FURTHER EVIDENCE THAT MEMBRANE THICKNESS INFLUENCES VOLTAGE-GATED SODIUM CHANNELS

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ABSTRACT The short-chain phospholipid, diheptanoyl phosphatidylcholine, at 520 μM, reduced the maximum inward sodium current in voltage-clamped squid giant axons by >50%. Analysis of these currents by means of the Hodgkin-Huxley equations showed this reduction to be mainly the result of a large depolarizing shift in the voltage dependence of the steady state activation parameter, m_∞. The voltage dependence of the steady state inactivation parameter, h_∞, was also moved in the depolarizing direction and the axonal membrane capacitance per unit area measured at 100 kHz was increased. A longer chain length derivative, didecanoyl phosphatidylcholine, had no significant effect on the axonal sodium current at concentrations of 3.7 and 18.5 μM. Dioctanoyl phosphatidylcholine was intermediate in its effects, 200 μM producing approximately the same current suppression as 520 μM diheptanoyl phosphatidylcholine, together with depolarizing shifts in m_∞ and h_∞. These effects may be contrasted with those of the normal and cyclic alkanes (1–3), which tend to move both m_∞ and h_∞ in the hyperpolarizing direction and to reduce the capacitance per unit area at 100 kHz. The above results are all consistent with the hypothesis that small hydrocarbons thicken, while short-chain phospholipids thin, the axonal membrane. Thus membrane thickness changes may be of considerable importance in determining the behavior of the voltage-gated sodium channel.

INTRODUCTION

It has been suggested that the actions of a wide variety of neutral molecules on the sodium current of the squid giant axon may be accounted for in terms of physical effects exerted predominantly on the lipid of the axonal membrane (1–5). One of the more detailed mechanisms proposed is that small hydrocarbons, e.g., n-pentane and cyclopentane, move the voltage dependence of the steady state inactivation parameter, h_∞, in the hyperpolarizing direction by increasing the thickness of the axonal membrane around the sodium channel. Such an increase in thickness would reduce the electrical field across the membrane, which is equivalent to a positive shift in the membrane voltage. The slope of the curve of h_∞ vs. membrane potential is reduced at the midpoint by treatment with small hydrocarbons, which is also consistent with an increase in axonal membrane thickness. A quantitative theory linking the shifts and slope changes caused by hydrocarbons to membrane thickness changes has been developed, in which it is assumed that the membrane electrical field is linear (1, 2, and, for a full discussion, see reference 3). One prediction of this theory is that the adsorption of substances that would thin the membrane should cause a shift of the h_∞ curve in the opposite, i.e., depolarizing, direction.

Preliminary experiments with the short-chain length phospholipid dioctanoyl phosphatidylcholine indicated that this was the case (4). The involvement of membrane thickness changes in these effects has now been further explored by investigating the action of two other members of the phospholipid series, diheptanoyl and didecanoyl phosphatidylcholine. These would be expected to produce a greater and a lesser thinning, respectively, of the axonal membrane than the dioctanoyl derivative and, therefore, greater and lesser effects on the sodium current. The effects of phospholipids on the axonal sodium current are also of interest in that since such substances are lipids, they are not likely to act in a manner any more specific than the lipids already present in the membrane. Any effect that such substances may have, therefore, should be relatively easy to understand in physical terms and may contribute to an understanding of the action of neutral anaesthetics.

METHODS

Experiments were performed using giant axons of the squid, *Loligo forbesi*. The methods used in voltage-clamp studies of the axonal sodium current and the analysis of these currents in terms of the parameters of the Hodgkin-Huxley equations were as described by Haydon and Kimura (1) and Haydon and Urban (2). Measurements of axonal impedances at the resting potential and their conversion into measurements of axonal membrane capacity per unit area were as described by Haydon and Urban (2, 7) and Haydon et al. (6).

For measurements of axonal impedance and voltage-clamp experiments on intact axons with unblocked potassium channels, the external

solution consisted of (concentrations in millimoles per liter): NaCl, 430; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 50; trizma base, 10 (full Na artificial sea water [ASW]). The pH was adjusted to 7.4 by adding HCl. In the other voltage-clamp experiments the axonal potassium channels were blocked, either by external addition of 1 mM 3,4 diaminopyridine (3, 4 DAP) or by internal perfusion of the axons with a solution containing CsF. In experiments using 3,4 DAP-treated axons, the external NaCl concentration was reduced to 215 mM and 215 mM choline chloride added (1/2 Na ASW). In experiments on perfused axons the external NaCl concentration was 107.5 mM and 322.5 mM choline chloride added (1/4 Na ASW). The internal perfusion fluid consisted of (in millimoles per liter): CsF, 345; sucrose, 400; NaCl, 5; HEPES buffer, 10. The pH was adjusted to 7.3 by adding Cs_2CO_3. Leakage and capacity transient currents were removed by computer subtraction of currents obtained in the presence of 0.3 μM tetrodotoxin (TTX).

Compensation for the series resistance was applied in all voltage-clamp experiments and checks were made during each experiment to ensure that >95% compensation was achieved throughout. Phospholipids were obtained from the Sigma Chemical Co. Ltd. (Poole, Dorset, The United Kingdom). Solutions of lipid were made up ~10 min before use. All experiments were performed at 6 ± 1°C.

RESULTS

In the experiments reported below the phospholipids were introduced via the external solution. Internal addition, via the perfusion fluid, was found to be unsatisfactory owing to the apparent loss of lipid through the axonal membrane into the large external volume. Thus, although qualitatively similar results were obtained, current reductions were smaller, consistent with smaller but unknown concentrations.

Fig. 1 *a* illustrates the suppression of sodium current by 520 μM diheptanoyl phosphatidylcholine in an intact axon with the potassium channels blocked by treatment with 3,4 DAP. In this experiment the maximum inward current was reduced by 64% and, as is shown in Fig. 1 *b*, the voltage that elicited maximum current was moved some 15 mV in the depolarizing direction. The small shift in the potential for zero current was probably the result of leakage currents, which have not been subtracted in these records. Such a shift was not observed in the other experiments. Reversibility of maximum inward current suppression by diheptanoyl phosphatidylcholine, where attempted, was always >90%.

Sodium currents from axons in which the potassium channels had been blocked, and from which leakage and capacity currents had been subtracted (see Methods) were

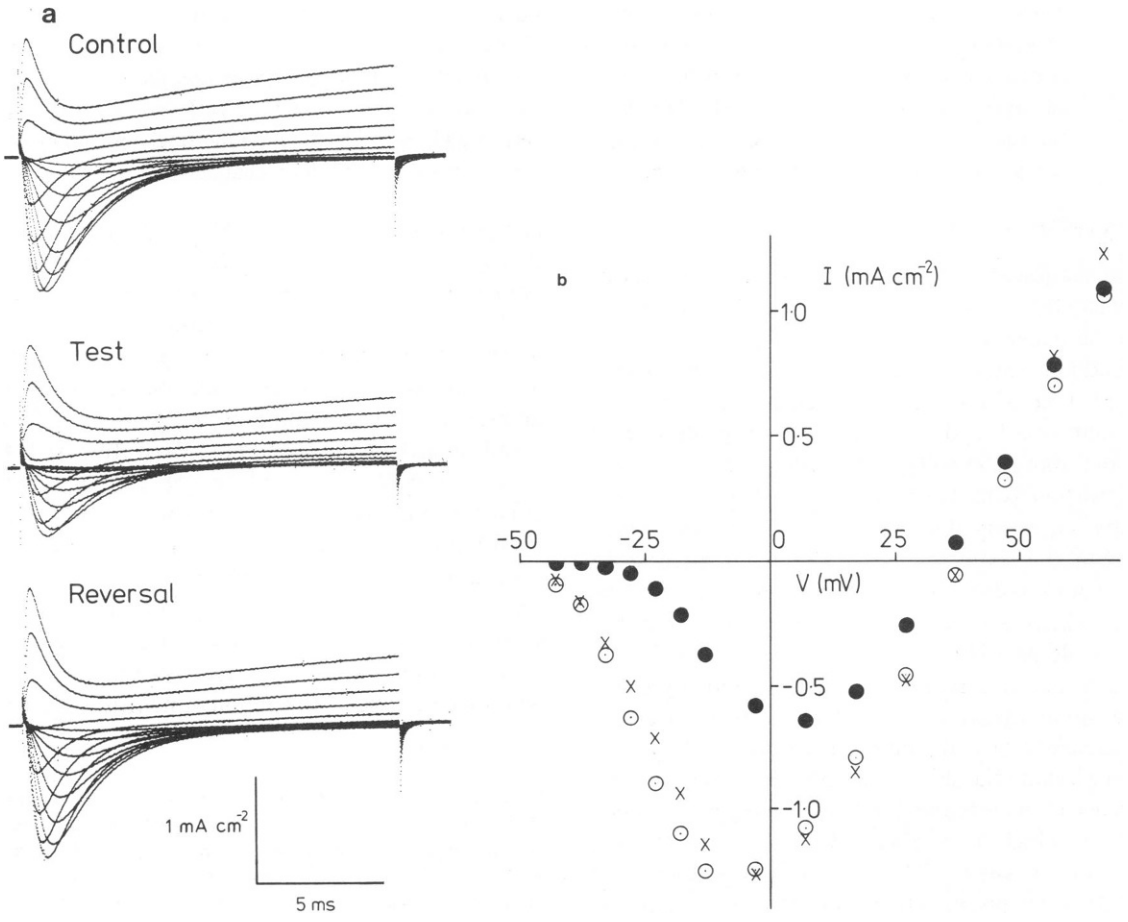

FIGURE 1 (*a*) Ionic currents before (*control*), during (*test*), and after (*reversal*) exposure of a 3,4 DAP-treated intact axon to 520 μM diheptanoyl phosphatidylcholine. The depolarizations from the holding potential of −63 mV were 20–50 mV in 5 mV steps and 50–130 mV in 10-mV steps. A hyperpolarizing prepulse 20 mV in amplitude and 50 ms long was applied immediately before each depolarization. Axon 614. (*b*) The peak current (I) vs. membrane potential (V) for the records of *a*. ⊙, control; ●, 520 μM diheptanoyl phosphatidylcholine; x, reversal.

TABLE I
THE EFFECTS OF DIHEPTANOYL AND DIOCTANOYL PHOSPHATIDYLCHOLINE ON THE SODIUM CURRENT IN SQUID GIANT AXONS

Axon	Conditions	I'_p/I_p	$\bar{g}'_{Na}/\bar{g}_{Na}$	ΔV_h	ΔV_m	$[\tau_h]'_p/[\tau_h]_p$	$[\tau_m]'_p/[\tau_m]_p$
				mV	*mV*		
520 μM diheptanoyl phosphatidylcholine							
613	‡	0.36	—	5.5	—	—	—
614	§	0.36	0.86	8.0	14.5	—	1.02
616	§	0.66	1.02	5.5	6.5	—	1.01
655	‖	0.41	0.68	9.8	17.0	0.48	0.90
683	‖	0.52	0.78	6.5	14.5	0.67*	0.91
200 μM dioctanoyl phosphatidylcholine							
593	‡	0.47	—	4.0	—	—	—
594	‡	0.37	—	5.5	—	—	—

The ionic conditions are described in the Methods section. I'_p/I_p gives the reduction in maximum inward sodium current and $\bar{g}_{Na}/\bar{g}_{Na}$ the reduction in maximum sodium conductance. ΔV_h and ΔV_m are the shifts in the midpoints of the h_∞ and m_∞ curves, respectively, and $(\tau'_h/\tau_h)_p$ and $(\tau'_m/\tau_m)_p$ denote the changes in the peak height of the τ_h and τ_m curves.
*not reliable.
‡Intact axon, full Na ASW.
§Intact axon, ½ Na ASW, 3,4 DAP.
‖Perfused axon, ¼ Na ASW.

analyzed according to the relationships of Hodgkin and Huxley (8), i.e.,

$$I_{Na} = I'_{Na}\,[1 - \exp(-t/\tau_m)]^3 \{h_\infty\,[1 - \exp(-t/\tau_h)] + \exp(-t/\tau_h)\} \quad (1)$$

$$I'_{Na} = \bar{g}_{Na} m_\infty^3\,(V - V_{Na}). \quad (2)$$

The effects of diheptanoyl phosphatidylcholine on the maximum inward sodium current (I_p) and on various parameters of the Hodgkin-Huxley equations (the maximum sodium conductance, $\bar{g}_{Na}$, the voltage dependences of h_∞ and m_∞, and the peak values of the time constants of inactivation, τ_h, and activation, τ_m) are given in Table I. (The possibility that a substantial fraction of the m_∞ shift observed was the result of inadequate series resistance compensation was examined by analyzing the effects of low concentrations of TTX. The results indicated that the maximum error in assessing voltage shifts in m_∞ in a perfused axon with 50% suppression of I_p was <1 mV). Fig. 2 shows the effects of 520 μM diheptanoyl phosphatidylcholine on the voltage dependence of m_∞, h_∞, τ_m, and τ_h.

The effects of diheptanoyl phosphatidylcholine were similar in all experiments, although the current suppression seen with axon 616 was markedly less than that produced in the other four axons. The maximum sodium conductance was reduced in most cases but the major factor that reduced the peak current was the large (generally ~15 mV) depolarizing shift in the voltage dependence of m_∞. Where recovery was attempted this voltage shift reversed to within 2 mV. The depolarizing shift in the voltage dependence of h_∞ was always less than the shift in m_∞ and again reversed to within 2 mV. No clear overall effect of diheptanoyl phosphatidylcholine on the slope at the midpoint of the h_∞ curve was discerned. Individual results obtained varied between the slight decrease shown in Fig. 2 and an increase noted with axon 614. The τ_h and τ_m curves were also moved in the depolarizing direction, but whereas the peak value of τ_h was noticeably reduced, that of τ_m was barely affected.

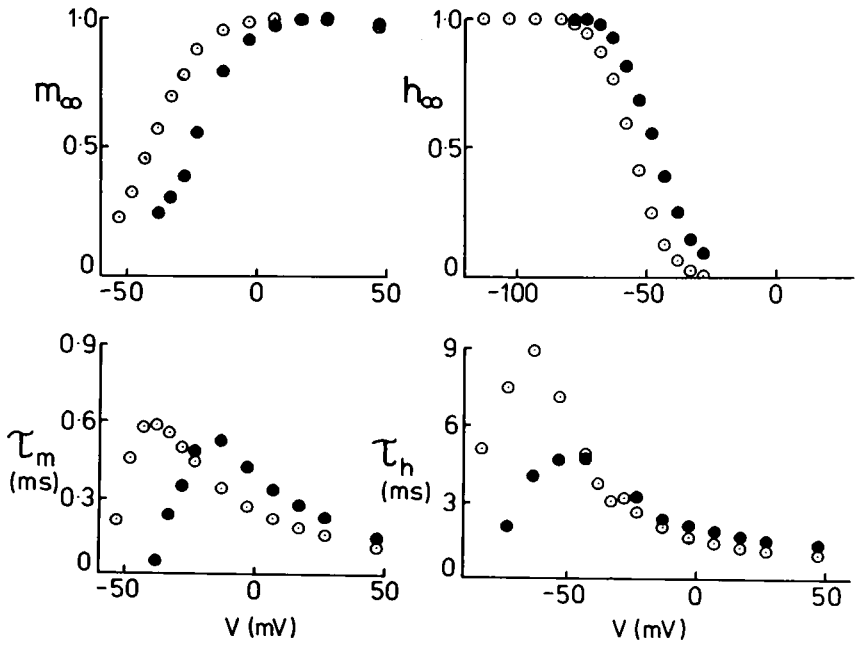

FIGURE 2 The effects of 520 μM diheptanoyl phosphatidylcholine on various parameters of the Hodgkin-Huxley equations; m_∞, h_∞, τ_m, and τ_h, in a CsF perfused axon. V is the membrane potential. ⊙, control; ●, test; axon 655.

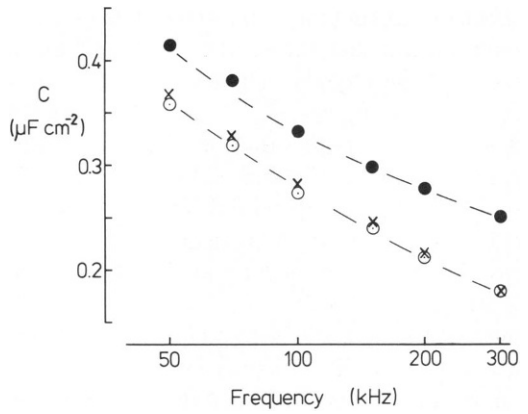

FIGURE 3 The effect of 520 μM diheptanoyl phosphatidylcholine on the axonal membrane capacity per unit area (C) at various frequencies. ⊙, control; ●, test; x, reversal; axon 691.

Fig. 3 shows the effect of 520 μM diheptanoyl phosphatidylcholine on the axonal membrane capacity per unit area (C), at various frequencies of measurement. The short-chain phospholipid clearly increased the capacity per unit area at all the frequencies used and that increase was reversible. In the experiment shown (axon 691) the capacity per unit area measured at 100 kHz increased by 22% and 85% of that change reversed. In a separate experiment (axon 689) a 30% increase in C at 100 kHz was obtained, of which 56% reversed.

The effects of dioctanoyl phosphatidylcholine on the squid giant axon sodium current have been reported by Haydon and Urban (4). Two further experiments with a higher concentration of lipid (200 μM) were performed, the results of which are given in Table I. This concentration of dioctanoyl phosphatidylcholine reduced I_p by over 50% and moved the midpoint of the h_∞ curve some 5 mV in the depolarizing direction. The peak of the I-V curve was also moved in the depolarizing direction by 14–15 mV. The effects of dioctanoyl phosphatidylcholine were less reversible than those of diheptanoyl phosphatidylcholine.

Didecanoyl phosphatidylcholine had no observable effect on the sodium current. Application of 3.7 μM for ~50 min and of 18.5 μM for ~40 min resulted in no significant change in the voltage dependence of h_∞ and a ≲5% reduction in I_p.

DISCUSSION

Suppression of Current

Diheptanoyl and dioctanoyl phosphatidylcholine reversibly suppressed the inward sodium current in squid giant axons. These phospholipids would be expected to partition into the lipid region of the axonal membrane rather than to have a specific effect on the channel protein itself. The experiments reported here therefore strongly suggest that sodium channel function may be perturbed by alterations in the composition and physico-chemical properties of the surrounding lipid.

Shifts in Voltage-dependent Parameters

Small hydrocarbons decrease the capacity per unit area of axonal membranes (1, 3, 6), suggesting that the membrane thickness has increased, and move the voltage dependence of h_∞ and m_∞ in the hyperpolarizing direction. Diheptanoyl phosphatidylcholine caused a reversible increase in axonal membrane capacity per unit area (see Fig. 3), and moved the voltage dependence of h_∞ and m_∞ in the depolarizing direction. The theory advanced by Haydon and Kimura (1) linking shifts in h_∞ to membrane thickness changes is therefore supported by these observations. In particular, the possibility that the actions on the channel occur via the surrounding lipid is strengthened. Thus it could be argued that the hydrocarbons act by adsorbing into hydrophobic regions of the sodium channel protein, rather than the lipid, so causing swelling or increase in thickness. However, it is less easy to imagine how the adsorption of the phospholipid into the protein could produce a thinning. A quantitative analysis of lipid-induced–capacity changes in terms of membrane-thickness changes is hindered by the fact that some of the observed increase must be the result of an increase in membrane area.

Small hydrocarbons clearly reduce the slope of the h_∞ curve at the midpoint (1–3), while diheptanoyl phosphatidylcholine has no obvious effect on the slope. A depolarizing shift in the voltage dependence of h_∞ without a change in slope could arise from a membrane thinning if the h_∞ sensor were a dipole that originated from a protein helix, which initially spanned the whole of the membrane field. Then a reduction in thickness would increase the membrane field but at the same time could reduce the effective dipole moment of the sensor in the same proportion, simply because less of the helix would be in the membrane and experience the field. The result would be equal and opposite effects on the slope.

Dioctanoyl phosphatidylcholine caused a smaller shift in h_∞ than diheptanoyl phosphatidylcholine and didecanoyl phosphatidylcholine had no effect. The effects on h_∞ therefore declined as the expected effects on membrane thickness declined. The concentrations of the three lipids used were chosen for the following reasons. 520 μM diheptanoyl phosphatidylcholine was found to produce ~50% suppression of the sodium current and was therefore a convenient concentration to use. It is also well under the critical micelle concentration for this lipid (9). Comparable adsorption of the dioctanoyl and didecanoyl phosphatidylcholines into the axonal membrane might be expected at concentrations that bear the same ratio to the corresponding critical micelle concentration as for the diheptanoyl derivative. On this basis the concentrations of the dioctanoyl and didecanoyl phosphatidylcholines should have been 100 and 1.9 μM, respectively (9). In fact, 200 μM dioctanoyl phosphatidylcholine produced just more than 50% suppression of I_p and 18.5 μM didecanoyl phosphatidylcholine had no significant effect. Thus a fraction of the critical micelle concentration of the didecanoyl derivative

some ten times larger than that of the diheptanoyl derivative was inactive. This indicates either a failure to adsorb to the membrane according to the rules established for lipid bilayers, which seems unlikely, or a reduced effectiveness of the longer chain derivative, which is as predicted from the thickness theory. Since low aqueous concentrations of lipid soluble molecules are liable to be depleted by adsorption to the considerable lipid sinks present, such as the Schwann cells, this could constitute an alternative explanation. Such an explanation can, however, be ruled out since 22 μM n-decanol reduced the peak sodium current by 20% after 13 min exposure and 10 μM n-dodecyl (oxyethylene)$_3$ alcohol produced a 10% reduction after 18 min.

The shift at the midpoint of the m_∞ curve caused by diheptanoyl phosphatidylcholine was always larger than the shift in the h_∞ curve. One explanation for this is that the m gate sensed a greater change in membrane field on adsorption of lipid than the h gate, perhaps because the membrane thinning was greater around the m gate. An increase in the external calcium concentration in nerve preparations also shifts the activation and inactivation systems in the depolarizing direction, and also appears to affect the activation system more than the inactivation system (10, 11) so this action of phospholipids is not unique. (In frog muscle membranes, however, Hakin and Campbell [12] have reported equal shifts in activation and inactivation for a given change in calcium concentration.) In the squid giant axon the m system is affected by compounds such as the n-alkanols, which do not greatly affect the h system (3, 4). This effect has been postulated to arise from an asymmetric action on the membrane dipole potential to which the h gate is insensitive. If adsorption of phospholipid increased the external surface dipole potential (i.e., by increasing the surface concentration of lipids, which have very large dipole potentials), then that may add an additional shift to that caused by thinning in the m system.

Maximum Sodium Conductance

Diheptanoyl phosphatidylcholine reduced the maximum sodium conductance, $\bar{g}_{Na}$ by ~16%. Larger reductions in $\bar{g}_{Na}$ have been reported for the small hydrocarbons (1–3) and these have been suggested to be the result of membrane thickness increases. It has recently been reported that decreasing the thickness of artificial planar lipid bilayers below a certain optimal value reduces the lifetime of gramicidin channels in that bilayer (13) and it is therefore possible that a membrane thinning caused by the short-chain phospholipid is responsible for the observed reduction in $\bar{g}_{Na}$.

Time Constants

Most anaesthetic-like molecules reduce the peak values of the time constants, τ_m and τ_h, and this has been tentatively attributed to an increase in membrane fluidity (1–5). Adsorption of a short-chain phospholipid could also be expected to increase membrane fluidity and it was therefore surprising to find that the peak value of τ_m was virtually unaffected by diheptanoyl phosphatidylcholine, while peak τ_h was considerably reduced. A differential effect on peak time constants is also produced by a number of other substances (2–5) but its origin is not clear.

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