were compared to the already reported acidification induced by loading the axon with Ca in the presence of high K (Fig. 8). The alkalinization induced by hexanol was concentration-dependent and the larger dose (8 mM) induced about the same decrease in H^+ as 30 mM pentanol. Notice that at these concentrations pentanol and hexanol had about the same potency in increasing internal Ca^{++}, (see Fig. 2).

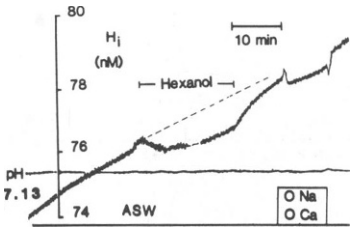

FIGURE 7 The alkalinization induced by 10 mM hexanol is clearly shown by the difference spectrum recorded in a phenol red injected axon. The axon was also impaled with an H^+-sensitive microelectrode and the pH tracing is shown simultaneously.

Other Hydrocarbon Derivatives

General anesthetic properties are not limited to alcohols, but rather alkanes and Br-alkanes appear even more potent in direct relation with their oil-gas partition coefficient. The effects of hexane (100 μM), Br-hexane (66 μM) and chloroform (1 mM) were compared to the effects of hexanol (8 mM). All three substances increased markedly the arsenazo absorbance with Br-hexane being more potent even at this concentration (not shown). Application of a saturated octane solution (5 μM) induced a very slow rise in resting absorbance while returning to ASW triggered a large, poorly reversible, increase in signal (not shown).

Special attention was given to hydrocarbon derivatives with an amine group since NH^+_4 is known to induce alkalinization (Boron and De Weer, 1976). Fig. 9 shows, in an axon injected with arsenazo III and impaled with Na

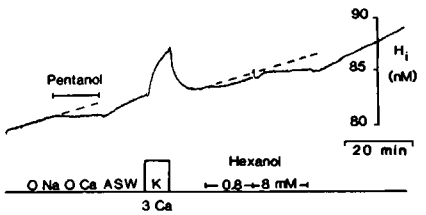

FIGURE 8 In an axon injected with phenol red and that showed a continuous slow acidification, pentanol (30 mM) and hexanol (0.8 and 8 mM) induced alkalinizations even in the absence of Na and Ca in the external solution. A fast reversible acidification developed on applying a 450 K, 3 Ca ASW.

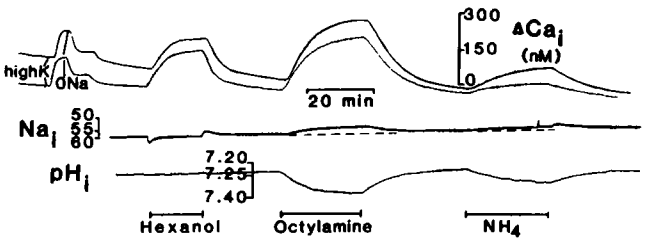

FIGURE 9 The axon was injected with arsenazo III and impaled on one side with an Na^+-sensitive electrode and on the other with an H^+-sensitive, together with the reference electrode. It was treated successively with 450 K then 0 Na ASW and to ASW added with hexanol (8 mM) octylamine (0.25 mM) and NH_4Cl (0.5 mM). Due to the H^+ sensitivity of arsenazo III, there was an apparent increase in resting absorbance of unequal amplitude of the two dual wavelength difference spectra. The increase in absorbance induced by octylamine had a larger amplitude and, like with hexanol, the two difference spectra varied in parallel, indicating that the pH alteration contributes weakly to this effect.

and H electrodes, the results of applying ASW containing first hexanol (8 mM), second octylamine (0.25 mM) and third NH_4Cl (0.5 mM). The pH electrode shows that with octylamine, pH increased from 7.24 to 7.33 (a reduction of 10.8 nM H^+) and with NH^+_4 from 7.20 to 7.27 (a similar reduction of 9.3 nM), while the reduction with hexanol was of the order of 1 nM. The two dual wavelength differences for arsenazo III signals are shown, they show that the alkalinization induced by NH_4 altered the arsenazo III response. The electronic magnification was set such that the variation in the two signals were identical with small entry of Ca. With NH^+_4 not only were the responses different in amplitude but they were slightly positive, particularly the 685–660 signal, while it is known that alkalinization decreases resting Ca (Mullins et al., 1983). This is consequent to the direct effect of H^+ on arsenazo III. However, this provides a test that allows one to compare the octylamine effect on resting absorbance. While 0.25 mM octylamine induced about the same change in H content as 0.5 mM NH^+_4, the variations of the two arsenazo III signals were much larger, indicating a large increase in free Ca. This increase appeared as fast and large as that with similar concentration of octanol and was fully reversible. The half times of on and off effect were 4 min or less. It thus appears that octylamine molecule has the dual role of releasing internal Ca like other hydrocarbons and of inducing alkalinization due to its amine function. Furthermore, octylamine induced a small decrease in Na_i up to 3 mM, which was also seen in the two other experiments using octylamine in which an Na electrode was used. Such changes in $[Na]_i$ were not observed with the use of other hydrocarbons derivatives. A particular feature of the octylamine effect is its stability when compared to NH_4 effect. While the alkalinization developed with about the same half time, pH was steadily maintained in the presence of octylamine. In one experiment where 0.5 mM octylamine was applied, the pH increased from 7.49 to 7.61 (−7.8 nM H^+) and was still 7.56 after 160 min. It then decreased to 7.43 on switching back to ASW (+9.6 nM H^+). In the presence of octylamine the usual acidification was of the order of 0.02 nM min^{-1}, much less than usually observed. During this period the resting absorbance of arsenazo III was markedly increased suggesting a rise in $[Ca]_i$ by >1 μM. The effect was rapidly reversed on removing octylamine. Furthermore, the free Ca content in the presence of the substance appeared "clamped" since hexanol (8 mM) and Na-free or high K solutions hardly changed its level. This was unexpected, since alkalinization by NH^+_4 promotes Na-dependent Ca influx (Mullins et al., 1983) and since the effects of hexanol on pH recording and arsenazo III signal were not significantly altered by the presence of NH^+_4 (5 mM) and propionate (5 mM) (not shown).

DISCUSSION

All of the general anesthetics that we have tested produce at appropriate dose a release of Ca ions of the order of 100–500 nM and an absorption of protons of a very much smaller magnitude (1–2 nM) and both of these processes have an identical time course. This small apparent alkalinization, occurring together with the increase in resting Ca, is to be compared to observations in the absence of alcohols where a large Ca entry acidifies axoplasm (see Mullins et al., 1983 for related references and discussion). The Ca release is a negligible fraction of the total analytical Ca (68 μmol/kg axoplasm) (Requena et al., 1979). It is also known that in fresh axons (those not exposed to high Ca sea water) the mitochondria have a negligible fraction of the total analytical Ca stored (Brinley et al., 1977) so that it has usually been assumed that it is the smooth endoplasmic reticulum of the axon that stores the bulk of the Ca. This assumption has not been tested because there has been no method by which Ca release from this structure could be brought about. Our experiments with ryanodine and with caffeine show that these substances do not release Ca, nor does the inhibitor of phosphorylation, vanadate, have any marked effect. Since this latter substance ought to block ATP-driven pumping of Ca into the smooth endoplasmic reticulum, we have to conclude that either the endoplasmic reticulum is an extremely tight structure, so that Ca leakage is negligible, or that the bulk of the axoplasmic Ca may be stored in other, so far not identified structures.

As originally pointed out by Ferguson (1939) much of the action of hydrocarbons and substituted hydrocarbons in inhibiting cell functions can be predicted by assuming that all of these substances act at equal thermodynamic activity (when the pure liquid is the standard state) (see Mullins, 1973). A simplified way of looking at this is to note that the thermodynamic activity is 1.0 in a saturated aqueous solution and activity declines roughly linearly with a dilution of such a solution. We have worked with solutions of substances in the range of 5–20% saturation (activities of 0.05–0.2). The entire dose-response curves

are not experimentally accessible because higher concentrations of anesthetic agents will cause the collapse of the plasma membrane, and thus prevent any further measurement.

The hydrocarbon derivatives used in this study were, roughly, equally potent in releasing Ca and binding H if one considers their fractional saturation in sea water, i.e. their thermodynamic measure of concentration. However, electrical activity block and Na-Ca exchange inhibition did not follow this rule. As suggested by Haydon and Urban (1983*a*) the concentration of alkanols that produced 50% suppression of the Na current indicates that equal concentrations of molecules in the membrane produce the same response. This yielded a standard free energy of transfer for CH_2 groups from the aqueous phase to the membrane that was constant (3.04 kJ mol^{-1}).

The results of this study cannot be discussed without reference to some framework that will explain how it is possible for alcohols and other general anesthetics to produce a reversible Ca release-H absorption by membranes. This decrease in membrane affinity for Ca ions can be explained by a conformation change in membrane protein, but can also be explained by an altered spacing of the negative sites of phospholipids toward the optimum spacing of 9.6 Å for binding the hydrated calcium ions (Hauser and Dawson, 1968). Since the relative affinity of the binding sites for Ca vs. H can be altered by the addition of lipophilic substances such as alcohols, it suggests that the structure releasing Ca is phospholipid in nature. The fact that Ca release can occur at anesthetic concentrations well below those blocking nerve conduction suggests that it may not necessarily be the plasma membrane that is the target but rather that other phospholipid structures within the axoplasm could well be the sites of Ca storage.

The alcohols used in this study are known to be incorporated into phospholipid bilayers in model systems (Haydon and Urban, 1983*a*) and the paraffins (hexane, Br-hexane, octane) that are also effective in producing Ca release-H absorption are also able to thicken phospholipid bilayers (Haydon and Urban, 1983*b*). These substances in appropriate doses are known to block nerve conduction. It is therefore logical to suppose that phospholipid bilayers are the appropriate target for the compounds that we have used. Now, in addition to the conventional surface membranes of axons, there are also membranous structures in the axoplasm such as mitochondria and smooth endoplasmic reticulum that have many times the area of the surface membrane. It is likely therefore that some or all of these phospholipid structures bind Ca^{2+} in relation to ambient Ca concentration and that H^+ also is bound to such structures. If general anesthetics perturb the phospholipid structures, then it is to be expected that this perturbation can well release Ca and that H are absorbed by the sites vacated by Ca thus reproducing our experimental findings. Assuming uniform distribution of phospholipids in the surface membrane and that all of them are bridged by Ca ions, one might expect up to 10^{14} Ca ions bound per cm^2, i.e. an increase of up to 70 μM if all of them were released in the axoplasm. This is of the order of the total Ca content in a fresh axon and ~200 times the increase we generally observe. Obviously, not only Ca ions are bound on phospholipid heads, but on the other hand the internal membrane structures are much more extended than the axolemma.

Ca release and H absorption were both larger with increasing concentrations of the drugs and their time courses were very similar. However, even if we cannot give precise features, the relative variations in both ion concentrations with a given alcohol appeared markedly dependent upon the Na load of the axon; a decrease in one H^+ was associated roughly to an increase of 50 Ca^{++} in a low Na-loaded axon (≃18 mM) and to an increase of up to 500 Ca^{++} in a high Na-loaded axon (≃50 mM). However, there was no significant variation of the Na content during the alcohol effects, they were only seen when using octylamine. It should also be noted that if the usual buffering capacity were not impaired, an increase in free Ca of the order of 300 nM would correspond to a release of 6 μM Ca, taking into account that only one Ca^{++} in 20 entering could contribute to the ionized Ca of a fiber (Brinley et al., 1977). On the other hand the measurements of Boron and De Weer (1976) indicate that 10^4 protons are bound for each one free at normal values for pH_i; this would mean that the 1–2 nM H^+ change we observed in a fresh axon corresponds to a 10–20 μM H binding. Such approximations suggest an equivalence between Ca release and H absorption close to 1 Ca^{++} to 2 H^+ in a fresh axon. In a Na-loaded axon (which also means Ca-loaded) this ratio might be different, or rather, the buffering capacity for Ca is changed such as 1 Ca out of 50 up to 2,000 remains free (Brinley et al., 1977).

Octylamine possesses two modes of action on cell pH. Besides the strong alkalinization due to its amine group, octylamine induced a release of Ca and an absorption of H. The amplitude of these latter effects should be related to the nonionic form that, taking into account the dissociation constant at the internal pH, suggests that for each mM of octylamine only 0.1 μM becomes nonionic intracellularly. Then, octylamine is only 20- to 50-fold more potent than octane in releasing Ca ions. Its much faster on and off effects can be attributed to the faster diffusion in the membranes because of the higher total concentration of octylamine.

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