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Molecular Motion Underlying Activation and Inactivation of Sodium Channels in Squid Giant Axons

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Summary. Measurements of the changes in birefringence associated with changes in membrane potential were made with internally perfused squid giant axons in low sodium solutions at 0–8°C. The time course of the birefringence changes share many properties of the 'gating' (polarization) currents previously studied in this nerve. Both can be demonstrated as an asymmetry in the response to voltage pulses symmetrical about the resting potential which is not present about a hyperpolarized holding potential. Both have a rapid relaxation, which precedes the sodium permeability change. Both exhibit an initial delay or rising phase. Both are reversibly blocked by perfusion with 30 mM colchicine; neither are altered by changes on sodium concentrations or 300 nM tetrodotoxin. The birefringence response has a decrease in the amplitude of the rapid relaxation associated with the appearance of a slow relaxation. This is similar to the immobilization of fast gating charges which parallels sodium current inactivation.

The amplitude of the birefringence and the gating current responses is consistent with a change in the alignment of several hundred peptide bonds per sodium channel.

Key Words birefringence · optical retardation · nerve impulse · action potential · sodium channels · excitability · colchicine

Introduction

There is a transient change in the birefringence of a nerve which is nearly coincidental with the passage of an action potential. Following its discovery (Cohen, Keynes & Hille, 1968; Cohen, Hille & Keynes, 1970) a voltage-clamp study was made, but the molecular origin(s) of the phenomena were not determined. The feeling was expressed that this was not a good prospect for obtained much direct information, at the molecular level, of conformational changes associated with activation and inactivation of sodium channels (Cohen, Hille, Keynes, Landowne & Rojas, 1971). Several recent experiments have led me to an alternative hypothesis, that the birefringence change permits a measurement of the amplitude and time course of the motion of or within the sodium channel molecules.

The sodium channel molecule is a large glycoprotein oriented in the nerve membrane with an external neurotoxin (tetrodotoxin or saxitoxin) binding site. The surface density of sodium channels in squid axons is 170–550 binding sites/μm^2 (Levinson & Meves, 1975; Strichartz, Rogart & Ritchie, 1979). The eel electroplaque sodium channel has been isolated, purified and reconstituted into lipid membranes where it behaves quantitatively and qualitatively as native sodium channels (Rosenberg, Tomika & Agnew, 1984). The sequence of its 1820 amino acids has been published (Noda *et al.*, 1984).

For a protein without visible color the principle contribution to the index of refraction or the retardation of the passage of light is from the π-π^* transition of the peptide bonds linking the amino acids. For visible and near infrared light polarized parallel to the transition dipole, about 15° from the N—C axis in the N—C=O plane, the retardation of these bonds is proportional to their surface density (Cantor & Schimmel, 1980).

$$R = S \cdot e^2/2\varepsilon_o m\omega_o^2 = S \times 2.5 \times 10^{-15} \text{ pm}.$$

S is the number of peptide bonds/cm^2, e and m are the charge and mass of the electron, ε_o is the permitivity of space and ω_o is the resonance frequency of this electronic oscillator, which is also responsible for the 190-nm absorption band. Cohen et al. (1970) estimated that the change in birefringence or the difference in retardation to light polarized in the two directions was about 0.15 pm. 60,000 peptide bonds/μm^2 rotating 90° from lying in the plane of the membrane to perpendicular to it, or a larger number rotating through a smaller angle, would produce a signal of this magnitude. Divided by the sodium channel density, this means the movement of a few hundred bonds/channel could be responsible for the birefringence change.

Electrical recording techniques have greatly in-

creased our understanding of the properties of sodium channels. Measurements of the asymmetry of displacement current transients have shown there is a voltage-dependent intramembraneous movement of charge associated with the activation of sodium channels (the gating current) which is reduced or immobilized with a time course that parallels inactivation (Armstrong & Bezanilla, 1973, 1977; Almers, 1978). This movement precedes the sodium conductance change, so it would not have been described as 'conductance-dependent' had it been seen in the initial birefringence experiments. The published records were made at too slow a sweep speed to resolve this rapid movement. The investigators were influenced by the birefringence response to hyperpolarization, which clearly is not directly associated with the activation of sodium channels, and therefore did not study the events during depolarizing pulses in more detail.

In order to test this molecular motion hypothesis I have measured the birefringence response in voltage-clamped squid axons with sufficient temporal resolution to observe events with the time course of gating currents. By using low temperatures, low external sodium concentrations, internal perfusion and an improved internal electrode assembly, many of the problems of the earlier experiments have been avoided. An alkaloid has been found which reversibly reduces the ionic sodium current, the gating current and the birefringence response to a depolarizing voltage-clamp pulse (Landowne, Larsen & Taylor, 1982; Matsumoto, Ichikawa & Tasaki, 1984). The results presented here suggest that most of the birefringence response to modest depolarizing pulses is associated with the activation and inactivation of sodium channel molecules. Preliminary reports have appeared (Scruggs & Landowne, 1980; Landowne, 1984*a*,*b*).

Materials and Methods

15-mm segments of well-cleaned squid giant axons were cannulated, internally perfused and voltage-clamped using standard techniques. In order to measure and control the absolute potential across the membrane, the double spiral platinized-platinum internal electrode assembly used previously (Cohen et al., 1971) was replaced with a KCl-filled 80 μm glass tube with an electrically floating Pt wire inside to sense the voltage. A 50- to 60-μm bundle of 10-μm carbon fibers (about 20 fibers) was glued to the glass tube to provide current over a 5-mm length on both sides of the tip of the voltage electrode. Conducting silver paint made the electrical connection between the carbon fibers and a fine copper wire.

The axons were mounted in a 10 × 2 × 2 mm slot milled into a block of silver. The interior of this cavity was electroplated with gold and then platinized. Outside the slot the axon passed through airgaps and then rested on plastic bridges. In the central 4 mm the side walls were electrically isolated from the rest of the chamber, connected to a current-to-voltage converter (virtual ground) and use as the current-measuring electrodes. A second KCl-filled electrode sensed the bath potential through a hole in one of the walls. The floor of this guarded region was a 1-mm thick glass plate with a 5-mm f.l. cylindrical lens cemented to its lower surface. A removable coverslip formed the top of the chamber. With seawater in the chamber this combination focussed the light beam into a 1 × 4 mm rectangle at the top of the chamber without striking the walls.

The external solutions were a mixture of Na and TMA (tetramethylammonium) artificial seawaters referred to in the text by the percentage of Na. They contain (in mM) 500 Na or TMACl, 10 KCl, 50 $CaCl_2$ and 2 HEPES (N-2-hydroxyethylpiperizine-N'-2-ethanesulfonate) buffer, pH 7.4. They flowed continuously at about 0.1 ml/min through the chamber. The internal solution contained 400 K^+, 320 glutamate, 50 F^-, and 30 mM phosphate buffer, pH 7.4, flowing under a pressure of about 10 cm of water. A chilled water-methanol mixture cooled the block; the temperature was monitored with an indwelling thermistor. Cool dry flowing nitrogen kept the lens and coverslip from fogging.

The light source was a 12-V 100W tungsten-iodide bulb powered by a regulated DC supply. The beam was partially collimated and then passed through a Glans-Thompson crystal polarizer mounted at 45° to the long axis of the axon. No heat filter was used on the beam. The collimator was adjusted so a portion of the filament was focussed on the coverslip, thus the axon was critically illuminated to provide the greatest intensity. About 4 mW or 100 mW/cm^2 was measured at the coverslip.

After striking the axon the light was collected with a 10 × 0.4 n.a. microscope objective and passed through a second polarizer set perpendicular to the first one. At the image plane a ground glass plate was used to observe the axon and position stops just beyond its edges and over the image of the electrode. The plate was replaced with a YAG-444 photodiode (E.G.&G.) used in the photoconductive mode. This device is responsive from 400 to 1100 nm. The voltage changes developed across a 100 K load resistor were amplified and then digitized and stored with a Nicolet 1170 signal averager and an Apple II computer. With the amplifier bandwidth set to 30 kHz the system responded to a light pulse with an 8 μsec half time. A second channel of the averager was used to simultaneously record the current flowing through the central portion of the axon. Typically records were made by measuring the resting (DC) light intensity and then averaging 1024 changes in light intensity in response to a repeated voltage pattern impressed across the membrane. The results are expressed as the change in light intensity, $\Delta I/I_{rest}$, in parts per million (ppm).

In many experiments a secondary or 'state 2' response develops irreversibly. To assess the condition and stability of the axons, each experiment began with two or three control measurements. Except for the experiments designed to study the secondary response all of the results reported here come from bracketed experiments (control, test, recovery) made after this initial control period.

To measure the resting retardation the electrode assembly was removed and the axon was tied at both ends of the chamber so that it remained inflated with perfusion fluid. The axons were removed from the chamber and mounted between a pair of coverslips in 1 mm of seawater. This assembly was placed on a Zeiss polarization microscope with crossed polarizers and a rotating stage. Under 540-nm illumination the axons were rotated to an intensity minimum and then an additional 45° to the intensity

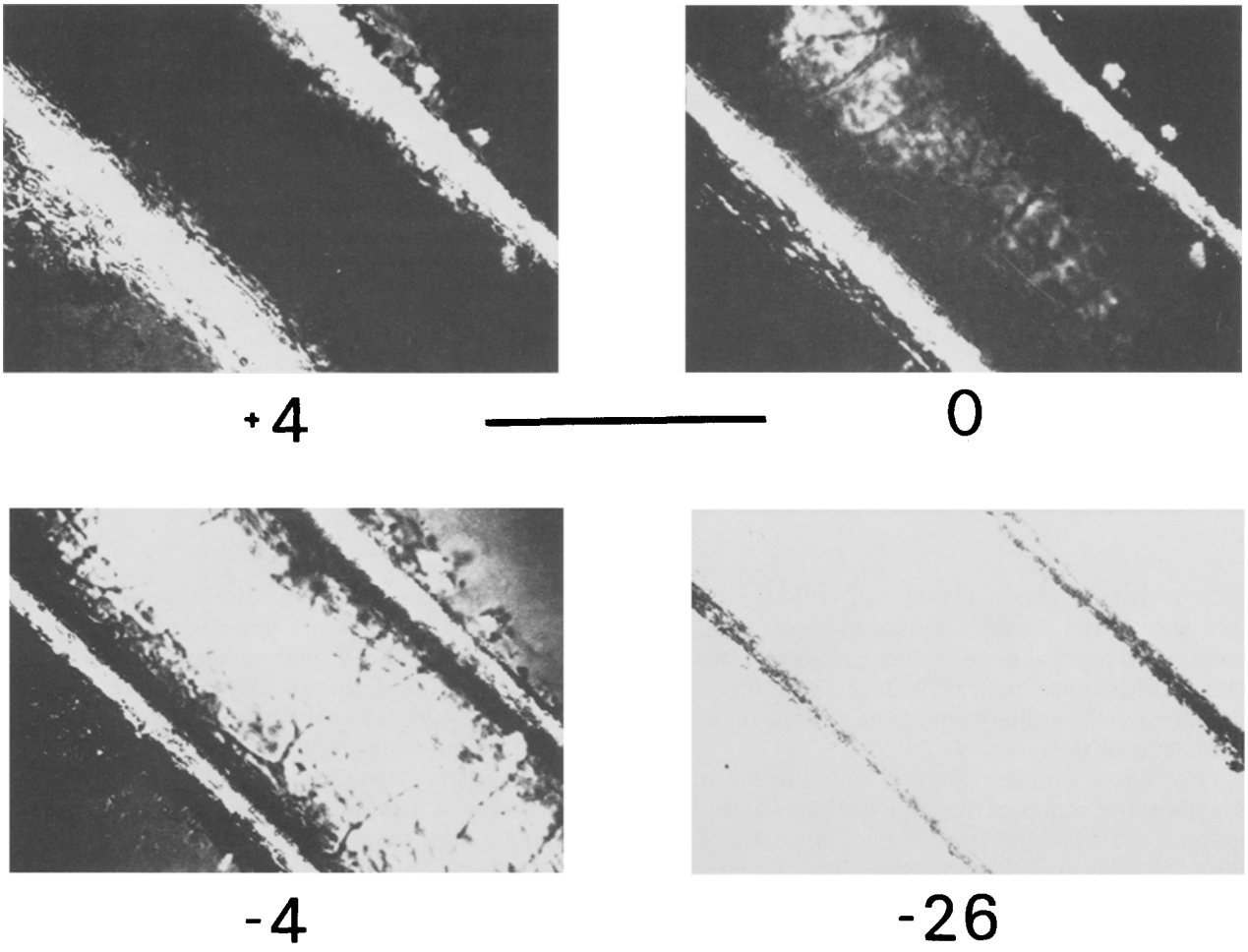

Fig. 1. The optical retardation of an internally perfused squid giant axon. In the frame marked *O* the axon is seen at 45° between crossed polarizers. The bright bands are the Schwann cells and connective tissue sheath. Dark individual cell nuclei can ben seen near the lumen of the axon. In the lower right frame −26 nm of optical compensation was added in series with the axon. The sheath appears dark and the background is bright, In the upper left frame +4 nm compensation was added, darkening the center of the axon. The horizontal bar represents 500 μm

maximum. A Brace-Koehler rotating plate compensator with a maximum compensation of 56 nm was inserted in the optical path and rotated to the darkest background. With further rotation the background became lighter and portions of the axon became dark (*see* Fig. 1). The retardation of the thus darkened regions is 56 nm · sin $2u$, where u is the amount of further rotation. The measurements at the center and edges of the axons were repeated for all four background minima and averaged.

Results

Resting Retardation

At 45° between crossed polarizers an internally perfused axon appears as two bright 50-μm bands about 0.5 mm apart (Fig. 1, upper right). There is also a fainter more diffuse band in the center of the axon. With added external compensation the central light band becomes wider and more prominent and several small blood vessels can be seen (Fig. 1, lower left). With this axon the addition of 26 nm of retardation produced the dark edges, indicating complete compensation of the edges (Fig. 1, lower right). Similar measurements on 10 axons gave an average resting retardation of 32 nm (SD ± 8, range 20–44), which is less than the 56 nm reported by Cohen et al. (1971) for intact *L. forbesi* axons. The slow axis, with the higher index of refraction, is along the geometrical axis of the cell. With respect to an optical axis perpendicular to the membrane this is a negative birefringence. Since the nerve fiber retardation is much larger than the 400 pm reported

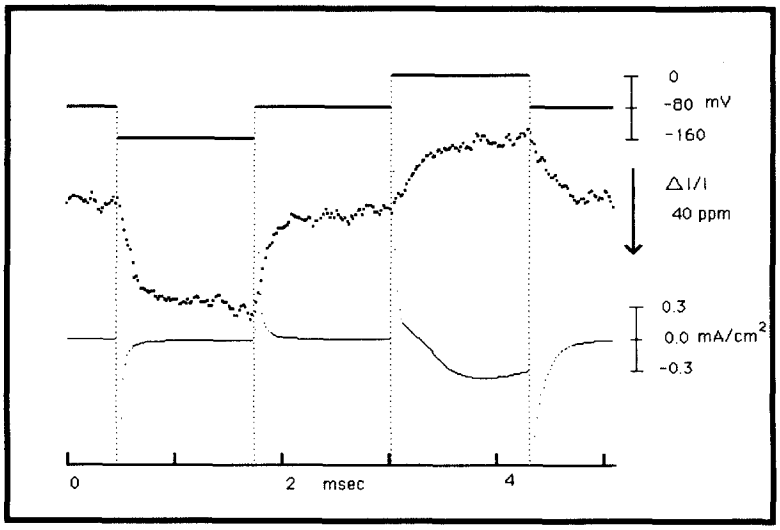

Fig. 2. The birefringence response of an internally perfused squid axon. The upper trace is the membrane potential; a −80 mV holding potential with a hyperpolarizing pulse to −160 mV and a depolarizing pulse to 0 mV. The center trace shows the change in intensity of light passing through the axon and the crossed polarizers. During the hyperpolarization the light level increased (a downward deflection) by 42 ppm. The bottom trace shows the membrane current. During the depolarization the peak inward current was 0.28 mA/cm². Temperature, 8°; 20% NaSW; optical recording bandwidth, 30 kHz

for erythrocyte ghosts (Mitchison, 1953), presumably most of the resting retardation comes from the enveloping Schwann cells and connective tissue. This birefringence pattern is as if there are more optical dipoles parallel to the axon surface than perpendicular to it.

If 4 nm of compensation is added in the other direction, the center of the axon becomes dark (Fig. 1, upper left). The birefringence in the center of the perfused axon has the opposite sign compared to the edges. This indicates a component of net dipole orientation perpendicular to the long axis of the axon cylinder. The entire picture is consistent with dipoles oriented parallel to the membrane surface tilted to slightly less than 45° to the plane of a transverse section of the axon. This is an average picture, by focussing on the upper surface of the axon discrete birefringent fibrils at various orientations can be seen.

The Change in Retardation During Brief Pulses

When the electrical field across the cell membrane is changed there is a change in the amount of light passing through the crossed polarizers. When the axon is depolarized, the light measured by the photodiode decreases. This corresponds to a net increase in the number of dipoles oriented parallel to the electrical field change, that is, perpendicular to the membrane surface. In 23 freshly mounted axons when the membrane potential was depolarized by 80 mV the light level decreased by 27 ppm (SD ± 8, range 14–70) measured at the time of the peak sodium current. This fractional change in intensity is about twice as large as value reported by Cohen et al. (1971) for a 50-mV hyperpolarizing pulse. From the voltage dependence of the birefringence, the expected change for an 80-mV depolarization is about 90% as that for a 50-mV hyperpolarization. In terms of the change of light intensity the present responses are larger. However the change in differential retardation, one half the change in light intensity times the resting retardation, is about the same for the two sets of experiments because the resting retardation values found here are only about half the earlier value.

Figure 2 shows an average response of an axon stimulated with 1024 pairs of symmetrical 80-mV pulses at 20/sec from a −80 mV holding potential. These pulses are short with respect to the time course of inactivation of the sodium channels. During the hyperpolarization the light level increased (a downward deflection in the figure) by 42 ppm with a 70-μsec half time (the optical recording apparatus half time is 8 μsec). In five freshly mounted axons the half rise time was 81 μsec (SD ± 12, range 70–92). This is slower than the 20–40 μsec time constants reported by Cohen et al. (1971), which may reflect the lower temperature used here. During the depolarization the light level decreased by 32 ppm with a 220-μsec half time, threefold slower than the response to hyperpolarization. The five axons had a mean half rise time of 205 μsec (SD ± 31, range 120–250). At the end of the depolarizing pulse the light returned towards the resting level more slowly than after the hyperpolarizing pulse. There are also small slow components of the signals, which will be discussed below.

Asymmetry Controls

This difference in the time course of the birefringence response is reminiscent of the gating cur-

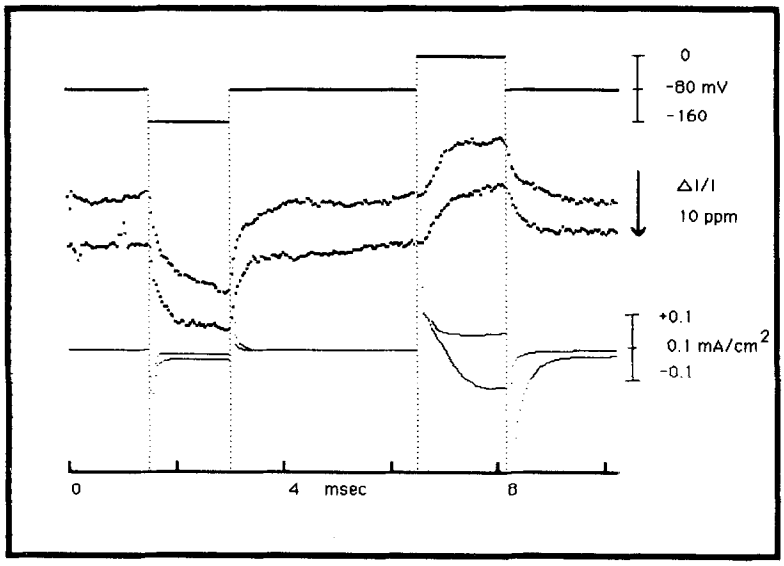

Fig. 3. Tetrodotoxin does not significantly alter the birefringence response. Upper traces: birefringence before and 20 min after external application of 300 nM tetrodotoxin. Lower traces: the currents. 0°, 10% NaSW, 30 kHz

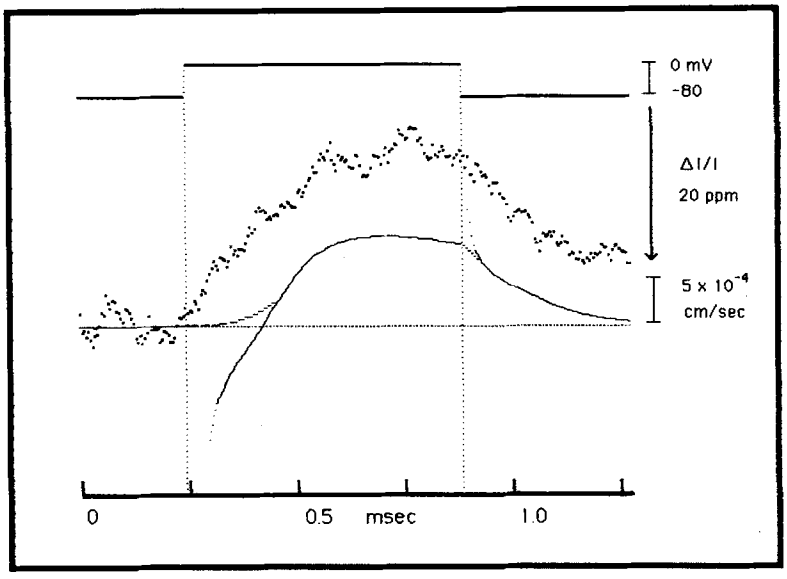

Fig. 4. Activation precedes opening of sodium channels. Upper trace is the birefringence; lower trace, sodium permeability change derived by dividing the current by the driving force on sodium ions. The dashed line is an estimate during the capacity and gating current transients. The peak sodium permeability is 7.5×10^{-5} cm/sec derived from a current of 0.72 mA/cm². 8°, 20% NaSW, 30 kHz

rents. Several experiments indicate the asymmetry of the optical record are not artifactually dependent on the flow of ionic currents. The gating currents are unchanged by alterations in the concentrations of permeant ions or the addition of tetrodotoxin. Figure 3 shows tetrodotoxin blocking the ionic sodium current without changing the birefringence response. In other experiments (not shown), at different voltages or sodium concentrations, the asymmetry of the birefringence response was not dependent on the size or direction of the ionic sodium current.

When the experiment of Fig. 2 was repeated from a −160-mV holding potential, the response to symmetrical 80-mV pulses had superimposable rapid time courses, similar to the response to an 80-mV hyperpolarization from a −80-mV holding potential (Landowne, 1984*a*, Fig. 2). This is also consistent with the gating current studies; the asymmetrical charge movements occur in the voltage range which opens sodium channels and not when the membrane is hyperpolarized outside that range.

Relation to the Permeability Change

Comparing the time courses of the birefringence change and the sodium conductance (Fig. 4) illustrates other similarities between the optical signal and the gating currents. There is a delay of about 150 μsec after the birefringence change at the onset

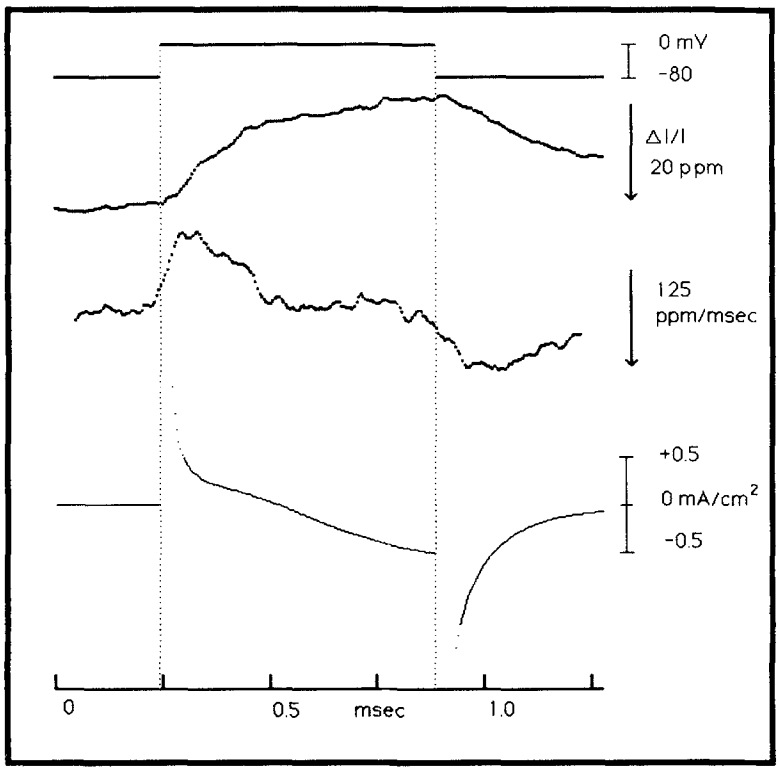

Fig. 5. Initial delay of the change in birefringence. The upper trace is the birefringence here the average of 3072 sweeps; the middle trace is its time derivative, computed as the difference between points separated by 0.1 msec; and the lower trace is the membrane current. 7°, 20% NaSW, 30 kHz

of the pulse before the increase in sodium conductance. At the end of the pulse both the birefringence and the ionic currents decline roughly exponentially with the same 120-μsec time constant. Likewise, the on-gating currents relax more rapidly than the rise of the ionic sodium current and the off-gating currents have the same time course as the sodium tails. With larger depolarizations the delay between the fast birefringence change and the ionic current is briefer, as is the delay between the time integral of the gating currents and the conductance change (Keynes, Greeff, & van Helden, 1982).

An Initial Delay

During a depolarizing step the birefringence decreases sigmoidally. With more extensive signal averaging (Fig. 5) an initial delay in the birefringence change can be seen, corresponding to the rising phase of the gating current. The time derivative of the birefringence record (middle trace) does not reach its maximum until 50–60 μsec after the beginning of the depolarization. This delay in the optical response should not be associated with an artifact arising from the currents charging the membrane capacitance because it does not equally appear at the termination of the pulse, nor at either end of hyperpolarizing pulses. The rising phase of the gating current has been attributed to a distributed series resistance arising from a restricted space in the cell layer wrapping the axon (Stimmers, Bezanilla & Taylor, 1984).

Colchicine Treatment

The addition of 30 mM colchicine to the internal perfusion fluid inhibits the rapid birefringence change during a modest depolarizing pulse. The gating currents and the ionic sodium currents are also inhibited, whereas potassium currents are only slightly reduced. These effects are dose-dependent and reverse within seconds of switching to colchicine-free media (Landowne et al., 1982; Matsumoto et al., 1984).

Large depolarizing pulses, to levels above +160 mV, produce a birefringence response which is initially in the opposite direction, an increase in light intensity or a downward deflection compared with the figure in this paper. At these large voltages the effect of colchicine is to make this downward deflection larger, as if a superimposed upward deflection was inhibited (Landowne, 1984*a*; Fig. 3). Figure 6 is a plot of the normalized amplitude of the colchicine-sensitive portion of the birefringence response as a function of membrane potential for depolarizing pulses. Records made in colchicine were

subtracted from the average of before and after control records. Within the experimental recording limits colchicine did not affect the birefringence response to hyperpolarizing pulses. The colchicine-sensitive birefringence response increases with the size of the depolarization and may saturate with larger pulses. This is similar to a plot of the time integral of the gating currents *vs.* membrane potential.

Analogs of Colchicine.

Because much larger concentrations of colchicine are required to block sodium channels than to inhibit mitosis or to depolymerize microtubules, it seemed possible that the effects on the axon were similar to the neutral lipophilic anesthetics, such as the alkanes (Haydon, Elliott & Hendry, 1984). To explore this, several compounds with structures that resembled a portion of the colchicine molecule were tested. 30 mM tri-methoxybenzoic acid (4 axons), 30 mM tropolone (3 axons) and 3 mM colchicinic acid (2 axons) did not alter the birefringence response to brief depolarizations, whereas 1 mM demecolcine reduced the response by about one third. All of these treatments reduced the ionic sodium currents. The effects of the first three substances differed from colchicine and demecolcine in that potassium currents decreased more rapidly than the sodium currents. Of these compounds, demecolcine structurally best resembles colchicine and is the only other potent anti-mitotic agent.

Wavelength Dependence

The relationship between light retardation and molecular dipoles described in the introduction is for frequencies of light that are small with respect to ω_o, the resonant frequency of the dipole oscillator. In this longer wavelength region the retardation should be almost the same for all wavelengths. Cohen et al. (1971) reported no change between 400 and 700 nm for responses to hyperpolarizing pulses for three axons in state 2 (giving the secondary response described below, p. 180). I have repeated this experiment with depolarizing pulses for axons in the fresh (state 1) condition. The fractional birefringence response measured with light between 400 and 710 nm was compared to bracketing measurements made with infrared light between 750 and 1100 nm. Eleven comparisons on 4 axons gave a ratio (infrared/visible) of 1.08 ± 0.29. Ten comparisons for hyperpolarizing pulses gave a ratio of 1.03 ± 0.10. Thus there is no significant difference between measurements made with visible light and those made in the near infrared.

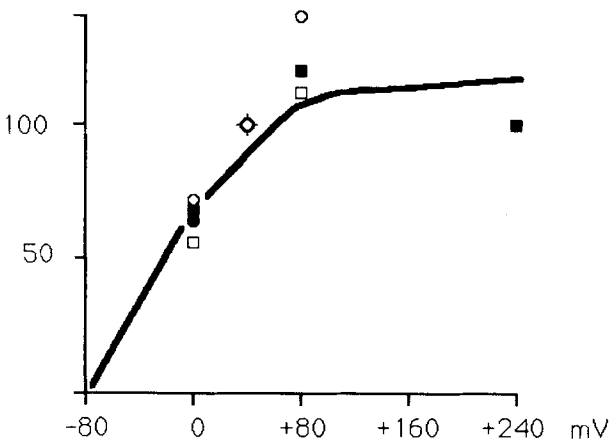

Fig. 6. The relative amplitude of the colchicine-portion of the birefringence response as a function of membrane potential. Measurements were made by subtracting records made in the presence of 30 mM colchicine from the average of records made before and after drug treatment. The amplitudes have been normalized to a measurement made on each axon at +40 mV. The different symbols refer to different axons

LONG PULSES AND COMBINATIONS OF PULSES

When recordings were made on a longer time base (Fig. 7) a slow upward slope to the birefringence trace can be seen to follow the rapid changes of Figs. 2–5. Four birefringence traces were recorded at 3-min intervals and superimposed in this figure. At the start of the 7.92-msec conditioning pulse to 0 mV the light level decreased by 40 ppm during the first 300 μsec and then slowly by an additional 15 ppm during the rest of the pulse. At the end of the pulse there was a rapid recovery of 28 ppm in the first 300 μsec followed by a slow recovery with a 6–7 msec time constant.

The rapid change at the end of the conditioning pulse is smaller than the initial rapid rise. This is similar to the immobilization of gating charge, the rapid gating current transient after a long pulse is smaller than the rapid transient at the beginning (Armstrong & Bezanilla, 1973, 1977). Test pulses to 0 mV applied shortly after the conditioning pulse produce a smaller rapid birefringence response than the conditioning pulse. With longer intervals between the conditioning pulse and the test pulse the amplitude of the rapid phase recovers. The rapid phase of the gating currents also recovers with longer intervals paralleling the recovery from inactivation.

Sodium channels recover from inactivation or

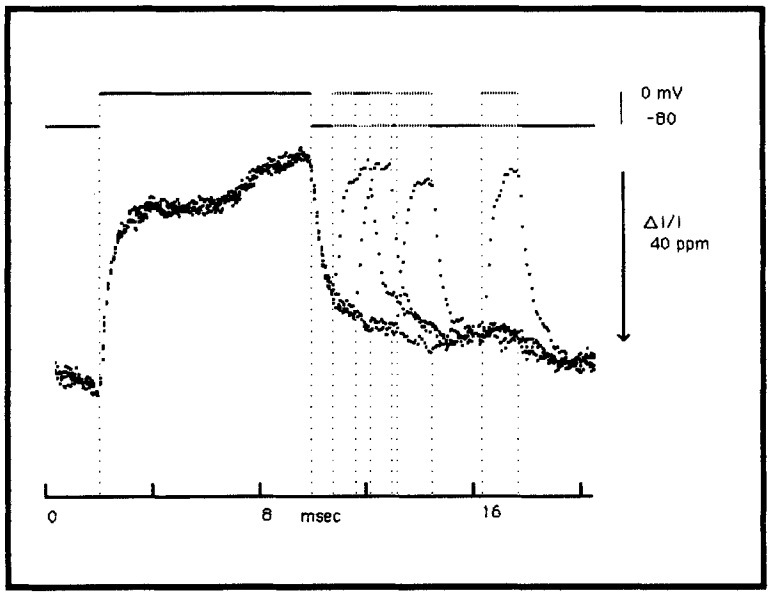

Fig. 7. Birefringence records showing activation, inactivation and recovery of sodium channels. Four records are superimposed, each with a 7.92 msec conditioning pulse and a 1.28 msec test pulse. The recovery intervals were 0.86, 1.6, 3.2 and 6.4 msec. The upper traces were the membrane potential; the lower traces are birefringence. 12°, 20% NaSW, 10 kHz

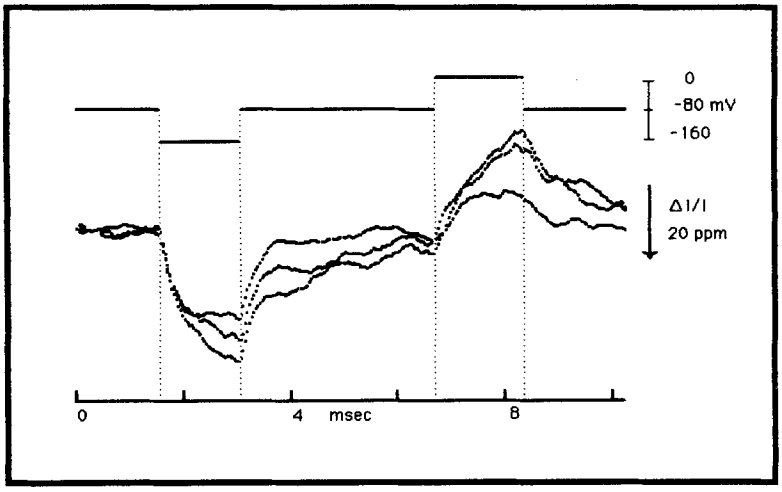

Fig. 8. The secondary birefringence response. Upper trace: voltage Middle family, birefringence records at 0, 4 and 7 min, smallest first. Bottom trace: membrane current for the first record. 2°, 20% NaSW, 3 kHz

immobilization with a rate that increases as the membrane potential is made more negative. Correspondingly, the slow recovery of the birefringence is more rapid when the membrane is hyperpolarized. During the conditioning pulse the slow phase becomes more rapid for larger depolarizations which also have a larger effect on the response to subsequent test pulses. As with the gating currents, depolarizing conditioning pulses reduce but do not eliminate the birefringence response.

Secondary or State 2 Responses

Cohen et al. (1971) concluded there was more than one source of voltage-dependent changes in axon retardation. A fast phase seen in freshly mounted axons and a slow phase or state-2 response appeared irreversibly, either spontaneously or after a large variety of manipulations. They also describe a third response, a very slow 'rebound,' which was extremely labile and apt to disappear. In all of the results described earlier in this paper, the axons exhibited the fast response to hyperpolarization characteristic of state 1. The state-2 response is a secondary response with quite different properties and does not appear to be related to the normal functioning of sodium channels. Because the secondary response is larger than the primary one and obscures the types of observations made above, I usually take its appearance as a sign of pathology and begin a new axon.

Figure 8 shows the progressive appearance of

the secondary response. When first mounted, this axon exhibited the smaller response which reaches a fairly constant level within 0.5 msec after the membrane potential was changed. Five minutes later the slower secondary response can be seen, it has a time constant of several milliseconds and does not reach a steady level during these 1.5-msec pulses. The secondary response continues to grow and can eventually become about five times larger than the primary response and completely obscure it. Unlike the primary response, the secondary response appears symmetrical both in amplitude and time course. No treatment has been found that alters or reverses the secondary response; it persists in morbid axons that have lost the high resistance resting state and the characteristic transient sodium currents. Colchicine, which dramatically reduces the primary birefringence response during depolarization, has no obvious effect on the secondary response.

In this axon the secondary response seemed to appear spontaneously. In many experiments its appearance seemed to be correllated with accidental mechanical or electrical trauma. Contrary to the report of Cohen et al. (1971) tetrodotoxin does not seem to induce the transition. In nine axons external application of 300 nM tetrodotoxin did not produce a secondary response (Fig. 3). Tetrodotoxin does not obviously alter the appearance of the birefringence response under normal conditions or in the additional presence of colchicine. Similarly tetrodotoxin does not alter the gating currents.

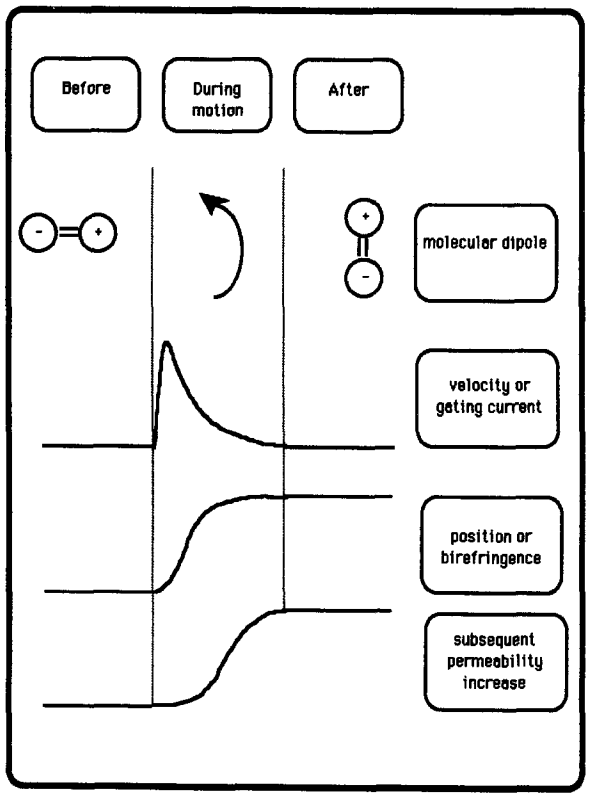

Fig. 9. The relationship between dipole rotation, polarization current and birefringence related to activation of nerve sodium channels

Discussion

Since the classical work of Hodgkin and Huxley (1952) it has been known that the primary event underlying excitation and conduction in nerves is a transient voltage-dependent increase in sodium permeability. This in turn "suggests that the permeability changes arise from the effect of the electrical field on the distribution or orientation of molecules with a charge or dipole moment." A reorientation of molecular dipoles should be accompanied by a displacement current and a change in birefringence.

Although progress has been made since 1952, the composition of the excitable membrane is still largely unknown. Certainly there is a lipid bilayer and imbedded sodium channel proteins but there are also many other proteins associated with the membrane, e.g. other ion channels, sodium and other pumps, and cytoskeletal anchoring proteins.

Armstrong and Bezanilla (1974) found that most of the nonlinear portion of the displacement current which flows when a membrane is depolarized could be associated with gating of sodium channels "with the understanding that the term gate . . . has no very precise definition." It is in the context of an unknown composition and an imprecisely defined structure that I will discuss the relationships between the birefringence results presented above, the gating currents and the ionic sodium currents.

When working with data from electrical measurements it is natural to think in terms of the movement of gating charges. Associating a birefringence change with the function of sodium channels implies there is movement of molecular dipoles. This is not inconsistent with the electrical findings and was proposed as an alternative by both Hodgkin and Huxley and Armstrong and Bezanilla. The relationship between the orientation of a molecular dipole in response to a change in the electrical field within the membrane and the electrical and optical detection of this motion is shown symbolically in Fig. 9.

There are many properties shared by the gating currents and the birefringence changes and there are a few apparent contradictions. I will first review the similarities and then discuss the differences.

Both the birefringence change and the gating currents show an asymmetry about the resting potential which is not seen about a hyperpolarized holding potential. They each precede the increase in sodium conductance when the membrane is depolarized, and they have the same time course as the decline of the sodium conductance when the membrane is repolarized. There is a rising phase of the gating currents and a corresponding initial delay in the birefringence change. Both show a reduction of their rapid phase following a prolonged depolarization and the concomittant appearance of a slow phase. The recovery of the amplitude of the rapid phase parallels the decline of the slow phase and the recovery from inactivation.

A portion of the gating current and of the birefringence response are reversibly blocked by application of colchicine with half-maximal block at about 10 mM internally. Neither the electrical nor the optical signal is affected by reduction of the sodium concentration or the application of tetrodotoxin. All of these treatments reversibly reduce the sodium conductance.

Some of the contradictions between the electrical and the optical view of the phenomena following a depolarization of the axon membrane may arise from the details of the measurement techniques. Some might be resolved by more experiments with squid axons, others may be inherent in the complexity of the preparation. The linear subtraction technique which was so important for the gating current studies is not *a priori* applicable to the optical experiments. The linear displacement currents arise from the movements of ions up to the surfaces of the membrane and the redistribution of the electrons of the apolar lipid molecules. The optical effects of this electronic polarization are not detectable at the present level of sensitivity.

The fast birefringence response during a hyperpolarizing pulse should have a detectable displacement current analog. Unfortunately it is so rapid that it might easily be lost in the much larger geometric and electronic displacement currents. Because of the subtraction techniques used in the gating current studies the molecular polarization which underlies the response during a hyperpolarization would appear as a rising phase of the gating current. This rising phase has been seen, although its significance has been debated (Stimmers et al., 1984).

The displacement current corresponding to the slow 'creep' of the birefringence trace during a prolonged depolarization (Fig. 7) would be difficult to detect in the gating current experiments. It would appear as a steady elevation of the baseline and would not be distinguishable from the nonlinear leak current which is routinely subtracted in these experiments. The slow symmetrical structural changes underlying the secondary birefringence response would not be seen using the gating current subtraction techniques. Given its symmetry, its slow time course, its insensitivity to colchicine and the uncertainty of its appearance, the secondary response is unlikely to be important in the activation of sodium channels.

The significance of the fast, colchicine-insensitive, birefringence changes seen with hyperpolarizations or large depolarizations are also unexplained. They could arise from functionally unimportant movements of the sodium channel proteins or conformational changes of any other molecules in the electrical field. Presently the existence of this fast response to hyperpolarization is confusing because it is difficult to be sure how much it also contaminates the response during depolarization. The amplitude of the response to hyperpolarization is not linearly dependent on the size of the voltage step so a simple linear subtraction is not appropriate. The amplitude of the fast response which remains after colchicine treatment appears to vary as the voltage squared with the origin near 0 mV (Landowne, 1984*a*). The fast response in colchicine at 0 mV is less than one fifth the size of the response without colchicine and its time course has not been reliably determined. Four possible lines of experiments suggest themselves to better dissect the components of the optical response. A chemical or pharmacological technique might be discovered with removes the fast response to hyperpolarization while preserving the function of the sodium channels. More sophisticated optical experiments might reveal signals specific to the two responses. More extensive signal averaging can improve the signal-to-noise ratio and permit a mathematical separation. Finally, a reconstituted system could greatly improve the signal-to-noise ratio and permit positive identification of the molecular origins of these signals.

Strictly speaking, none of these birefringence or gating signals have been experimentally tied to the sodium channel protein molecules. The possibility that a structural change of the lipid portion of the membrane produces these phenomena seems unlikely but has not been rigorously excluded. More specific optical measurements could test this idea, the polarization of the amide infrared absorption bands of the peptide bonds could be measured, although this might be technically difficult.

A Kinetic Scheme

There are two relaxations in the birefringence changes and gating currents and at least one addi-

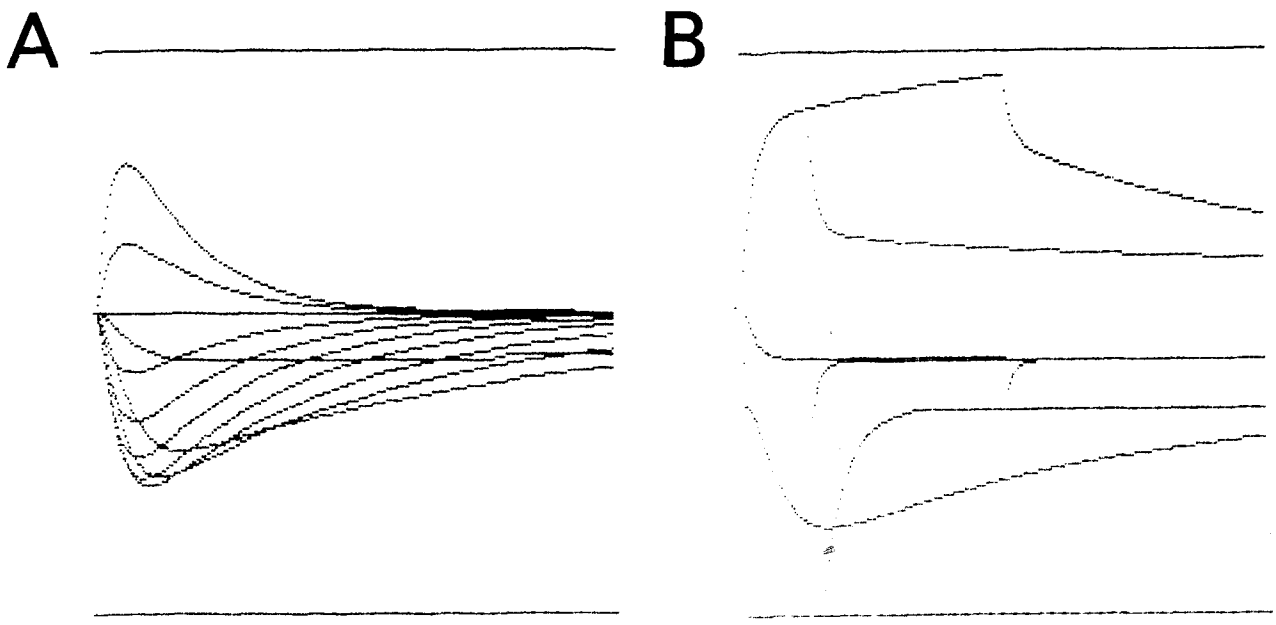

Fig. 10. Simulated sodium channel ionic and polarization currents and changes in birefringence predicted from the kinetic scheme described in the text. (*A*) A family of ionic currents at −40 to +70 mV in steps of 10 mV. (*B*). Upper trace: change in birefringence; middle trace: polarization current for a pulse to 0 mV for two different durations. Lower trace: the ionic current for the short pulse and for a pulse which persists. These figures were computed using the kinetic scheme described in the text. Seven of the nine rate constants were modelled by $k = k_o \pm m(V - V_o)$, + in the forward direction and − for the back reactions. V_o was −30 mV. If the sweep duration is taken as 6.4 msec the rate constants k_o (in sec^{-1}) and their voltage coefficients (in mV^{-1} sec^{-1}) were: resting-activated, k_o, 1600, m, 240; activated-inactivated, k_o, 10, m, 4; resting-inactivated, k_o, 10, m, 4; activated→ open (forward only), k_o, 100, m, 8; open→ activated, k_o, 1000, m, 0; open→ inactivated, k_o, 4000, m, 0. These constants were chosen to illustrate the general form of the currents predicted by this scheme. They are only rough approximations to the actual molecular rate constants

tional relaxation in the ionic sodium currents. Thus a kinetic scheme describing the birefringence response, the gating currents and the ionic currents must include at least four states. I propose naming these the *resting state*, an *activated state*, a separate *open state* and an *inactivated* (and closed) *state*. Depolarization initiates the transition from the resting state to the activated state accompanied by the fast birefringence change and gating current. Channel opening is a subsequent step which is not seen in the birefringence or the gating current records and thus does not involve reorientation of large portions of the molecule or the movement of charges in the direction of the electrical field. This step is slowed by replacing the H_2O with D_2O, which alters the ionic current without changing the gating current (Meves, 1974; Schauf & Bullock, 1979). The D_2O effects suggest the open channel is aqueous and its kinetics are solvent-dependent. Tetrodotoxin may block this step or plug the pore preventing the flow of ions through an otherwise patent channel.

The birefringence data also supports the view that there is a second, slower transition into an inactivated state which is reversed when the membrane is repolarized. The slow off-gating current may also report the transition from the inactivated to the resting state. As discussed above, the slow on-gating current which is predicted by the birefringence measurements would be difficult to separate from a nonlinear ionic leak current. The kinetic scheme can be represented by a simple state diagram.

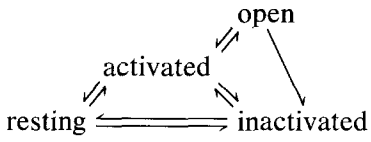

Figure 10 shows predictions based on this scheme with voltage-dependent rate constants for the molecular motion and channel opening and voltage-independent rate constants leading from the open state. The initial delay seen in Fig. 5 is not included because it is brief and may reflect a relaxation in series with the sodium channel molecule (Stimers et al., 1984). Adding an additional intermediate state would make this scheme appear formally similar to the basic model proposed by Armstrong and Bezanilla (1977) (*cf.* Armstrong & Gilly, 1979).

However, two distinctions arise when the molecular motion hypothesis is contrasted with the gating charge models.

Activation is used here to describe the molecular rearrangement which underlies the rapid birefringence change and gating currents and leads to an activated but not necessarily open channel. In the basic model, as in Hodgkin and Huxley's model, activation referred to the actual opening. In the present scheme the molecular motion occurs during the activation process and no major reorientation of dipoles is associated with the subsequent formation of the conducting pathway.

Inactivation here refers to a molecular conformational change to a configuration which does not support the transition to the open state. It is accompanied by the slow birefringence change and gating current and is distinct from the collapse of the conducting pathway. The inactivation motion reported here appears to be voltage-dependent which differs from the proposal of Armstrong et al. More recently Horn, Vandenberg and Lange (1984) and Stimers, Bezanilla and Taylor (1985) have suggested there is a slight voltage dependence to the inactivation process. The rate of leaving the open state, which would determine the single-channel lifetimes, is constant in the model, corresponding to the findings of Aldrich, Corey and Stevens (1983).

The Magnitude of the Motion

Quantitative arguments presented in the introduction suggest that 100–300 peptide bonds/channel molecule rotating 90° could produce a birefringence response of the magnitude seen during a nerve impulse or a depolarization to +40 mV. The dipole moment of formamide, which contains the same N—C=O dipole as the peptide bond, is 3.7 Debye (Kurland & Wilson, 1957). Thus the movement corresponds to the rotation of 400–1100 Debye/channel. Dividing by six charges, the number thought to be responsible for the conductance increase and the gating currents, gives the theoretical distance which experiences the electrical field, about 70–180 Å. This is larger than can reasonably be expected for a lipid bilayer. In order for the proposed hypothesis to be correct a larger dipole, with more peptide bonds, is rotating through a smaller angle or the channel molecule protrudes out of the bilayer. Further work is needed to reduce the experimental uncertainties in these estimates.

In a different membrane channel which facilitates communication between cells, x-ray and electron microscope data have been used to construct a model with six roughly rod shaped subunits, each with a mol wt of 25,000–30,000 daltons. These subunits appear to rotate an average of 5° and slide over one another with a shutter action (Unwin & Zamphigi, 1980). 1500 (6 × 250) peptide bonds rotating 5° would be expected to produce the same birefringence signal as 1500 sin 5° ≈ 130 bonds rotating through 90°, which is within the range of estimates made above for the movement of the sodium channel.

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