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THE ASYMMETRICAL EFFECTS OF SOME IONIZED *n*-OCTYL DERIVATIVES ON THE SODIUM CURRENT OF THE GIANT AXON OF *LOLIGO FORBESI*

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SUMMARY

1. The effects of octyltrimethylammonium ions ($OTMA^+$), octyl sulphate ions (OS^-) and octanoic acid (OA) on the sodium current of the voltage-clamped squid giant axon have been investigated using intracellular and extracellular application of the test substances.

2. $OTMA^+$ applied externally at concentrations of 0·8–5·0 mM produces a small reversible increase in the peak inward sodium current in both intact and CsF-perfused axons. Intracellular application of $OTMA^+$ at 0·8 mM to CsF-perfused axons causes a reversible 50 % suppression of peak inward sodium current.

3. The inhibition of peak inward current by internal $OTMA^+$ arises largely from a shift of the steady-state activation parameter (m_∞) in the depolarizing direction along the voltage axis. There is little use dependence of the current suppression by $OTMA^+$

4. OA applied either internally or externally is more effective at suppressing peak inward sodium current at pH 6·0 than at pH 7·4. At pH 6·0 external application of 5 mM-OA to perfused axons causes approximately 60 % suppression. This is associated with a depolarizing shift of m_∞ of about 13 mV and a hyperpolarizing shift of the steady-state inactivation (h_∞) curve of about 4 mV. The effects of internal and external OA are broadly similar except that the h_∞ shift is not seen with internal application.

5. OS^- at concentrations above 2·0 mM produces complete irreversible loss of sodium current. At 2·0 mM, OS^- produces 10 % current suppression and a small depolarizing shift of the m_∞ curve. Internal and external applications of OS^- differ little except that external OS^- causes a 25 % increase in the time constant of activation (τ_m).

6. The possible origins of these effects are discussed. It is proposed that the shift of m_∞ caused by internal $OTMA^+$ is due to a diminution of the lipid dipole potential at the internal surface of the membrane caused by $OTMA^+$ adsorption. This effect could also account for the m_∞ shift caused by OA.

7. The results showing that OA produces shifts of opposite sign in the voltage dependence of m_∞ and h_∞ are discussed with respect to their implications for models of sodium channel gating.

INTRODUCTION

A number of recent studies have examined the effects of various non-ionic anaesthetic molecules on the ionic currents of squid and crayfish nerves (Haydon, Requena & Urban, 1980; Haydon & Kimura, 1981; Bean, Shrager & Goldstein, 1981; Haydon & Urban, 1983*a–c*). Many of these observations can be accounted for on the basis of a small number of physico-chemical interactions between the anaesthetic and some lipid portion of the axonal membrane. Perturbations in bilayer thickness, surface tension and surface dipole potential caused by anaesthetic adsorption have been invoked as possible explanations for their nerve blocking action (Haydon, Elliott & Hendry, 1984). There appear to be significant differences between the effects of non-ionic general anaesthetics and the actions of clinical local anaesthetics on the sodium current in nerve. Local anaesthetics, such as lignocaine, appear to act by direct binding to a site within the ion pore of the sodium channel; they are most effective as cations acting from inside the axon (Hille, 1977; Cahalan, 1978). The experiments reported in this paper were designed to explore the differences further by using ionized and ionizable test molecules which are closely related in structure to the non-ionic anaesthetics already examined.

The three substances chosen for study were octyltrimethylammonium ($OTMA^+$), octyl sulphate (OS^-) and octanoic acid (OA). A number of non-ionic octyl derivatives have already been investigated on squid axons, e.g. *n*-octane, octan-1-ol, methyl octanoate, *n*-octyl (oxyethylene)$_3$ alcohol, methyl heptyl ketone and dioctyl lecithin (Haydon & Urban, 1983*a*,*b*; J. R. Elliott, D. A. Haydon & B. M. Hendry, in preparation). It is therefore relatively simple to examine the extent to which these ionic surfactants are similar in their mode of action to their non-ionic counterparts. The use of relatively impermeant ionic substances also allows questions concerning the asymmetry of the axonal membrane and its response to anaesthetics to be considered. Experiments were carried out to compare intracellular and extracellular routes of application for the three substances. For OA the relative activities of its neutral and anionic forms were examined by varying the pH of its aqueous solution.

The sodium current records from experiments with CsF-perfused axons were analysed by means of equations similar to those proposed by Hodgkin & Huxley (1952). In this way the effects of test substances can be described and discussed in terms of alterations in the steady-state parameters (m_∞, h_∞), time constants (τ_m, τ_h) and maximum sodium conductance ($\bar{g}_{Na}$) used in the Hodgkin–Huxley formulation. The results for these ionic test molecules and OA are of the same form as those for the non-ionic molecules and most of the data can be interpreted with ideas based on the earlier work. The asymmetry of membrane sensitivity to $OTMA^+$ suggests that the axonal membrane possesses an asymmetrical distribution of lipids at its internal and external interfaces, and that the non-ionic anaesthetics may act predominantly on the inner surface of the membrane.

METHODS

Giant axons were dissected from the mantles of freshly killed *Loligo forbesi*. The axons were finely cleaned and were usually between 600 and 1000 μm in diameter.

The external bathing solution for most experiments with intact axons was of the following

composition (mM): NaCl, 430; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 50; Trizma base, 10. The pH was adjusted to 7·4 or 6·0 by the addition of HCl. To check for pH artifacts related to the choice of buffer some experiments were performed using 10 mM-piperazine-*N*,*N*′-bis (2-ethane sulphonic acid) (PIPES) in place of Trizma. At a given pH the change of buffer made no difference to the results. For experiments on perfused axons the external NaCl concentration was reduced to 107·5 and 322·5 mM-choline chloride added. Sodium currents were suppressed where necessary by addition of 0·3 μM-tetrodotoxin (TTX).

The usual internal perfusate was of composition (mM): CsF, 345; sucrose, 400; NaCl, 5; HEPES, 10. The pH was adjusted to 7·3. The pH 6·0 internal perfusate was of composition (mM): CsF, 225; sucrose, 490; NaCl, 5; citric acid, 40; CsOH, 120.

Details of the chamber in which the axons were mounted, the electrodes, the perfusion technique and the means of introducing the external bathing solutions have been described previously (Haydon *et al.* 1980). The perfusion capillary had an external diameter of approximately 450 μm. In order to change the internal perfusate from a control to a test solution, or vice versa, the axon was re-perfused by at least two insertions of the capillary. The voltage clamp and data acquisition procedures were as in Kimura & Meves (1979) and the analysis of the sodium currents was as described by Haydon & Kimura (1981). Compensation for the series resistance was applied in all experiments.

The experiments were carried out at 6 ± 1 °C. Solutions of OS^- were made up as the sodium salt less than 5 min prior to use. OA was Grade 1 from Sigma (Poole, Dorset). $OTMA^+$ was synthesized as the bromide. Control experiments were performed using NaBr to ensure that none of the apparent effects of $OTMA^+$ were due to bromide ions.

RESULTS

Intact axons were employed whose resting potentials were in the range -50 to -60 mV. They were voltage clamped to -55 or -60 mV. Prior to the depolarizing test step, a pre-pulse to -80 mV was applied for 50 ms to remove fast inactivation. The test pulse for measurement of peak inward current was that which gave the maximum current under control conditions. This pulse was usually to -10 or zero mV. CsF-perfused axons were voltage clamped at -70 mV and a pre-pulse of 50 ms duration at -90 mV was employed. Again, the test pulse for monitoring peak inward current was established in control conditions and then remained fixed for each experiment. The final records in each experiment on perfused axons were obtained in the presence of TTX. These TTX-insensitive currents were subtracted prior to the analysis of sodium currents. The sodium currents were analysed as in earlier work (Haydon & Urban, 1983*a*) according to an equation derived from the relationships of Hodgkin & Huxley (1952), i.e.

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

The value of τ_h in the vicinity of its peak was determined by a variation of the method introduced by Gillespie & Meves (1981) as described in Haydon & Urban (1983*a*). This analysis allows the effects on peak inward current to be dissected into the alterations in the Hodgkin–Huxley parameters. In Table 1 appears a summary of the data on perfused axons; it includes the effects on peak inward current (I_p), m_∞, h_∞, τ_m, τ_h and $\bar{g}_{Na}$. The figures for I_p and $\bar{g}_{Na}$ are means of the fractional suppressions. The figures for m_∞ and h_∞ are means of the voltage shifts in the mid-point of each curve (ΔV_m and ΔV_h respectively). The figures for the time constants are the fractional change of the peak value; this measure was employed so that voltage shifts of the time constant curves can be discounted.

TABLE 1. The influence of octyltrimethylammonium ions ($OTMA^+$), octanoic acid (OA) and octyl sulphate ions (OS^-) on parameters of the Hodgkin–Huxley equations. In column 2, i and o indicate internal and external application respectively. The figure in column 3 gives the pH of the solution containing test substance. I_p^t/I_p gives the reduction in the peak inward current and $\bar{g}_{Na}^t/\bar{g}_{Na}$ is the corresponding change in maximum Na conductance. In columns 6 and 7 are shown the shifts ΔV_m and ΔV_h in the mid-points of the m_∞ and h_∞ curves respectively, while in columns 8 and 9 $(\tau_m^t/\tau_m)_p$ and $(\tau_h^t/\tau_h)_p$ give the changes in the peak height of τ_m and τ_h. The figures in parentheses are regarded as unreliable

Substance	Concn. (side) mmol/l	pH	I_p^t/I_p	$\bar{g}_{Na}^t/\bar{g}_{Na}$	ΔV_m(mV)	ΔV_h(mV)	$(\tau_m^t/\tau_m)_p$	$(\tau_h^t/\tau_h)_p$	No. of axons
$OTMA^+$	0·8 (o)	7·4	1·09	1·10	+1·5	+2·2	0·99	0·90	4
$OTMA^+$	0·8 (i)	7·3	0·51	0·77	+6·5	+0·1	0·66	0·88	8
OA	5 (o)	6·0	0·36	0·97	+12·7	−3·7	0·75	0·69	4
OA	5 (o)	7·4	0·83	1·02	+5·1	−1·0	0·94	0·89	3
OA	5 (i)	6·0	0·63	0·92	+4·7	+0·4	0·96	(1·0)	3
OA	5 (i)	7·3	0·84	0·94	+2·8	(+2·0)	1·03	0·82	2
OS^-	2 (o)	7·4	0·92	1·06	+1·7	+2·0	1·24	1·03	2
OS^-	2 (i)	7·3	0·88	1·05	+3·0	−1·65	0·96	1·07	3

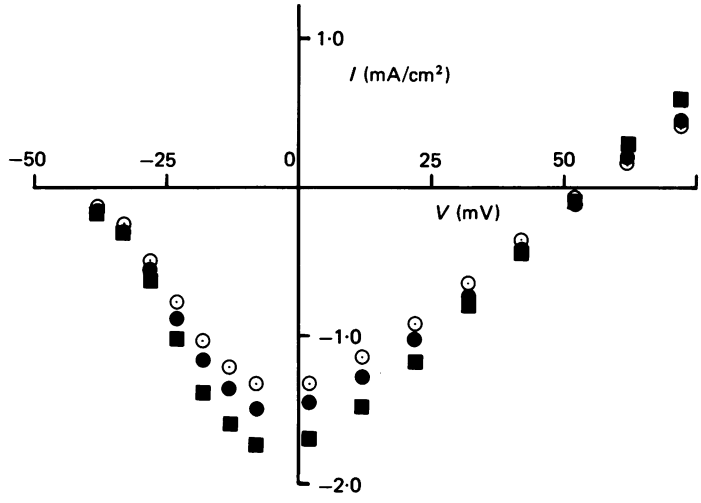

Fig. 1. The effect of external $OTMA^+$ on the I–V relationship for sodium current in an intact axon. The open circles are control data, 0·5 mM-$OTMA^+$ is represented by filled circles and 2·0 mM-$OTMA^+$ by filled squares. The reversal points are omitted for clarity but are coincident with the control values. V is the membrane potential, I is the membrane current. Axon 370.

$OTMA^+$

External application of $OTMA^+$ at 0·5–5·0 mM produced small but reproducible increases in the peak inward sodium current in both intact and perfused axons. Fig. 1 shows the I–V curves for peak inward current in an intact axon exposed to 0·5 and 2·0 mM-$OTMA^+$. The reversible increase in I_p occurs with no change in the reversal potential for sodium current. Analysis of records from perfused axons show the sodium current increase to result from an increase in $\bar{g}_{Na}$ (Table 1). 0·8 mM-external

$OTMA^+$ produces a 10 % increase in $\bar{g}_{Na}$. There are also small ($\simeq$ 2 mV) depolarizing shifts of m_∞ and h_∞ along the voltage axis. There is no significant effect on the time constants of activation (τ_m) or inactivation (τ_h).

Internal application of $OTMA^+$ in CsF-perfused axons causes inhibition of sodium current. 5·0 mM-$OTMA^+$ internally produces a complete suppression of peak inward current. 0·8 mM-$OTMA^+$ inside the axon causes a reversible 50 % suppression of I_p. Fig. 2 shows the effect of internal $OTMA^+$ on the I–V relationship in one axon. The

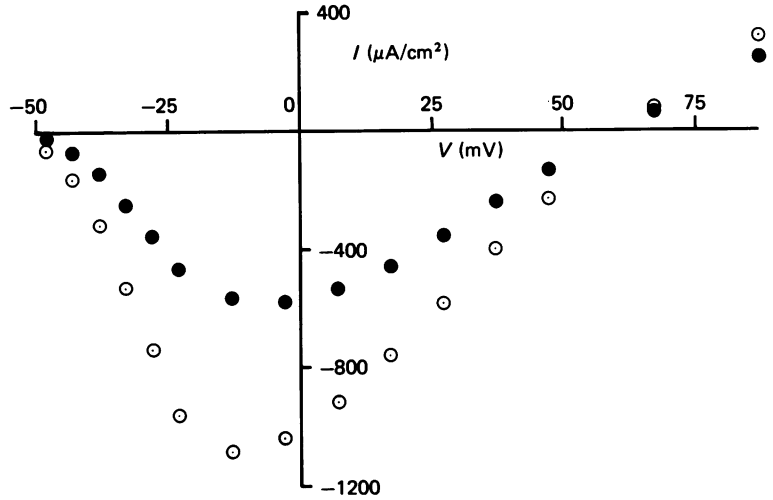

Fig. 2. The effect of 0·8 mM-internal $OTMA^+$ on the I–V relationship of a CsF-perfused axon. Control values (○) were obtained after the test data (●) following re-perfusion with $OTMA^+$-free CsF. V is the membrane potential, I is the membrane current before subtraction of TTX-insensitive current. Axon 524.

reduction in I_p is associated with a depolarizing shift in the voltage for peak inward current. There is no significant change in the potential for zero current. The data for this axon analysed in terms of the Hodgkin–Huxley parameters are displayed in Fig. 3. There is a depolarizing shift of m_∞ of about 7 mV and a reduction in τ_m at the vicinity of its peak. The inactivation parameters h_∞ and τ_h are little affected except that τ_h is reduced at voltages greater than −10 mV. In this axon $\bar{g}_{Na}$ was reduced to 0·85 of control by $OTMA^+$. Mean values for all these effects appear in Table 1. The major contributors to the suppression of I_p are the positive m_∞ shift and the reduction in $\bar{g}_{Na}$. It proved difficult to perform an experiment in which control, test and reversal data were obtained on the same axon. Accordingly some axons were initially perfused with $OTMA^+$ and then re-perfused with $OTMA^+$-free solution so that control records could be obtained prior to TTX addition. Three of these experiments are included in the averages of Table 1 and their data did not differ significantly from the results obtained with initial control experiments. The effects of internal $OTMA^+$ therefore appear fully reversible.

In the presence of internal $OTMA^+$ there is a small increase in the use dependence of the peak inward current produced by repetitive voltage-clamp stimuli. As usual the holding potential was −70 mV and each 15 ms depolarizing pulse to −10 mV

was preceded by a 50 ms pre-pulse to −90 mV. In control conditions stimulation at 5 Hz with this pulse sequence reduced I_p by 4% by the tenth pulse. At 10 Hz the reduction by the tenth pulse was 7%. In the presence of 0·8 mM-internal $OTMA^+$ the corresponding reduction in I_p at 5 Hz was 5% and at 10 Hz it was 12%. These effects are small compared to the 50% reduction in first pulse amplitude caused by this internal concentration of $OTMA^+$.

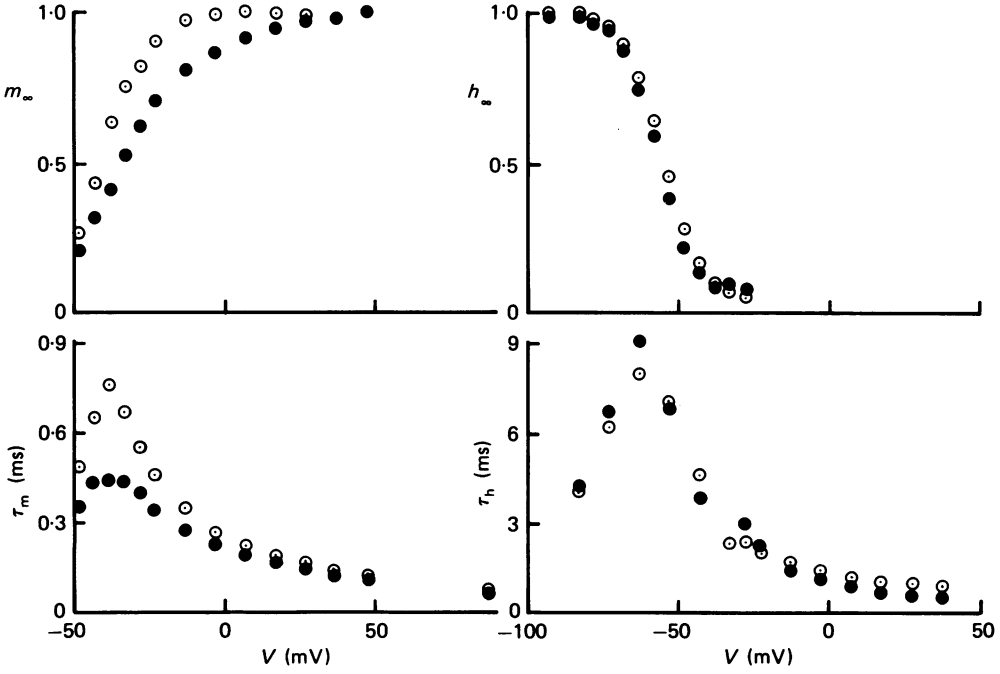

Fig. 3. The effects of 0·8 mM-internal $OTMA^+$ on the various Hodgkin–Huxley parameters. Control values (○) were obtained after the test data (●). V is the membrane potential. Axon 524.

OA

External application of 5·0 mM-OA to intact axons at pH 6·0 or 7·4 caused partial suppression of inward sodium current which was fully reversible. Fig. 4 shows the effect of OA on I_p for two intact axons each of which was exposed to OA at both pH 6·0 and 7·4. The current suppression is greater at pH 6·0. The effect of pH change alone from 7·4 to 6·0 was less than 5%. The currents often failed to reach a steady state but continued to drift downwards in the presence of OA. Exposure for more than 40 min was associated with increasingly irreversible current loss. In intact axons the mean fractional suppression of I_p in 5·0 mM-OA at 30 min exposure was 0·52 (five axons) at pH 6·0 and 0·80 (four axons) at pH 7·4. The effect of 5 mM-external OA on the I–V curve for sodium current in an intact axon is shown in Fig. 5. There is a reversible suppression of current and a depolarizing shift in the voltage stimulus eliciting maximum current. The current reversal potential is unaffected.

Similar results were obtained with external application of 5 mM-OA to CsF-perfused

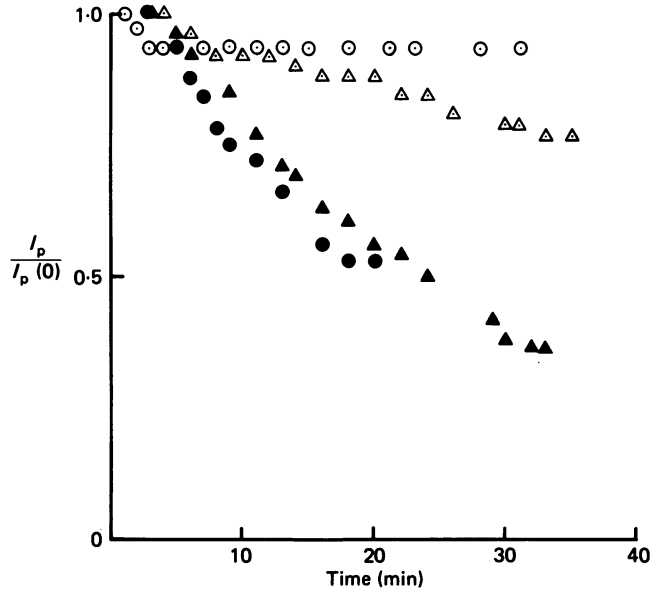

Fig. 4. The effects of 5·0 mM-OA at pH 6·0 and 7·4 on the peak inward current (I_p) in two intact axons. The ordinate is I_p expressed as the fraction of its initial value. The abscissa is time in minutes since change to OA. Filled symbols are OA at pH 6·0 and open symbols at pH 7·4. The circles are axon 382, the triangles are axon 388. These effects were fully reversible.

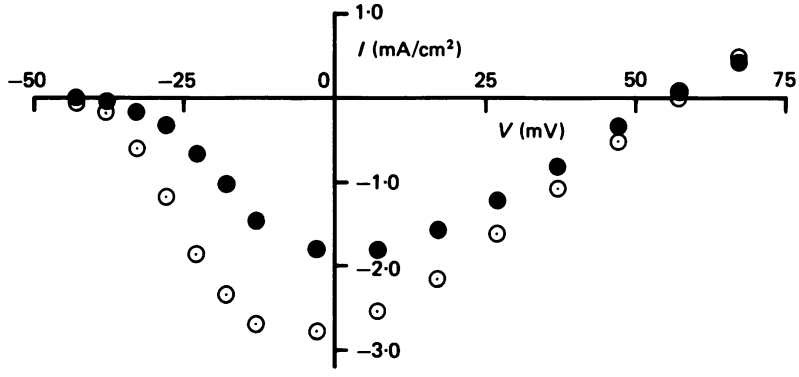

Fig. 5. The effect of 5 mM-external OA at pH 6·0 on the *I–V* relationship for sodium current in an intact axon. The open circles are control data at pH 6·0, the filled circles are test data. Reversal points are omitted for clarity but are coincident with the control points. *V* is membrane potential and *I* is membrane current. Axon 382.

axons, except that the degree of reversibility was more variable. Again OA is more effective in suppression of I_p at pH 6·0 than pH 7·4. The mean changes in I_p at 30 min exposure are shown in Table 1. The effects of 5 mM-external OA at pH 6·0 on the Hodgkin–Huxley parameters for one axon are shown in Fig. 6. There is a depolarizing shift of m_∞ of about 13 mV and a hyperpolarizing shift of h_∞ of 4 mV. The h_∞ curve

also shows a reduction in the slope at its mid-point. Averaged effects of OA are shown in Table 1. The major contribution to the suppression of I_p is the depolarizing shift of m_∞.

Internal perfusion of axons with OA produced results similar to external application. Again the effects were greater at pH 6·0 than at pH 7·3. For experiments involving internal OA at pH 6·0 control data were also obtained at an internal pH of 6·0. The variation of internal pH from 7·3 to 6·0 produced small effects on the parameters of

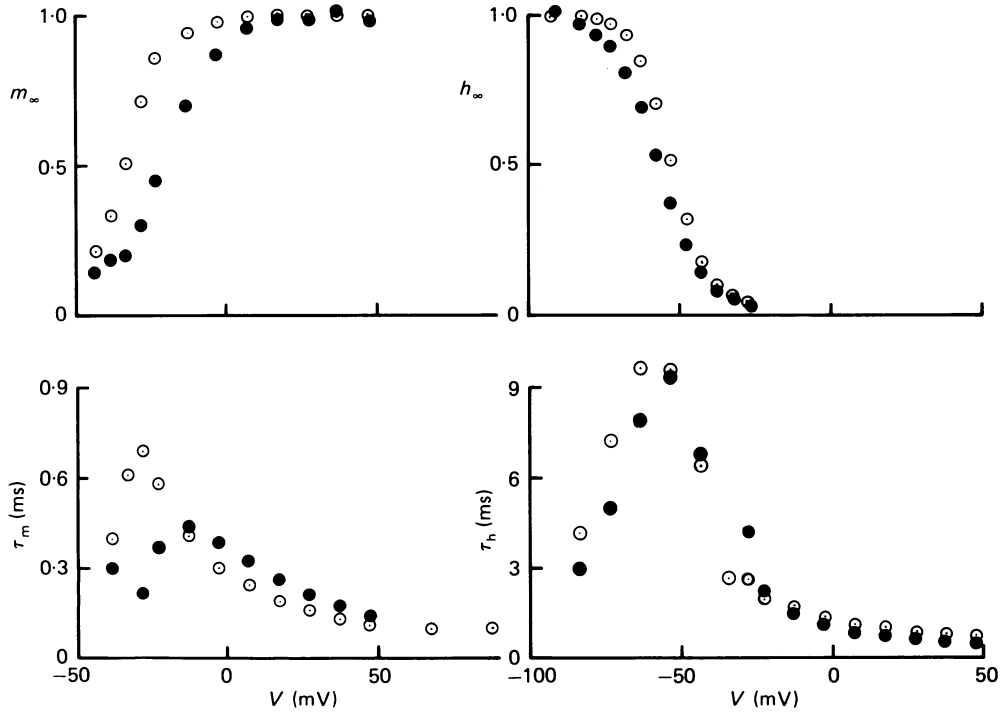

Fig. 6. The effects of 5 mM-external OA at pH 6·0 on the various Hodgkin–Huxley parameters. The open circles are control data while the filled circles are test data taken at 30 min exposure. V is the membrane potential. The reversal in this experiment was over 80%. Axon 504.

h_∞, m_∞, τ_h and τ_m. These were in agreement with the results of Wanke, Carbone & Testa (1980) and Carbone, Testa & Wanke (1981) who also used citrate as internal buffer. One qualitative difference between internal and external application of OA was that internal OA at 5 mM had little effect on h_∞. The other parameters in Table 1 were on the whole affected similarly, but internal OA produced smaller changes. This may be related to problems of depletion of OA from inside the axon by diffusion across the membrane into a large OA-free external sink.

OS^-

At concentrations of 3–5 mM external application of OS^- produced complete irreversible loss of sodium current in both intact and CsF-perfused axons. This loss was associated with a gradual rise in the holding current required to voltage clamp the axon at −55 to −70 mV. At 1·0 mM, OS^- had no significant effect on intact axons. Some small reversible effects could be obtained at 2·0 mM-OS^- but these were variable

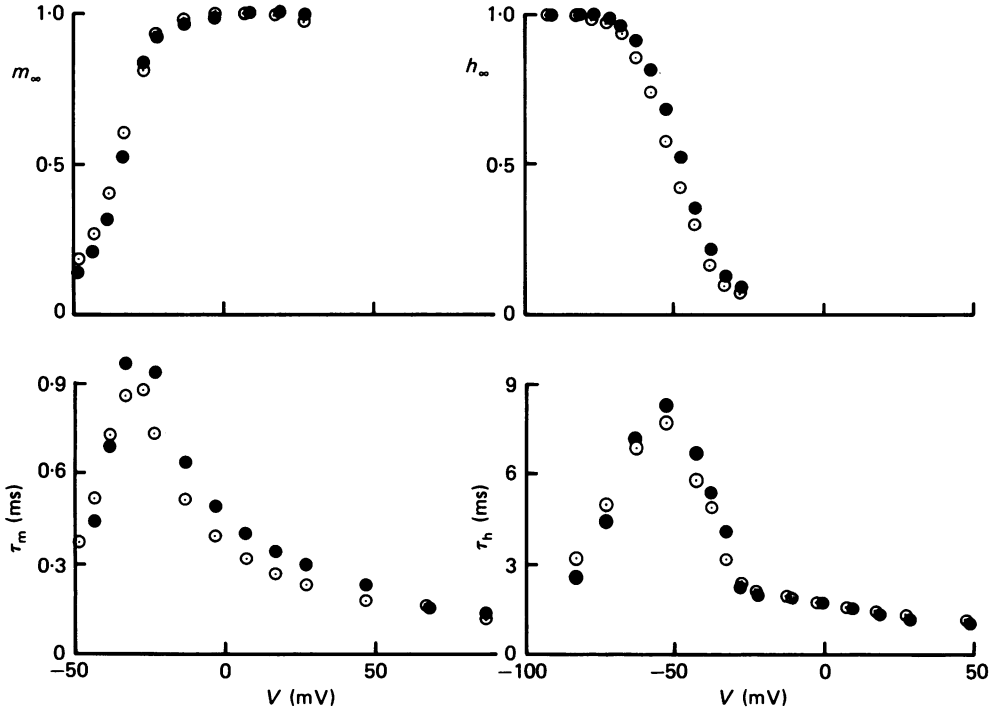

Fig. 7. The effects of 2 mM-external OS^- on the various Hodgkin–Huxley parameters. The open circles are control data while the filled circles are test data obtained after 20 min exposure to OS^-. *V* is the membrane potential. The reversal points are omitted but are coincident with the control points. Axon 465.

in size and usually associated with irreversible changes. It was therefore difficult to obtain data for OS^- which could be directly compared with the results for $OTMA^+$ and OA. Five experiments in which OS^- was applied at 2·0 mM without pronounced effects on the holding current are summarized in Table 1. The suppressions of I_p by internal or external OS^- are similar at about 10%. This is associated with a positive shift of m_∞ and external OS^- also causes an increase in τ_m. Data from one axon in which the effect of external OS^- was fully reversed are shown in Fig. 7.

DISCUSSION

OA and pH effects

The existing data for non-ionic *n*-octyl derivatives indicate that 50 % suppression of peak inward sodium current in the squid axon occurs at concentrations below 0·5 mM (Haydon & Urban, 1983*b*). It was therefore interesting to find that OA concentrations of the order of 5·0 mM were required to produce significant suppression of sodium currents. Also, despite the high concentration, relatively long times were required to reach a steady-state suppression. These initial observations led to further experiments designed to compare internal and external routes of application of OA and to compare data for pH 6·0 with those for pH 7·3–7·4. The effects of OA were greater at pH 6·0 than at pH 7·3–7·4, whether application was internal or external. The pK_a of OA at 25 °C is 4·85, and the pH changes used here would not have affected the concentration of the anionic octanoate moiety by more than a few per cent. The neutral OA molecule would have been at some twenty times higher concentration at pH 6·0 than pH 7·3. The results suggest, therefore, that the neutral form is more active than the anionic species. The long time course of action when 5·0 mM-OA is applied externally can be explained on the basis that non-ionic OA molecules which enter the axon are effectively entering a large diffusion sink arising from intra-axonal ionization. However, if the internal and external axonal membrane interfaces are assumed to remain in equilibrium with their respective bulk phases, then the long time course seen with external OA would imply that it acts at the internal interface. External OA at a concentration of 5·0 mM applied at pH 6·0 produced about 60 % suppression of I_p. The concentration of neutral OA molecules at this pH would have been approximately 0·35 mM. This value may be compared to concentrations for 50 % suppression of I_p of 0·29 mM for octanol, 0·35 mM for methyloctanoate, 0·44 mM for *n*-octyl (oxyethylene)$_3$ alcohol (Haydon & Urban, 1983*b*) and 0·3 mM for methyl heptyl ketone (J. R. Elliott, D. A. Haydon & B. M. Hendry, unpublished observations). The assumption that the non-ionic form of OA is the active species is therefore consistent with data for structurally related non-ionic molecules. The effects of OA on the steady state Hodgkin–Huxley parameters are also consistent with results for these other molecules. OA behaves as if it were intermediately placed between octanol and methyl octanoate (Haydon & Urban, 1983*b*). All these molecules produce an appreciable positive m_∞ shift. A likely explanation for this effect is that based on preferential internal interfacial adsorption and consequent lipid dipole potential changes (Haydon *et al.* 1984). These molecules also produce negative shifts in h_∞ in the order methyloctanoate > methyl heptyl ketone > OA > octanol. Where significant the h_∞ shifts are associated with a reduction in slope of the h_∞ curve at its mid-point ($h_\infty = 0{\cdot}5$). This slope reduction can be seen in Fig. 6. The mean slope change caused by 5 mM-external OA at pH 6·0 is 0·93. This combination of a negative shift of h_∞ and slope reduction is prominent in the results for hydrocarbon anaesthetics (Haydon & Kimura, 1981; Haydon & Urban, 1983*a*). An explanation based on bilayer thickness changes could explain both shift and slope effects for these non-ionic non-polar molecules (Haydon *et al.* 1984). The basis of this explanation is that a membrane thickness increase will reduce the intramembrane electric field at a given

value of membrane voltage. Artificial bilayer studies suggest that octanol does not cause membrane thickening (Elliott & Haydon, 1979) and this conclusion is likely to hold for the other surface active octyl derivatives. The shift and slope change seen with a substance like methyloctanoate therefore suggest a direct interaction between the anaesthetic and the sodium channel, perhaps causing a local membrane thickening in the vicinity of the channel. A more detailed discussion of the relationships between anaesthetic structure and h_∞ effects will be presented in a following publication (J. R. Elliott, D. A. Haydon & B. M. Hendry, in preparation). For present purposes it is sufficient to note that OA behaves in a manner consistent with the chemical structure of its neutral form. The effects of OA on the Hodgkin–Huxley time constants are also consistent with the effects of octanol and other related molecules (Haydon & Urban, 1983*b*).

The effects of OA applied internally were less marked than those found with external OA. The simplest explanation for this finding is that significant depletion of OA from the axon took place by transmembrane diffusion into the large external fluid volume. The permeability of bilayer membranes to lipophilic anions is several orders of magnitude higher than their permeability to similarly lipophilic cations (Szabo, Eisenman, McLaughlin & Krasne, 1972) so even the anionic form of OA may leave the axon at a significant rate. When OA was applied externally at 5·0 mM, effects on the inward sodium current occurred over a period of about 30 min. Similar diffusion rates will govern the loss of OA from the axon when applied internally, and its concentration may therefore have been halved within 10 min. One qualitative difference between internal and external OA appears to be that internal OA does not produce a negative h_∞ shift. An attractive explanation for this is that the negative shift is cancelled out by a surface charge effect arising from adsorption of octanoate anions at the internal interface. This hypothesis would predict an h_∞ slope change without a voltage shift. No such slope change is apparent in the records of three experiments in which OA was added internally at pH 6·0. The predicted change of slope would, however, be very small ($< 2\%$).

Asymmetrical effects of $OTMA^+$ on the sodium current

The most striking asymmetry in membrane sensitivity revealed in the present results was exhibited by $OTMA^+$. External application of $OTMA^+$ produced small increases in sodium current while internal application produced current suppression. The reversible 10% increase in $\bar{g}_{Na}$ caused by 0·8 mM-external $OTMA^+$ was an unexpected finding. It is not clear whether this increase resulted from an increase in the surface density of available sodium channels or from an increase in their single-channel conductances. Adsorption of $OTMA^+$ at a site near the external mouth of the ion pore might electrostatically alter the energy profile experienced by a sodium ion passing through the channel, and so affect channel conductance. However, local positive charge at the channel entrance appears more likely to decrease rather than increase channel conductance. The alternative explanation that external $OTMA^+$ somehow recruits more sodium channels is not very attractive and has no independent support. The effect of external $OTMA^+$ on $\bar{g}_{Na}$ has, therefore, no obvious explanation. The small (~ 2 mV) depolarizing shifts of m_∞ and h_∞ caused by

external $OTMA^+$ are consistent with surface charge effects (Frankenhaeuser & Hodgkin, 1957; Gilbert & Ehrenstein, 1969).

The blockage of sodium current by internal application of $OTMA^+$ reported here is superficially similar to the actions of many local anaesthetics and amphipathic cations on the squid (Yeh, 1977; Cahalan, 1978). The usual explanation for this behaviour is that a blocking site exists within the channel itself which is accessible to positively charged molecules only from the axoplasmic side (Strichartz, 1973; Hille, 1977). The asymmetry in membrane sensitivity is therefore held to reside in the asymmetrical structure of the sodium channel itself. However, two features of the present data distinguish the effects of internal $OTMA^+$ from the effects of substances like procaine, tetracaine and QX-222 (Cahalan, 1978). First the sodium current block by internal $OTMA^+$ shows no significant use dependence at stimulation rates up to 5 Hz. This is in contrast to clear use-dependent block by the other substances at frequencies of 1 Hz and below. Secondly, the block by internal $OTMA^+$ arises largely from a positive shift of m_∞ along the voltage axis. This effect has not been noted for the other substances and is difficult to reconcile with a model of direct cation binding within the ion pore of the sodium channel. These considerations lead us to suggest that the axonal membrane asymmetry to the actions of $OTMA^+$ resides, at least partly, in an asymmetry in the adsorption of the $OTMA^+$ which, in turn, is related to the asymmetry of the membrane lipids and/or proteins. The m_∞ shift may be explained on the basis of alterations in the internal membrane lipid dipole potential caused by adsorption of $OTMA^+$ at the internal interface (see e.g. Haydon, 1975; Lundström, 1977).

The positive m_∞ shift caused by internal $OTMA^+$ is a description of the fact that the sodium current evoked by small depolarizing voltage pulses is blocked more by $OTMA^+$ than the current evoked by large depolarizations (e.g. Fig. 2). The voltage dependence of block by internal cations like QX-222 is of the opposite form; block increases with increasing depolarization (Cahalan, 1978). Thus the voltage dependence of block by $OTMA^+$ is inconsistent with a model in which membrane voltage affects the partitioning of the internal cation to a site within the ion pore. Shifts in m_∞ can also be produced by alterations in internal surface charge (Chandler, Hodgkin & Meves, 1965). However, a positive shift of m_∞ due to internal $OTMA^+$ is not in the expected direction. It is also not clear why the h_∞ curve should remain unaffected if internal surface charge effects are important, as other studies have shown both m_∞ and h_∞ to be approximately equally affected (Baker, Hodgkin & Meves, 1964; Chandler *et al.* 1965).

A semi-quantitative explanation for the m_∞ shift produced by internal $OTMA^+$, involving alterations in lipid dipole potentials, can be achieved using two basic assumptions. First, that the squid axonal membrane lipid exhibits interfacial dipole potentials of $\gtrsim 400$ mV. Secondly, that there is an asymmetry of composition between the internal and external membrane surfaces which results in a greater adsorption of $OTMA^+$ at the inner than at the outer interface. The transmembrane variation in voltage which might arise from lipid dipole potentials is illustrated schematically in Fig. 8, from which surface charge effects have been omitted for clarity. The existence of these large dipole potentials has been confirmed in a wide variety of

phospholipid bilayers and monolayers (Papahadjopoulos, 1968; Hladky & Haydon, 1973; Reyes & Latorre, 1979). It therefore is not unreasonable to suppose that they also exist in the lipid bilayer portions of the squid axonal membrane. Dipole potential magnitudes for phosphatidylcholine and phosphatidylethanolamine are in the range 400–450 mV (Papahadjopoulos, 1968; Hladky & Haydon, 1973). An asymmetrical distribution of electron density across the bilayer has been demonstrated by X-ray methods for the myelin membrane of frog sciatic nerve (Caspar & Kirschner, 1971).

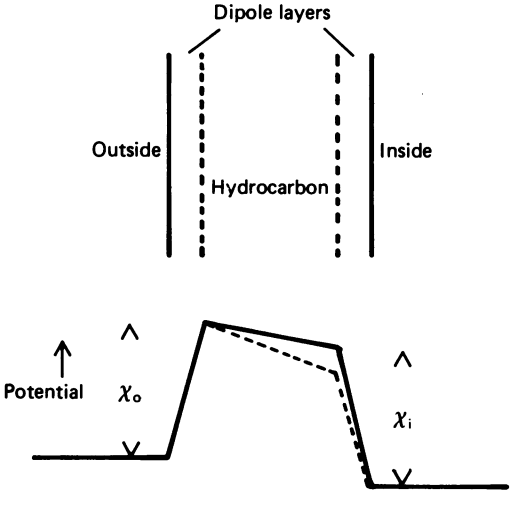

Fig. 8. A schematic diagram of the transmembrane variation in electrical potential due to the presence of the lipid dipole potentials. The inside and outside dipole potentials are of magnitude χ_i and χ_o respectively. The dotted line in the lower diagram represents the potential after $OTMA^+$ adsorption at the inside interface has reduced χ_i.

One interpretation of these results is that the outside half of the cell membrane is richer in cholesterol than the inside. Indirect evidence of surface charge differences between internal and external interfaces of the squid axonal membrane suggests lipid asymmetry, particularly in the distribution of phosphatidylserine (Fohlmeister & Adelman, 1982). Cholesterol inhibits the adsorption of a number of anaesthetics into lipid bilayers (Miller, Hammond & Porter, 1977; Haydon, Hendry, Levinson & Requena, 1977; Simon, Stone & Bennet, 1979; Smith, Porter & Miller, 1981). It is therefore quite conceivable that $OTMA^+$ is preferentially adsorbed at the axoplasmic interface of the squid nerve membrane.

The effect of such inner adsorption on the lipid dipole potential is illustrated by the dotted lines in Fig. 8. The dipole potential change caused by dodecyltrimethylammonium adsorption at an *n*-heptane/0·5 M-NaCl interface has been measured (Haydon, 1962). At a surface concentration of one ion per 100 $Å^2$ the dipole potential is −180 mV. This result can be used to estimate the effect of $OTMA^+$ adsorption at the inner interface of the squid axonal membrane. If an area per phospholipid headgroup of 70 $Å^2$ is assumed, along with an original lipid dipole potential of

+450 mV, then adsorption of one $OTMA^+$ molecule per 100 phospholipid molecules will reduce the dipole potential by 9 mV. This surface concentration of $OTMA^+$ would affect the surface potential arising from the ionic double layer by less than 2 mV. As shown in Fig. 8, the dipole potential change will alter the electric field within the membrane. To re-establish the original field strength after $OTMA^+$ adsorption, a corresponding increase in membrane voltage (V_m) is required (i.e. V_m must become less negative). If the activation gate of the sodium channel is an internal dipole which 'senses' this electric field within the membrane, then the positive m_∞ shift caused by internal $OTMA^+$ can be quantitatively accounted for by these lipid dipole potential alterations. Moreover the difference between the surface concentrations of $OTMA^+$ at the internal and external membrane interfaces, which must be invoked, is not large.

A dipole potential mechanism of this nature has already been advanced to explain the positive m_∞ shifts caused by a number of neutral anaesthetics in squid nerve (Haydon & Urban, 1983*b*; Haydon *et al.* 1984). The model introduced for neutral molecules also includes an asymmetry of adsorption secondary to an asymmetry of lipid composition. Such ideas may therefore have some general relevance in explaining the commonly found ability of anaesthetic molecules to shift m_∞ in a positive direction. One difficulty with an internal dipole potential model, however, lies in the fact that the h_∞ curve does not show a similar shift. This distinction between m_∞ and h_∞ occurs both for neutral molecules and $OTMA^+$ (Table 1). This apparent discrepancy could be resolved on the basis that an h gate or sensor is separated laterally from the lipid dipoles while the m sensor is within the membrane directly interior to them. There is no direct evidence for this idea but the selective sensitivity of the inactivation mechanism to internal pronase (Rojas & Rudy, 1976) is consistent with such a model.

Aside from the m_∞ shift, internal $OTMA^+$ at 0·8 mM also causes a 23% decrease in $\bar{g}_{Na}$, a reduction in τ_m at its peak and a reduction in τ_h which is increasingly apparent at more positive voltages (Table 1 and Fig. 3). For neutral molecules reductions in $\bar{g}_{Na}$ have been related to membrane thickness and tension changes (Haydon & Urban, 1983*a*) but such effects are unlikely in the case of $OTMA^+$. The $\bar{g}_{Na}$ effect may represent a direct channel blockade via the internal mouth of the ion pore. The voltage-dependent decrease in τ_h could result from a second component of inactivation due to direct $OTMA^+$ interaction with the open channel during the test pulse. If this exogenous inactivation proceeded with a time constant close to that of the intrinsic h mechanism then the two components would not be separately resolved by the present analysis using eqn. (1). Cahalan (1978) has measured rates of exogenous inactivation directly in pronase-treated axons containing cationic local anaesthetics. Certain local anaesthetics caused an 'inactivation' of the sodium current which proceeded with a time constant of about 0·8 ms. This is close to the value for τ_h measured in control axons at positive membrane voltages.

The effects of OS^-

The application of OS^- produced effects which were poorly reversible and irreproducible. Furthermore there was no concentration at which a reversible 50% suppression of I_p could be obtained. Concentrations of OS^- above 2 mM appeared to cause some gross irreversible disruption of membrane architecture such that non-specific increases

in membrane ion-conductance occurred at the same time as loss of sodium current. The presence of a net negative surface charge at both interfaces of the axonal membrane (Chandler *et al.* 1965; Fohlmeister & Adelman, 1982) mediates against anionic surfactant adsorption. It is perhaps not surprising, therefore, that neither OS^- nor octanoate appear to exhibit significant reversible anaesthetic activity in the millimolar concentration range. The adsorption of OS^- which does occur seems to involve gross structural changes. In those experiments where reversible OS^- effects were observed, a positive m_∞ shift was seen for both internal and external application. The activation time constant was, however, asymmetrically affected as 2·0 mM-external OS^- produced a 24% increase in τ_m while internal OS^- produced a slight decrease. If these τ_m effects arise from the physical presence of OS^- molecules near the activation voltage sensor then these results suggest that this sensor lies nearer the extracellular interface of the membrane than the axoplasmic interface.

Separate voltage probes for activation and inactivation

The mathematical success of the Hodgkin–Huxley equations in describing the voltage and time-dependent sodium conductance of the squid axon provides no compelling proof of any particular physical model of sodium channel gating. The original formulation was associated with a model in which activation and inactivation gates are independent and separately capable of sensing membrane voltage. The processes of activation and inactivation were considered to occur in parallel. Other models are also capable of correctly predicting the mathematical form of the voltage and time-dependence of g_{Na}. These models include some in which activation and inactivation are sequential rather than parallel and some in which inactivation has no direct voltage dependence but follows activation in a solely time-dependent manner. In this last class of model only one electric field probe is included in the gating structure. In the present paper the data for OA indicate that shifts in the voltage dependence of m_∞ and h_∞ occur in opposite directions (Fig. 6 and Table 1). This phenomenon has been reported for octanol, methyloctanoate and glycerol-1-monooctanoate (Haydon & Urban, 1983*b*) and is also seen with methyl heptyl ketone (J. R. Elliott, D. A. Haydon & B. M. Hendry, unpublished observations). This divergence of voltage dependence is most simply explained by the existence of two separate voltage-dependent probes, although it is not impossible to reconcile it with a single voltage sensor.

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