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Chloramine-T Alters the Nerve Membrane Birefringence Response

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Summary. The change in birefringence during depolarizing voltage-clamp pulses of internally perfused squid giant axons are biphasic. There is a rapid decrease in birefringence with a 220-μsec half time at 8°C followed by a slow decrease over the next several milliseconds. After the pulse there is a rapid recovery which is smaller than the initial rapid decrease followed by a slow recovery phase. The rate of change of the slow phase during the pulse is more rapid for larger depolarizations. After the pulse the rate of change is more rapid for more negative potentials.

3.6 mM chloramine-T, applied externally until the sodium currents were prolonged and inactivation was removed, removed the slow phase of the birefringence response both during and after the pulse and made the fast 'off' response as large as the fast 'on' response. Two anesthetics reduced the birefringence response by about 20%.

A rocking helix model is presented which relates the birefringence findings and earlier gating current experiments.

Key Words optical retardation · nerve impulse · action potential · sodium channel · excitability · squid giant axon · chloroform · N-methyl-strychnine

Introduction

When nerve fibers are excited there are transient changes in several of their physical properties. The action potential, the change in the electrical potential difference across the cell membrane, was the first known phenomena. The underlying changes in specific ion permeability control the electrical currents which cause the action potential to be self regenerating and propagating, supporting its information-carrying role in the cell (Hodgkin & Huxley, 1952). Other mechanical, thermal and optical changes are less well studied but offer the opportunity to gather independent information about the molecular changes at the basis of the excitation (Cohen, 1973; Watanabe, 1986).

The physical change that has been best linked with the action of nerve sodium channels are the polarization or gating currents (reviewed in Armstrong, 1981; Bezanilla, 1985) The birefringence response associated with membrane depolarization has also been associated with the change in sodium permeability (Landowne, 1985). Both of these have rapid components, which can be interpreted as showing a voltage-dependent change in molecular conformation which precedes the increase in sodium permeability. Both show an immobilization of a rapid component associated with inactivation of sodium channels and slow 'off' responses associated with the recovery from inactivation. In the birefringence records there is also a slow 'on' response which was associated with inactivation by a computer simulation, but no direct experimental evidence was provided.

This paper presents more kinetic data on the slow birefringence responses and examines the effects of chloramine-T, a mild oxidant, which prolongs sodium currents (Wang, Brodwick & Eaton, 1985) and prevents immobilization of gating charge (Tanguy & Yeh, 1988). Two other agents which modify sodium currents and gating currents have been tested for effects on the birefringence change. N-methyl-strychnine and chloroform reduce the sodium current and fast gating charge movement (Cahalan & Almers, 1979; Fernández, Bezanilla & Taylor, 1982). Both also reduce the fast birefringence response. Preliminary abstracts have appeared (Landowne, 1987, 1988)

Materials and Methods

The details of the techniques have been published (Landowne, 1985). Briefly, segments of squid giant axons were internally perfused and voltage-clamped in a chamber that permitted mounting the axon between crossed Glan-Thompson polarizers. The change in the intensity of a beam of light from a 100-W tungsten-halogen bulb passing through the axon and polarizers was measured and is expressed in parts per million (ppm). When comparisons of averaged data are made the values given are the

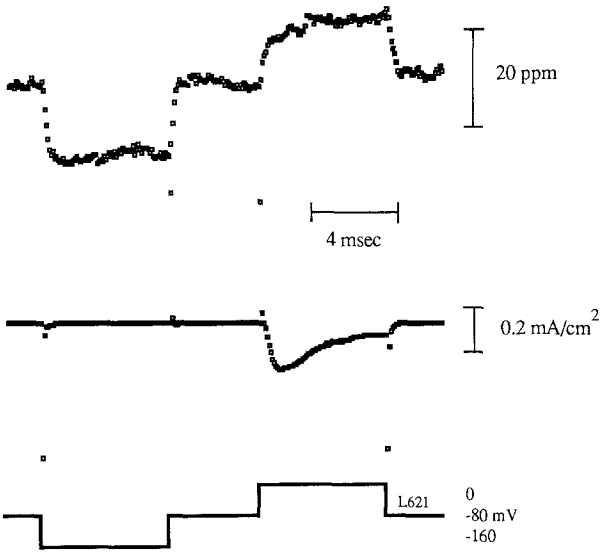

Fig. 1. The birefringence change of an internally perfused squid giant axon. The upper trace shows the change in light intensity produced by voltage-clamp pulses in an axon mounted between crossed polarizers. A decrease in light intensity is plotted upward. The middle trace is the corresponding membrane currents, and the bottom trace shows the voltage pulses. Temperature, 2°C

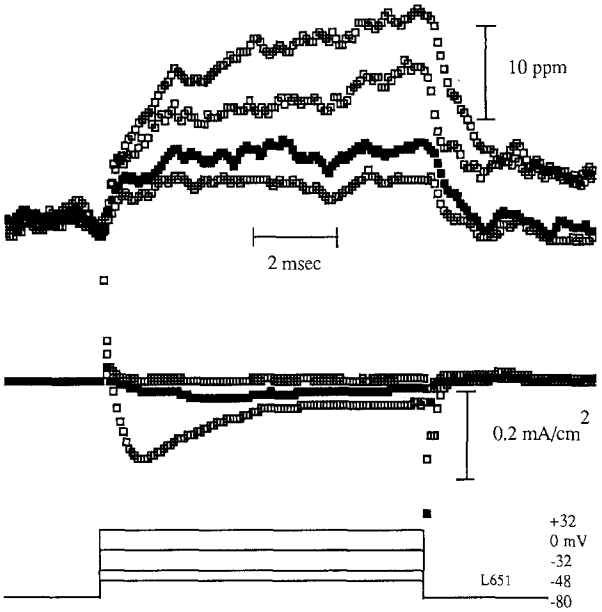

Fig. 2. The rate of the slow birefringence change is larger with larger depolarizations. The filled symbols are for the −32 mV pulse, the current for the −48 mV, and +32 mV pulses are near the baseline. Temperature, 2°C

mean ratio ± the SEM ratio. To permit expression of the retardation change in picometers, in some experiments the resting retardation was measured at the end of the experiment using a rotating 56-nm compensator and considering the visible light as 550 nm.

The external solution was an artificial seawater containing (in mM) 110 NaCl, 330 TMACl (tetramethylammonium), 50 $CaCl_2$ and 2 HEPES (N-2-hydroxyethyl-piperizine-N′-2-ethanesulfonate) buffer, pH 7.4. The internal perfusion fluid contained (in mM) 50 K or CsF, 150 K or Cs glutamate, 30 K phosphate buffer, pH 7.4 and 750 sucrose. Both internal and external solutions flowed continuously. The chamber was cooled to 2–12°C, an indwelling thermistor monitored the temperature and dry flowing nitrogen prevented fogging.

Chloramine-T was obtained from Sigma and dissolved in artificial seawater or perfusion fluid. The current response was monitored during application until about ¾ of the inactivation was removed, then the external solution was switched back to chloramine-free solution. Chloroform concentrations are computed from volume dilution and do not account for evaporation. Chloroform was dissolved in chilled artificial seawater in a Teflon beaker and only Teflon or glass was used to carry it to the axon. N-methyl-strychnine, a gift from Dr. M.D. Cahalan, was dissolved in the internal perfusion fluid. The perfusion fluid and artificial seawater were stored at −20°C; all other chemicals were added immediately before application.

Data collection was, as before, on a Nicolet 1170 signal averager with 4096 bins or, more recently, with a MacAdios board and a Macintosh II computer. The change in the optical signal and current were amplified, filtered at 30 kHz and digitized (12 bit) for 4096 10-μsec intervals while simultaneously controlling the voltage across the axon membrane. With the Nicolet, alternate points sampled the light and current signals. With the programmable computer, 16 optical sweeps were added, taken at $^{15}/_{240}$ (= $^{1}/_{16}$) sec intervals to average out residual 60 and 120 Hz interference. A separate sweep was taken sampling the current through the central, illuminated, portion of the membrane. This sequence was repeated 64–256 times. After collecting the data a constant value was added to the 32 bit sums to bring them near zero. The data was compressed by averaging over interval of 8 data points and retaining only the most significant 16 bits. Uncompressed 16 bit data was retained for 4 points before and 60 points following a change in voltage. In some experiments background records were made with the same timing but without changes in membrane potential to allow subtraction of systematic errors.

Results

When the nerve membrane is depolarized for several milliseconds the birefringence response has at least two distinct phases. There is a rapid decrease in birefringence with a halftime of about 200 μsec which precedes the rise in sodium current, and then a slower decrease, which continues for several msec after the sodium current (Figs. 1–3). After the pulse the birefringence traces return to base line with a biphasic time course. There is a rapid phase, which has a half time similar to the decline in sodium conductance. This is followed by a slower recovery with a time course that parallels recovery from inactivation (Landowne, 1985). In the experiment shown in Figs. 1 and 2, Cs internal perfusion fluid was used to record sodium currents without the normal potassium currents. Similar birefringence responses were obtained with K perfusion

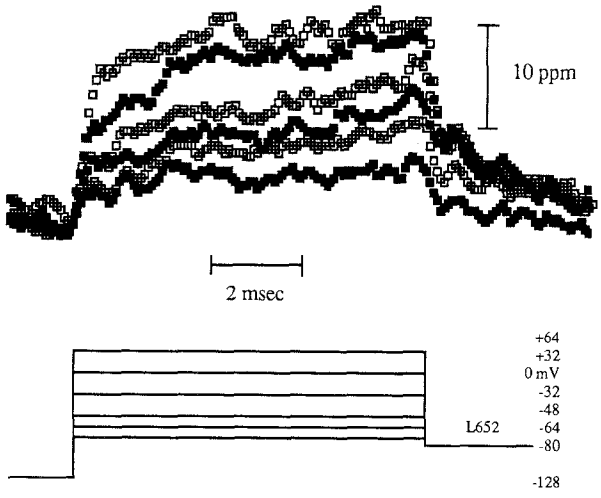

Fig. 3. A family of birefringence responses in another axon to illustrate the variability of the slow response. Alternate traces have filled symbols. Temperature, 3°C

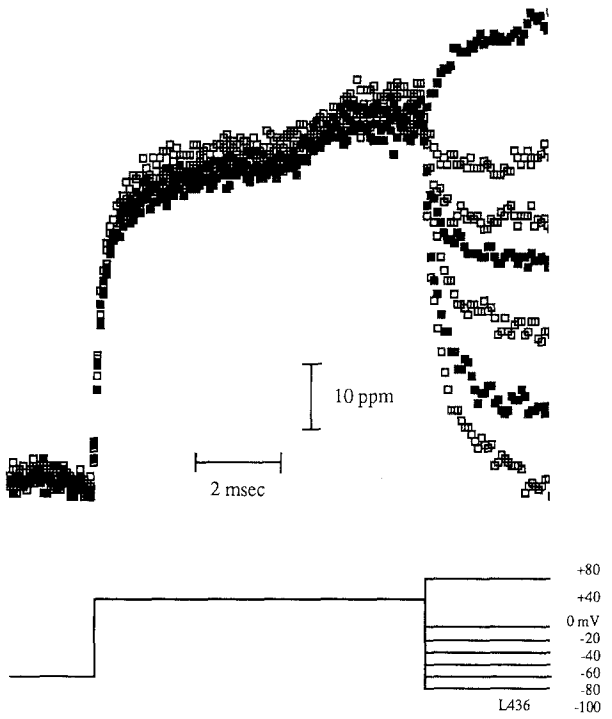

Fig. 4. The rate of recovery of the slow birefringence response increases with hyperpolarization. Traces with filled symbols are at +80, −40 and −80 mV. Temperature, 12°C, optical recording bandwidth, 10 kHz

fluid. The slow response during depolarizaton is more apparent with larger steps and thus can be said to be voltage dependent.

In axons in good condition, the birefringence response during a hyperpolarizing pulse is different from the response to depolarization. The rapid phase is faster and the slow phase is not apparent. The rapid phases are not well seen in the figures in this paper because of the slower time base; better examples at higher sweep speeds are shown in previous publications. The response during hyperpolarization is not blocked by colchicine in doses that block the response to depolarization and the gating currents (Landowne 1984, 1985; Matsumoto, Ichikawa & Tasaki, 1984). The rise seen near the end of the hyperpolarizing pulse and the dip during the depolarizing response (better seen in Fig. 4) come from imperfect subtraction of 60 Hz interference. Sometimes slow phases are seen during the hyperpolarizing pulse. These can either be because of incomplete removal of resting inactivation or a pathological secondary response. In the former case the slow phase can be removed by making the holding potential more negative.

The secondary or state 2 response may appear spontaneously and irreversibly during an experiment, often correlated with trauma to the axon. In the transition to state 2 in initial change in birefringence is prolonged, so that the traces no longer show the transition between a fast and a slow phase. State 2 is also characterized by a symmetry of the response for depolarizations and hyperpolarization (Cohen et al., 1971; Landowne, 1985). When the optical records show a transition to state 2, the capacity transients in the current records are prolonged. A similar effect on the capacity transient of obscure origin but associated with many voltage clamp pulses was reported by Greeff, Keynes and VanHelden (1982). Both the optical and the electrical phenomena suggest the transition to state 2 involves an increase of rotational freedom of dipoles, which then can move with changes in membrane potential. The secondary response is not sensitive to colchicine.

The slow response also differs from the long-lasting birefringence change which Watanabe and Terakawa (1976*a*,*b*) recorded following trains of action potentials. The long-lasting change lasts much longer and was reported to disappear when the axons were internally perfused.

The slow birefringence change during the depolarization usually appears as a slow upward slope which continues through the pulse duration. In Fig. 3 one gets the impression there is a slow exponential relaxation to a steady level. However, the distinction between these two descriptions cannot be made with the present data because of the relatively high noise level. One would very much like to see a clear experiment in which the slow response ends during a pulse of sufficient duration, but this has not been possible. It often appears that the birefringence is still changing after the sodium current has declined to a steady level due to inactivation (Fig. 5). With either description of the slow 'on' re-

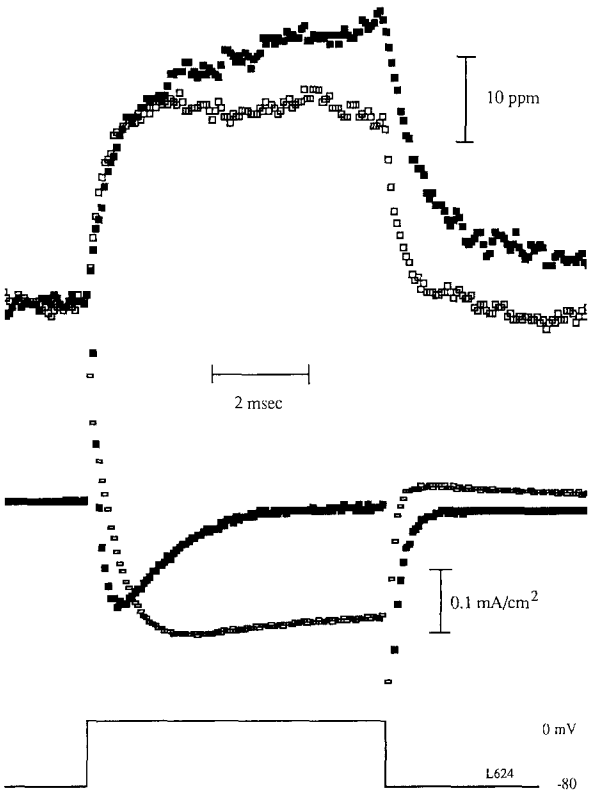

Fig. 5. Chloramine-T removes the slow birefringence responses. Filled symbols are before and open symbols are after external treatment with 3.6 mM chloramine-T. Temperature, 2°C

sponse, linear or slow exponential, the response is more obvious with larger depolarizations.

It is not easy to obtain better recordings because the primary noise source is the quantal nature of light. When the light level was reduced by half with a neutral density filter the signal/noise ratio was reduced by about one third, similar to a 29% reduction which would be expected if all the noise were photon shot noise. To improve the recording significantly requires either a brighter light source or more extensive averaging. The traces shown are each the average of thousands of pulses, typically 2 min for data collection is needed per trace. If longer pulses were used, it would be necessary to use a slower repetition rate. Thus, one should not expect to make much better recordings in this preparation with this technique until a brighter light source with photon-limited noise characteristics becomes available.

The Slow 'Off' Response is Voltage Dependent

If the membrane is repolarized to different potentials the slow relaxation of the birefringence change occurs at different rates. In the seven traces in Fig. 4 the axon was depolarized to +40 mV, then repolarized to a different levels. There is a fast 'off' response in the first 300 μsec after repolarization followed by a slow 'off' response for the remainder of the trace. During the slow phase following repolarization, the drop in the traces is accelerated at more negative potentials. The trace appears horizontal at −20 mV, increases slowly at 0, and at +80 mV it is clearly rising.

Chloramine-T Removes the Slow Phase

When 3.6 mM chloramine-T was included in the external solution the sodium currents were prolonged, indicating a removal of sodium inactivation as reported by Wang et al. (1985). Concomitantly the birefringence traces were altered, the slow phases were removed and the rapid phases at the beginning and the end of the pulse became the same size (Fig. 4). Chloramine-T removed both the slow 'on' and the slow 'off' phases of the birefringence change. In five experiments the slope of the slow 'on' phase was reduced to 0.21 ± 0.16 of control values.

Other methods of removing sodium inactivation were found to be too traumatic to allow birefringence experiments. Removal of sodium inactivation by internal perfusion with pronase (4 axons), N-bromo-acetic acid (5 axons) or chloramine-T (1 axon) precipitated the conversion of the birefringence response into the secondary response before showing a loss of the slow phase of the primary response. In the hope of finding a gentler way to treat the membranes, three axons were internally perfused with 5.3 mM sodium metabisulfite for 10 to 20 min before external application of chloramine-T and continuing during the chloramine-T treatment. Contrary to the report of Wang et al. (1985), metabisulfite seemed to protect the sodium channel molecule from the action of chloramine-T as seen in both the current records and the optical traces. In one axon the internal solution was switched back to Cs perfusion fluid and inactivation and the slow phases of the birefringence response were removed. Perhaps some subtle difference in perfusion characteristics can account for Wang et al.'s not seeing protection.

Chloroform and N-Methyl-Strychnine Reduce the Birefringence Response

Chloroform is a general anesthetic. In near saturating concentrations it will block the sodium current in squid axons and a part of the gating current (Fernández et al., 1982). Its effect on the birefrin-

gence response is modest. Figure 6 shows responses in a concentration of chloroform that completely blocked the sodium current but spared most of the potassium current. The amplitude of the birefringence response was reduced by 25% during both the depolarizing and the hyperpolarizing pulse. The effect appears as a reduction of all the responses; in Fig. 6 it appears as though there is a preferential decrease of the slow phase, but this was not a consistent finding. In six axons the birefringence response measured at the time of the peak of the sodium current during a 80-mV depolarization was reduced in 62 mM chloroform to 0.75 ± 0.06 of the preceding control record. The birefringence response measured at similar time during an 80-mV hyperpolarization was 0.80 ± 0.08 of control values. When the two consecutive control records before chloroform treatment were compared, their ratios were 1.01 ± 0.13 for depolarization and 1.03 ± 0.12 for hyperpolarization.

Because the consistency of this small decrease in the response in chloroform, experimental records made in 1981 on the effects of N-methyl-strychnine on the birefringence response was reviewed. In five axons 2 mM N-methyl-strychnine reduced the response to 0.80 ± 0.08 during depolarization and 0.71 ± 0.05 during hyperpolarization.

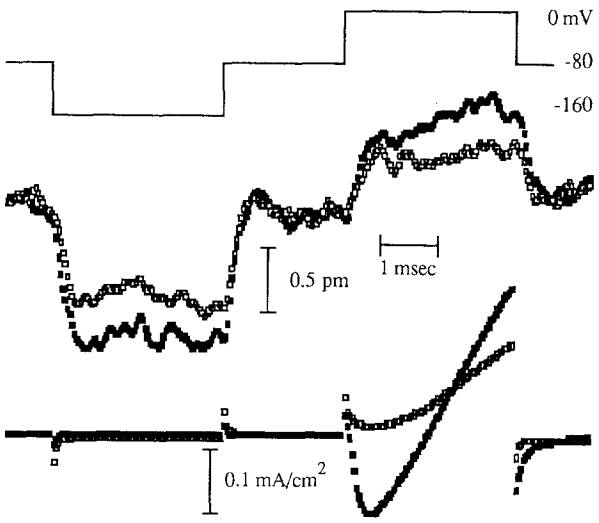

Fig. 6. Chloroform reduces the birefringence response. Filled symbols are control; open symbols are responses in 62 mM chloroform. Resting retardation, 22 nm. Temperature, 3°C

Discussion

The slow birefringence responses during and following depolarization of the squid axon membrane appear to be voltage-dependent and related to the inactivation of sodium currents. The slow birefringence response relaxes at different rates at different potentials and is removed by chloramine-T, which also removes sodium inactivation. These observations are consistent with the molecular motion hypothesis, which attributed sodium channel behavior to two conformational changes associated with activation and inactivation but not necessarily having the same time course as the rise and fall of the sodium conductance.

This conclusion is somewhat different from the model proposed by Armstrong and Bezanilla (1977), which suggests there is no gating charge movement associated with inactivation and, therefore, inactivation derives its voltage dependence from activation. This was based on the finding that the slow 'on' gating current (I_S) is 3–4 times faster than the decline in current and that I_S is not much affected by pronase. Pronase did reduce the slow "off' gating current and remove the immobilization phenomena. Tanguy and Yeh (1988) have reported that chloramine-T treatment also prevents immobilization.

The motion seen here associated with inactivation would be difficult to detect in a gating current experiment. The birefringence change is related to the amount of rotation of dipoles, whereas the gating current is related to their angular velocity. The time derivative of the slow birefringence change is small and would be difficult to distinguish from the pedestal which is seen in gating current experiments and ascribed to nonlinear leakage current. Without another method to determine the nonlinearity of the leak current there is no unbiased way to distinguish slow "on' gating currents.

There have been several reports that suggest there may be an asymmetrical polarization current with a time course similar to the slow birefringence change. Nonner (1980) has a figure in which time integrals of total gating current in frog myelinated nerve have the same form as the birefringence responses described here. He reports these slow gating currents were variable and not quantitatively analyzed. Bezanilla and Taylor (1978) show a figure of integrated gating currents with a slow phase and Keynes, Greeff and VanHelden (1982) report a palpable slow component in some experiments which was not consistent nor easily measured.

The I_S of Armstrong and Bezanilla (1977) is faster than the slow birefringence change reported here. Because of the signal/noise ratio no attempt has been made to resolve the fast birefringence change into more than one component to see if a part of its correlates with I_S. Without a pharmacological test it would be difficult to say that the birefringence response does or does not include components that correspond to both I_f and I_S.

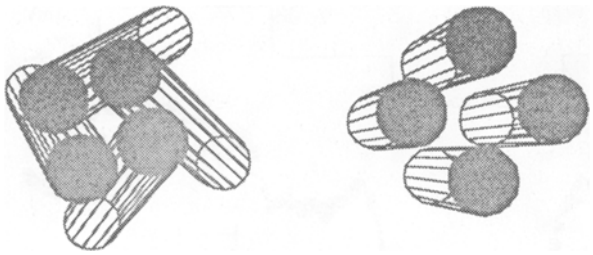

Fig. 7. A type of motion within the sodium channel molecule that could account for the changes in birefringence and the gating currents The cylinders are meant to represent transmembrane helices. Fourfold symmetry is expected from the sequence data; some higher multiple of four helices could be involved in the motion. Only four transmembrane segments are shown; the rest of the molecule has been omitted for simplicity. The helices on the left are in the resting state, tilted with respect to the plane of the membrane. The four on the right have been activated by the change in membrane potential and can support sodium conductance. They are more perpendicular to the membrane

Experiments on the effects of N-bromoacetamide on the statistics of single channel openings (Horn, Vandenberg & Lange, 1984) and simulations predicting the voltage dependence of the peak sodium conductance (Stimers, Bezanilla & Taylor, 1985) have led to a softening of the position such that there now appears to be a voltage dependence to the inactivation process. This implies there should also be a component of the gating current associated with inactivation.

The effects of anesthetics on the birefringence response are modest but consistent with gating current experiments. Fernández et al. (1982) reported that 62 mM chloroform reduced a rapid component of the gating current by about one half or the total charge movement by about 20%, which is the same as the reduction in the birefringence response. Cahalan and Almers (1979) did not tabulate the effect of N-methyl-strychnine on the amplitude of the gating current, but in three of their figures they report a decline of 26 ± 10%, which is consistent with the effects reported here. Colchicine produced a larger reduction of the birefringence response (Landowne, Larsen & Taylor, 1983) than chloroform or N-methyl-strychnine, which suggests a different mechanism of action may be involved.

The finding that chloroform reduces the birefringence response to hyperpolarization was unexpected and differs from the effect of colchicine. It may suggest there is a less specific mechanism of action for chloroform. Gating current is defined in terms of the nonlinear displacement current; its measurement involves the subtraction of currents produced by a pulse which is thought not to produce gating currents from one which contains them. Thus Fernández et al. (1982) used −150 mV as a subtraction holding potential for their smaller pulses used for subtraction. They report that the effects of chloroform were seen for both depolarizing and hyperpolarizing pulses from their −70 mV holding potential. With the birefringence data there is not a simple theory to justify a linear subtraction, which means comparison with gating currents remains somewhat qualitative. Taken together with Fernández's finding, there is the suggestion that at least a portion of the birefringence response during hyperpolarization should be associated with the gating currents.

Overall, there is general agreement between data on the motion of sodium channel molecules derived from gating currents and birefringence responses, except for the difficulty in recording a gating current associated with inactivation. Both have rapid 'on' responses which precede the ionic currents and rapid 'off' responses with the same time course as the sodium 'tail' currents which are reversibly blocked by similar doses of colchicine. Both show the phenomena of "immobilization' and in both immobilization can be removed with chloramine-T. Both are reduced by chloroform, and neither show a dramatic effect when tetrodotoxin is applied. Both show a slow 'off' response whose time course parallels the recovery from immobilization. The optical recordings thus confirm and extend the electrical recordings and with different artifacts. The optical recordings require that any explanation of sodium channel function includes the rotation of molecular dipoles or the movement of atomic nuclei. Strictly speaking, one might explain the gating currents as an internal redistribution of electronic charges within the sodium channel molecule.

To explain the birefringence data requires a model that includes dipole rotation towards a direction perpendicular to the membrane. This is a substantial modification of Armstrong's (1981) model in which subunits rotate about an axis which is perpendicular to the membrane. Rotating subunits about this axis would not directly produce the observed change in birefringence, although it could produce a charge movement. More specifically, the screwing helix model proposed by Catterall (1988) clearly would not produce the birefringence change. There could be some rotation of subunits about the perpendicular axis coupled through subunit interactions to a larger reorientation towards the perpendicular, which produces the birefringence response and most of the gating current. The reorientation of α-helices so they become more perpendicular to the plane of the membrane (Fig. 7) could produce both the gating currents and the birefringence changes. This type of conformational change would also provide the appearance of a concerted movement of a

large number of charges moving a small distance, which was an important consideration in Armstrong's model.

Structural predictions based on the amino acid sequence of the sodium channel molecule (Noda et al., 1984) have many transmembrane α-helices, including one type (S4) that is also seen in two other voltage-sensitive channels (Tanabe et al., 1987; Temple et al., 1987). Perhaps the change in membrane potential causes a small change in orientation of this helix which then triggers the reorientation of other helices, so that the channels assume a conformation that supports ion permeation. Alternatively, a change in electric field could rock tilted helices directly and the S4 helix could have some other function. The available optical and electrical data cannot distinguish among the helices. It is even possible that some other structural change happens involving the concerted reorientation of many dipoles. More sophisticated optical techniques, such as time-resolved, polarized Fourier transform infrared spectroscopy (Braiman & Rothschild, 1988), could resolve these questions if significant signal/noise ratios can be achieved.

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