INACTIVATION OF THE SODIUM CURRENT IN SQUID GIANT AXONS BY HYDROCARBONS

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ABSTRACT The voltage dependence of the steady state inactivation parameter (h_∞) of the sodium current in the squid giant axon is known to be shifted in the hyperpolarizing direction by hydrocarbons and it has been suggested that the shifts arise from thickness changes in the axon membrane, analogous to those produced in lipid bilayers (Haydon, D. A., and J. E. Kimura, 1981, *J. Physiol.* [*Lond.*], 312:57–70; Haydon, D. A., and B. W. Urban, 1983, *J. Physiol.* [*Lond.*], 338:435–450; Haydon, D. A., J. R. Elliott, and B. M. Hendry, 1984, *Curr. Top. Membr. Transp.*, 22:445–482). This hypothesis has been tested systematically by examining the effects of a range of concentrations of cyclopentane on the high-frequency capacitance per unit area both of the axonal membrane and of lipid bilayers formed from monoolein plus squalene. A similar comparison has been made for cyclopropane and *n*-butane, both at a pressure of 1 atm. The results are consistent with the notion that thickness increases in the axolemma produce the shifts in h_∞. Except at very high concentrations, however, the thickness changes in the lipid bilayer were too small to account for the h_∞ shifts. A possible explanation of this finding is discussed.

INTRODUCTION

Many hydrocarbons of low molecular weight suppress excitability in nerve axons (1–5). Recent examinations of this phenomenon have shown that the hydrocarbons perturb the voltage-dependent sodium current in the axon in several ways (4, 5). The most important of these is a (reversible) shift of the relationship between the steady state inactivation and membrane potential in the hyperpolarizing direction. This effect is produced apparently by all hydrocarbons and it can leave the nerve in a heavily inactivated condition. Another significant factor in many instances is the tendency to reduce the maximum sodium conductance achievable in absence of inactivation. This effect is also found for all hydrocarbons though to an extent that is variable and not always fully reversible. Discussions of the possible origins of these two effects have appeared in several earlier publications (3–5). The main conclusion arrived at so far is that an increase in thickness of the membrane on adsorption of the hydrocarbon could be an essential factor in each instance. The original reason for thinking that this might be so was that similar hydrocarbons, at similar concentrations to those that blocked action potentials, produced substantial thickness increases in lipid bilayers (6–9). Moreover, experiments with short-chain phospholipids have now added support to the notion that thickness changes in the axon membrane affect the gating processes (10).

For the detection of thickness changes in the axolemma the only feasible technique is that of capacitance measurement at high frequency (2, 4, 11). In contrast to the situation for lipid bilayers, however, there are ambiguities in the interpretation of the data. Nevertheless, the parallels found so far between the effects of hydrocarbons on the capacitances per unit area of axolemma and lipid bilayers are sufficiently similar that it has been thought worthwhile to compare the results for the two membranes over a range of hydrocarbon concentrations. The shifts in the voltage dependence of the steady state inactivation curve have also been measured for the same hydrocarbons over the same concentration range.

Prior to this investigation the available data suggested that thickness changes in a lipid bilayer could account quantitatively for the inactivation shift. The more systematic study with cyclopentane described below shows that, except at very high hydrocarbon concentrations, this is not generally true, the changes in the bilayer being too small. Cyclopropane and *n*-butane have also been examined, as earlier work (12) failed to detect any influence of these substances on lipid bilayers. Here, small but significant effects are reported.

EXPERIMENTAL

Methods

Giant axons were dissected from freshly killed specimens of *Loligo forbesi*. The axons had diameters in the range 500–800 μm, and were cleaned of connective tissue and surrounding small fibers. The chamber in which the axons were mounted, the arrangement of the electrodes, the temperature control, and the means of introducing the hydrocarbon solutions have been described in earlier publications (2–4). The bathing solution in which the hydrocarbons were dissolved consisted of 430 mM

NaCl; 10 mM KCl; 50 mM $MgCl_2$; 10 mM $CaCl_2$; and 10 mM Trizma base plus HCl to give pH 7.6. Sodium currents were suppressed where necessary by the addition of 0.3 μM tetrodotoxin. The voltage clamp and data acquisition procedures were essentially as described by Kimura and Meves (13). Compensation for the series resistance was >95% in all experiments. Impedence measurements were carried out at the resting potential of the axon (−50 to −60 mV) and involved essentially the equipment and procedure described previously (2, 4, 11). All axon experiments were carried out at 6 ± 1°C.

Black lipid films were formed over a 1 mm hole in a vertical Teflon vessel, and the capacitance and membrane area determined by standard methods as described elsewhere (14). The main difference from the earlier apparatus was that both the Teflon vessel and the perspex cell in which it was mounted were gas tight. Thus, membrane formation (by the feeder tube method [15]) and the introduction and withdrawal of all solutions were carried out through tubes sealed into a Teflon plug, which was in turn screwed into the Perspex outer cell and sealed by means of an O ring. All the lipid bilayer experiments were carried out in 0.1 M NaCl and at 25°C.

Materials

The inorganic electrolytes for both the squid axon and the lipid bilayer experiments were of A. R. grade. For the latter experiments the sodium chloride was roasted at 700°C to remove organic impurities and the water was doubly distilled. Monoolein was obtained from Nu-Chek Prep., Inc., (Elysian, MN) and was used without further purification. The liquid hydrocarbons (from Koch-Light Laboratories, Colnbrook, United Kingdom) and squalene (from Sigma Chemical Co., St. Louis, MO) were purified before use by passage through an alumina column. The cyclopropane and *n*-butane were from Cambrian Gases (Croydon, United Kingdom) and were used without further purification.

RESULTS

Steady State Inactivation of the Sodium Current

Measurements were carried out as far as possible under physiological conditions, i.e., the axons were intact (not internally perfused) and were bathed in normal artificial seawater, as described in Methods. This procedure was disadvantageous in that the possible effects of leakage and potassium currents on the results had to be considered. On the other hand, the effects of perfusion on the sensitivity of the sodium currents to anaesthetics, noted previously (4), were eliminated. The steady state inactivation parameter, h_∞, was determined by applying to the axon a range of 50 ms conditioning prepulses followed by a test pulse sufficient to give approximately the maximum inward current. The holding potential was usually −60 mV. The h_∞ parameter was then calculated by dividing the test currents by the test current for the most negative prepulse (i.e., that which effectively removed inactivation). No correction was made for the existence of leakage current but it was established that this neglect did not produce errors of >5% in the shifts (ΔV_h) in the midpoint of the h_∞ curve. The potassium current distorted the h_∞ curves at large depolarizing test pulses. At the midpoint and above, however, the curves were not significantly affected.

Fig. 1 shows the shift in the midpoint of the h_∞ curve produced by cyclopentane over a range of concentrations. The axons were exposed to the hydrocarbon for ~20 min, after which time the currents had reached a steady value. The hydrocarbon was then washed out for some 30 min, after which the currents had again reached a steady value. The points in Fig. 1 were obtained by comparing the midpoint of the h_∞ curve under test conditions with the mean of the values for the control and recovered axons.

Membrane Capacitance

Impedance measurements were carried out at frequencies between 50 and 300 kHz. In this range the results were effectively independent of the membrane potential, and of whether or not the sodium and potassium currents were blocked (2, 4, 11). The axons were at their normal resting potential and were essentially in a state comparable to those used for the inactivation measurements. The times required to reach a steady state both on exposure to, and on recovery from, hydrocarbon solutions were as found in the voltage-clamp experiments. The membrane capacitance at 100 kHz was calculated as described previously (2, 4). In brief, the axon membrane was considered to be a parallel combination of a capacitance and resistance, and to be in series with a resistance representing the axoplasm and the external bathing solution. The series resistance was estimated by plotting the impedance locus for 50–300 kHz and extrapolating it to infinite frequency (zero reactance). The membrane capacitance per unit area, which can then be calculated, is frequency dependent, as shown in earlier investigations (2, 4, 16), and is thus unlike the capacitance of a lipid bilayer (17–19). From a recent detailed examination of this result it has been concluded that the frequency dependence in the axon probably arises from the complex morphology of the Schwann cell and axon membranes rather than from any intrinsic structural feature of the axolemma itself (11). It was further concluded that the frequency range for which the capacitance was likely to correspond most closely to that of the axolemma was 50–100 kHz. Cyclopentane solutions at all concentrations reduced the capacitance per unit area uniformly over this frequency range. The hydrocarbon had only small and

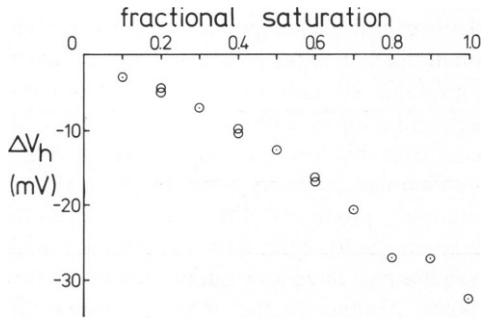

FIGURE 1 The shift ΔV_h of the midpoint of the steady state inactivation curve of the sodium current as a function of cyclopentane concentration. 11 different intact axons were examined, from two of which two data points each were obtained.

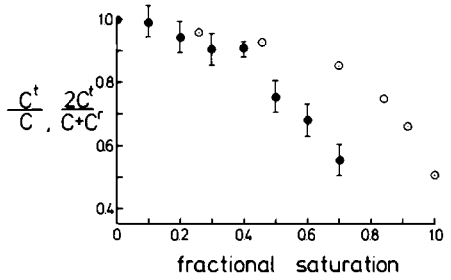

FIGURE 2 A comparison of the effects of cyclopentane at various fractional saturations on axonal membrane capacitance, and monoolein-squalene bilayer capacitance. For the axon, C, C^t, and C^r are, respectively, the capacitance per unit area at 100 kHz for the control, the axon exposed to the hydrocarbon solution, and the axon after recovery from hydrocarbon treatment. The points are mostly mean results for two or three experiments on different axons. In one instance (0.4 saturation) four experiments were carried out and the SEM calculated to be 0.026. In the other experiments twice this SEM has been arbitrarily assumed. For the bilayer, the control capacitance (C) (8.4 mM monoolein in squalene) was 0.751 μF cm^{-2}. Aqueous solution, 0.1 M NaCl.

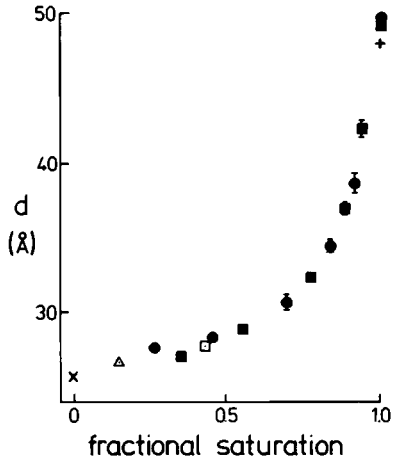

FIGURE 3 Hydrophobic layer thicknesses of monoolein-squalene bilayers (and one of dioleyl phosphatidylcholine) equilibrated with various hydrocarbons at different fractional saturations. X, squalene only; ●, cyclopentane; △, cyclopropane; ⊡, n-butane; ■, n-decane (23, 24); +, dioleoyl phosphatidylcholine plus n-decane (14). The thicknesses (d) were calculated from capacitances per unit area (C) by means of the expression $C = \epsilon_r\epsilon_o/d$ where ϵ_r is the relative permittivity and ϵ_o is the permittivity of free space. Values of ϵ_r were obtained as described in reference 29, with ϵ_r(monoolein) = 2.2, ϵ_r(n-decane) = 1.99; ϵ_r(cyclopentane) = 1.97; ϵ_r(n-butane) = 1.80; and ϵ_r (cyclopropane) = 2.2

nonsystematic effects on the series resistance, as estimated either from the impedance locus or from voltage-clamp compensation measurements. Fig. 2 shows the effect on the membrane capacitance of a range of cyclopentane concentrations. In all experiments impedances were measured before, during, and after exposure to the test solution. The results plotted in Fig. 2 are the ratios of the capacitance per unit area for the test (C^t) to the mean of the capacitances before (C) and after (C^r). The data shown in Fig. 2 are for 100 kHz, but a comparable plot for 50 kHz is not significantly different.

Lipid Bilayer Capacitance

The reference or control lipid bilayer was formed from monoolein and squalene. This structure has been well studied and is thought to contain very little (<5%) squalene (20). The effects of cyclopropane and of n-butane, both at a pressure of 1 atm, on the membrane capacitance per unit area are shown in Table I. Comparable data for cyclopentane at several concentrations are plotted in Fig. 2.

Care was taken in each instance to ensure that the membrane lipids had equilibrated with the aqueous phase and that there was no depletion of the hydrocarbon. In the cyclopentane experiments it was convenient to introduce the hydrocarbon via the membrane-forming lipid, such that the mole fraction in this phase was known. To a first approximation the fractional saturation of the aqueous phase can be equated to these mole fractions. However, the activity coefficients of low molecular weight hydrocarbons in squalene are not normally unity (21) and to compare accurately the cyclopentane data with the n-butane and cyclopropane results, some corrections are necessary. While there appear to be no activity coefficients tabulated specifically for cyclopentane in squalene, there exist extensive data for closely related hydrocarbons in squalene (22).

TABLE I

CAPACITANCE (C) AND HYDROCARBON THICKNESS (d) RATIOS FOR MONOOLEIN-PLUS-SQUALENE LIPID BILAYERS AND AXONAL MEMBRANES EQUILIBRATED WITH CYCLOPROPANE AND n-BUTANE AT 1 ATM PRESSURE

Hydrocarbon	Monoolein-squalene bilayer		Axonal membrane		Na current inactivation	
					d^t/d	
	C^t/C	d^t/d	$2C^t/(C + C^t)$	d^t/d	from ΔV_h (Eq. 1)	from slope ratio (Eq. 2)
Cyclopropane	0.979 ± 0.017	1.027 ± 0.019	0.921 (3)	1.09	1.12 (7)	1.10 (4)
n-butane	0.914 ± 0.008	1.074 ± 0.0127	0.922 (3)	1.08	1.16 (2)	1.22 (2)

t indicates test conditions and r, recovery. For the bilayers, thicknesses were calculated from capacitances per unit area by means of the expression $C = \epsilon_r\epsilon_o/d$, where ϵ_r is the relative permittivity and ϵ_o is the permittivity of free space. Values of ϵ_r were obtained as described in reference 29, using the values given in the legend to Fig. 3. Control values for capacitance and thickness (± sem) were 0.751 ± 0.004 μF cm^{-2} and 25.79 ± 0.13 Å, respectively. Axon data are taken from reference 4 and are means for the numbers of experiments given in parentheses.

There is also evidence that the activity coefficients in squalane and squalene do not differ greatly (22). From this information, it is possible to estimate the corrections required for the present systems (Needham, D., unpublished results). These have been incorporated into Figs. 2 *b*, 3, and 4. The corrections do not affect the general conclusions reached below, but they show that, when compared on a thermodynamic activity basis, the effects of various low-molecular-weight hydrocarbons on the thickenss of a simple bilayer are remarkably similar (Fig. 3).

DISCUSSION

A simple mechanism by which nonpolar molecules might produce voltage shifts of the Hodgkin-Huxley parameters of a nerve axon has been proposed in earlier papers (3–5). It was argued that such molecules would partition preferentially into the interior hydrophobic regions of membranes thus causing an expansion primarily in a direction normal to the surface, i.e., a thickening. At constant membrane potential this would tend to reduce the internal electric field experienced by the appropriate voltage sensor, and hence give rise to the observed shifts. Various pieces of evidence support this general type of explanation, one in particular being that hydrocarbons thicken lipid bilayers (6–9, 14). In fact, an extreme possibility is that the voltage sensors of the sodium channel are effectively embedded in a lipid bilayer, in which case it might be expected that the thickness change produced by hydrocarbons in lipid bilayers would account quantitatively for the h_∞ shifts.

For lipid bilayers, the estimation of thickness change is well established (9) and results for different hydrocarbons at various concentrations are plotted in Fig. 3. These data were all obtained from capacitance per unit area by means described in earlier papers, and with the dielectric constants given in the legend. Estimations of membrane hydrocarbon thickness from optical reflectance (8) and x-ray diffraction (25) have yielded values very similar to those of the capacitance method. As seen, for a given fractional saturation, the thickness increases do not vary greatly for the hydrocarbons tested. Of particular interest is the clinical general anesthetic, cyclopropane which, at 1 atm, gives a thickness increase of 0.7 Å, while *n*-butane, also at 1 atm, gives 1.9 Å. Franks and Lieb (12), using spectroscopic techniques, failed to detect a thickness change in phospholipid-cholesterol bilayers exposed to cyclopropane at 1 atm. A 0.7 Å change, however, seems likely to have been below the resolution of their measurements. Toward saturation, the thickness increase becomes much larger, approaching 25 Å or 100%. Fig. 3 includes one point for dioleyl phosphatidylcholine. This result, together with other data for monoolein and phosphatidylcholines equilibrated with different hydrocarbons (6), indicate that bilayers of monoolein accurately reflect the effects of the hydrocarbons on at least this type of phospholipid.

The cyclopentane results in Fig. 3 have been plotted in Fig. 4 as a ratio of the test to the control membrane thickness. Fig. 4 also plots the thickness ratios calculated for the axon membrane from the capacitance data of Fig. 2, assuming the formula for a simple geometrical capacitor to be applicable and that there is no change in the dielectric constant of the nonpolar region of the membrane on adsorption of the hydrocarbon. Finally, Fig. 4 shows the thickness ratios calculated from the shift ΔV_h by means of the expression proposed in earlier papers (3–5), i.e.,

$$\Delta V_h = V_h(d'/d - 1), \tag{1}$$

where V_h is the membrane potential at which $h_\infty = 0.5$ in the control axon, and d and d' are, respectively, the average thicknesses of the hydrocarbon region of the control axon membrane and the axon membrane in equilibrium with the hydrocarbon solution. The thickness ratios obtained from Eq. 1 are subject to an assumption which is equivalent to neglecting the possible asymmetry in surface potentials on the two sides of the membrane (5). However, the thickness ratio may also be obtained from the model, without making this assumption, by means of the equation (4, 5)

$$\left(\frac{dh_\infty}{dV}\right)_{V_h} \Big/ \left(\frac{dh'_\infty}{dV}\right)_{V'_h} = \frac{d'}{d}, \tag{2}$$

where the left-hand side is the ratio of the slopes of the h_∞ curves at their midpoints in control and test conditions. The accuracy of the results from Eq. 2 is poorer than from Eq. 1 but, as shown in Fig. 5, the two sets of thickness ratios are in fair agreement. It was shown in an earlier paper that the peak value of the inactivation time constant (τ_h) was shifted by hydrocarbons in the hyperpolarizing direction to approximately the same extent as the midpoint of the h_∞ curve (4). For the relatively small shifts at low cyclopen-

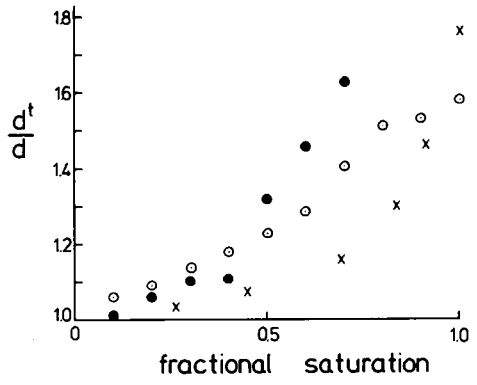

FIGURE 4 A comparison of thickness increases (relative to the control) produced by cyclopentane in axonal membranes (estimated by two different methods) and lipid bilayers. ●, for the axon from membrane capacitance; O, for the axon from h_∞ shifts (via Eq. 1) and the mean ΔV_h values of Fig. 1; X, for the monoolein bilayer from capacitance measurements.

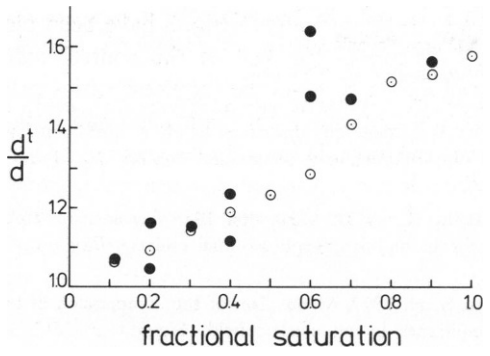

FIGURE 5 A comparison of axonal thickness ratios (test d^t, to control, d) deduced from the steady state inactivation (h_∞) curve by means of Eqs. 1 (○) and 2 (●). The open points are as in Fig. 4; the shaded points were calculated from the same experiments using Eq. 2 but the data have been averaged.

tane concentrations, the peak of the τ_h curve cannot be located sufficiently accurately to make a quantitative comparison of τ_h and h_∞ worthwhile. Nevertheless, in two experiments with 0.1 saturated cyclopentane, which were carried out for other reasons that will be reported elsewhere, the τ_h shifts were both about -3 mV, i.e., similar to ΔV_h in Fig. 1. The shifts found in τ_h are thus consistent with the thickening hypothesis. The time constant also undergoes other changes in hydrocarbons but, as previously discussed (5), these appear to be because it is a kinetic rather than an equilibrium parameter, and therefore subject to additional types of perturbation.

The data in Fig. 4 show that the thickness increases calculated from the axon membrane capacitance are in fair agreement with those required to account for the h_∞ shifts. There is a tendency for the capacitance results to be too low at low concentrations and too high at higher concentrations but, in view of the experimental errors and assumptions involved, these discrepancies may not be significant. In addition, whereas the capacitance results yield thicknesses that are averages for the whole axon membrane, the sodium channel proteins presumably sense only local thickness changes. The thickness increases in the lipid bilayer, except towards unit fractional saturation, are consistently smaller than those deduced from the axon capacitances, and are insufficient to account for the h_∞ shifts. The form of the curves of d^t/d vs. fractional saturation is nevertheless similar for both the bilayer and the axon, suggesting that the bilayer type of model is not wholly irrelevant for the interpretation of the data. Further evidence implicating lipid as the site of action comes from recent experiments in which short chain phospholipids (10, 26) have been shown both to increase the capacitance per unit area of the axon membrane (and hence presumably to decrease its thickness), and also to shift the h_∞ curve in the depolarizing direction. This is interesting because, while it is conceptually obvious that the adsorption of hydrocarbons into any nonpolar region of a membrane (i.e., lipid or protein) could increase its thickness, and that the adsoprtion of the short-chain phospholipid into a lipid layer would thin the membrane, it is not obvious how the adsorption of the phospholipid into a protein would produce a thinning.

The question therefore remains as to how, if the lipid is involved, the axon data may be accounted for. Biological membranes typically contain ~50% (wt/wt) lipid, the remainder being mostly protein or glycoprotein. In such a structure it is unlikely that much, if any, of the lipid would be in the form of an unperturbed bilayer, e.g., it would have either a larger or smaller area per molecule than normal, and be correspondingly either thinner or thicker than normal. The adsorption of hydrocarbons increases the thickness of lipid bilayers up to a value equal to approximately twice the extended chain length of the lipids involved (9, 14, Fig. 4). If, therefore, prior to the adsorption, the lipids were thinner than normal, the effect of the hydrocarbon could well be greater than that for a corresponding bilayer. A further factor is that the lipids of the axolemma seem likely to have an average chain length greater than the oleoyl lipids in the bilayer experiments (27, 28). The range of thicknesses achievable by hydrocarbon adsorption should therefore be larger than for oleoyl lipids.

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