

Charge Movement Associated with the Opening and Closing of the Activation Gates of the Na Channels

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ABSTRACT The sodium current (I_{Na}) that develops after step depolarization of a voltage clamped squid axon is preceded by a transient outward current that is closely associated with the opening of the activation gates of the Na pores. This "gating current" is best seen when permeant ions (Na and K) are replaced by relatively impermeant ones, and when the linear portion of capacitative current is eliminated by adding current from positive steps to that from exactly equal negative ones. During opening of the Na pores gating current is outward, and as the pores close there is an inward tail of current that decays with approximately the same time-course as I_{Na} recorded in Na-containing medium. Both outward and inward gating current are unaffected by tetrodotoxin (TTX). Gating current is capacitative in origin, the result of relatively slow reorientation of charged or dipolar molecules in a suddenly altered membrane field. Close association with the Na activation process is clear from the time-course of gating current, and from the fact that three procedures that reversibly block I_{Na} also block gating current: internal perfusion with Zn^{2+} , prolonged depolarization of the membrane, and inactivation of I_{Na} with a short positive prepulse.

INTRODUCTION

The sodium and potassium permeabilities of squid axon membrane are voltage dependent: both permeabilities are low at the resting potential, and they increase sharply when the membrane is depolarized (Hodgkin and Huxley, 1952). Regardless of the mechanism of ionic permeation through the membrane, the permeability increase must involve the movement of charged structures which change position or conformation in response to an alteration of the membrane field. No ion in either the external or the internal medium is essential for the maintenance of these voltage-dependent permeabilities (see Discussion), which leads one to expect that the moving structures are composed of molecules fixed within the membrane. There is

strong evidence that ions pass through the membrane by way of hydrophilic pores, and it is convenient to think of these pores as controlled by membrane molecules which serve as "gates," with the understanding that the term gate at present has no very precise definition.

In this view the gating molecules are charged or have dipole moments, which makes their conformation sensitive to the membrane field: the charges or dipoles move following a field change to new positions that minimize the overall potential energy of the gating structure. The charge movement associated with this process, or "gating current," should be measurable and there have been several attempts to observe it experimentally. Hodgkin and Huxley, who first predicted gating current, were unable to detect it in their experiments (1952), and concluded that the total charge movement, and the density of the gates in the membrane, must be low. Chandler and Meves (1965) attempted to measure gating current in internally perfused axons, after removing permeant ions both inside and out. Their procedure had the advantage that it virtually eliminated ionic current, but they were nonetheless unable to observe anything suggestive of gating current, and they concluded that there must be fewer than 100 Na channels/ μm^2 .

A small current presumably associated with gating was measured by Armstrong and Bezanilla (1973), who combined the techniques of internal perfusion, voltage clamp, and signal averaging. The existence of this current was soon confirmed by Keynes and Rojas (1973), who used essentially the same techniques, and added information helpful in identifying the observed currents with the Na channels. Additional properties of this current and strong evidence linking it to the opening and closing of the Na pores have been reported by Bezanilla and Armstrong (1974). In this paper we describe in detail some gating current properties, and a new technique (the "divided pulse" procedure) that allows us to study aspects of the current that were obscured by the original method.

THEORY OF THE METHOD

Membrane current following a positive step of membrane potential (V_m) is the sum of capacitative and ionic current, each of which can be subdivided into several components, as diagrammed in Fig. 1. Capacitative current, the flow of ions (a in Fig. 1) from one "plate" of the membrane capacitor, the external fluid, to the other plate, the internal fluid, is enhanced by charge redistribution in the membrane, which is the dielectric of the capacitor. This intramembranous charge movement has three parts. First is the rearrangement of charge along the apolar molecules that comprise the bulk of the membrane (b in Fig. 1). This movement is undoubtedly very rapid, and is complete by the time the "window" (described below) is opened, and it plays no part in our measurements. Concurrent with and following this

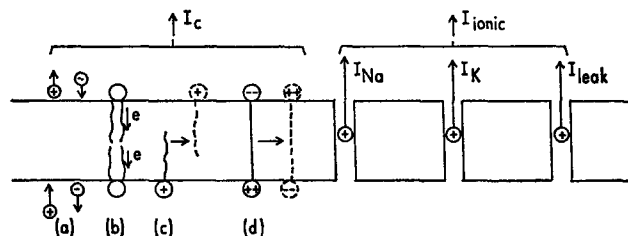


FIGURE 1. The components of membrane current after a change of V_m . Capacitative current (I_c) is enhanced by intramembranous charge movement due to (b) redistribution of electrons on the membrane lipid molecules, and reorientation of charged (c) or dipolar (d) molecules. (a) represents the ion movements that carry capacitative current from the membrane surfaces to the electrodes and the external (voltage clamp) circuit.

component is a slower component caused by the movement of charged groups tethered in the membrane (c in Fig. 1) or rotation of groups that have dipole moments (d in Fig. 1). A reasonable expectation is that gating is associated with one of these slower components, which are indistinguishable electrically. A small contribution to capacitative current may result from alterations of the membrane thickness (Mullins, 1959; Cohen et al., 1971).

Gating is a nonlinear property of the membrane (the activation gates open for positive steps from the resting potential, but not for negative ones), so it is reasonable to expect that gating current will be nonlinear, larger for positive steps than for negative ones. For this reason it is convenient to distinguish between the linear components of capacitative current, which are probably not associated with gating, and the nonlinear components, which may be. Our method, then, was to reduce ionic current as much as possible, and to eliminate linear components of the capacitative current, in the hope of revealing a nonlinear component of the latter. The second task was to prove that the nonlinear component is associated with gating.

All evidence indicates that the gating structures respond passively to changes of the membrane field. Axons that are perfused internally give many thousands of action potentials in the total absence of an energy supply other than the ionic gradients, and the gradients themselves can be reversed without affecting gating. The conclusion is that gating charge must move as dictated by changes of the membrane field: outward, after a positive step of membrane potential (V_m), when the gates are opening, and inward, during a negative step. Movement in the opposite direction would require the expenditure of energy.

Because Na activation is the fastest of the gating processes, we expected that gating current associated with it would be largest and most easily measured. It was possible to roughly estimate in advance the magnitude of the expected charge movement. From the dependence of the sodium conductance (g_{Na}) on V_m , Hodgkin and Huxley (1952) estimated that each Na

channel must have about six electronic charges associated with it. Taking 50 channels/ μm^2 as a rough estimate of channel density, there should be movement of 300 electronic charges/ μm^2 across the membrane after a step which opens all the pores. (In fact, as has been reported by Keynes and Rojas, 1973, total charge movement is substantially larger than this.)

Elimination of the Linear Component of Capacitative Current

This was accomplished by adding the current produced by a positive step to that from a negative step of exactly equal magnitude. The sum of all linear components of capacitative current is zero with such a procedure. To improve the signal to noise ratio, we usually applied 10–100 positive steps, and an equal number of negative steps and averaged. Equality of negative and positive steps was carefully adjusted by applying the steps to a test circuit composed of resistors and capacitors, and adjusting the pulses until the current sum for an equal number of positive and negative steps was zero. This procedure has the disadvantage that it mixes current from the negative step into the positive step current, which is the more interesting current since it is associated with the opening of the gates. To alleviate this difficulty, we sometimes used the divided pulse procedure, which is described in the Results section.

Elimination of the Ionic Currents

Most of our experiments were performed on axons perfused internally with CsF, and immersed in an external medium containing the impermeant ion Tris instead of Na^+ . Cs and Tris ion are essentially impermeant through the Na and K channels. In most of the experiments tetrodotoxin (TTX) was used to eliminate any residual current through the Na channels. Most of the ionic current that remains in these conditions is "leakage" current. The subtraction method just described eliminates most of the leakage current, leaving a base line that is determined by the nonlinearity of the leakage. If this current is time invariant, any nonlinearity presents no problem, for it produces a steady base line in the records that can easily be subtracted away. Complete time invariance of the leakage current cannot be assumed, but strong evidence is presented to show that the currents we record in these conditions are not ionic. Hence, variation of the leakage current with time is not a problem, except during steps to extremely negative membrane voltages, as described in the Results section.

METHODS

Experiments were performed on giant axons from the squid *Loligo pealei*. After cleaning away the surrounding small fibers, the axons were mounted in a chamber (for a description, see Armstrong et al., 1973), and internally perfused by a modification of the Tasaki technique (Tasaki et al., 1962; Rojas and Ehrenstein, 1965; Bezanilla and Armstrong, 1972). The internal fluid in a typical experiment was exchanged several times per minute. During the perfusion procedure a dual electrode was introduced into the fiber, one electrode for measuring the internal voltage, and the other, a platinized wire, for passing current to control the voltage. These electrodes and their external counterparts were connected to a voltage clamp circuit, which

could on command complete a step of membrane voltage in about 10 μ s. To improve accuracy and frequency response, the voltage clamp automatically compensated for the small resistance of the fluid path from electrodes to the membrane.

The names and compositions of the internal and external solutions are given in Tables I and II. In the text the name of the external solution is given, and followed, after two slashes, by the name of the internal solution. The osmotic strength of all solutions was checked with an osmometer, and internal and external solutions were

TABLE I
EXTERNAL SOLUTIONS

Name	Na ⁺	K ⁺	Tris ⁺	Ca ²⁺	Mg ²⁺	Cl ⁻	TTX
	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>M</i>
ASW	440			10	50	560	
ASW 10K	430	10		10	50	560	
5% Na SW	22		418	10	50	560	
Tris SW			440	10	50	560	
Tris SW TTX			440	10	50	560	3×10^{-7}
120 Ca Tris SW TTX			391	120		631	3×10^{-7}
Ca SW			7	367		741	
Ca SW TTX			7	367		741	3×10^{-7}

TABLE II
INTERNAL SOLUTIONS

Name	K ⁺	Ca ⁺	TEA ⁺	Zn ²⁺	F ⁻	Br ⁻	Cl ⁻	Sucrose
	<i>mM</i>	<i>mM</i>	<i>mM</i>		<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>
275 K	275				275			400
275 K 15 TEA	275		15		275	15		380
290 Cs		290			290			400
95 Cs 248 K	248	95			343			300
100 CS 180 K	180	100			280			400
185 Cs 100 K	100	185			285			400
185 Cs 100K 10 Zn	100	185		10	285		20	380

osmotically matched to within 1%. Solutions were buffered with 1 mM HEPES (*N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and pH was adjusted to 7–7.2 at room temperature.

DATA RECORDING The apparatus we used for averaging and recording membrane current is diagrammed in Fig. 2. The external current electrode in the measuring region of the experimental chamber was connected to the summing junction of an operational amplifier wired as a current to voltage transducer. The output of the transducer was AC coupled (150-ms time constant) to the first of a series of three amplifiers. The first amplifier in the series could be programmed for (a) a continuous gain of 1 \times , or (b) a gain of 1 \times during positive voltage steps, and a gain of 4 \times during negative steps. The latter program was used in the divided pulse procedure, which is described in the Results section. The output of the first amplifier was con-

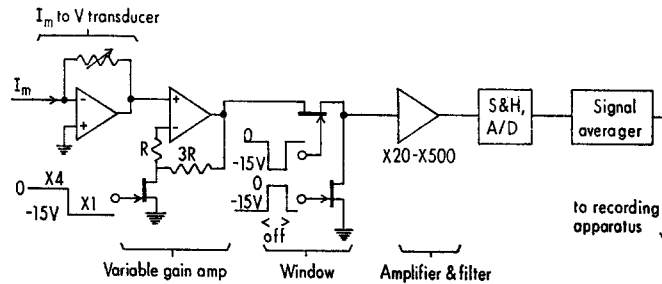


FIGURE 2. Apparatus for averaging and recording gating current. The window is a device for blanking out the large capacitive current signal that follows a step change of V_m . The variable gain amplifier was set for a continuous gain of 1 X except in the divided pulse procedure (see text). S & H is a sample and hold amplifier, and A/D an analogue to digital converter.

nected to a window, which grounded the input to the second amplifier when voltage steps were being applied to the membrane. The purpose of the window was to blank out the large signal representing the fast portion of capacitive current, to prevent saturation of the second and third amplifiers. The window was normally open, but closed for 5–65 μs just before the application of a step. The blanking interval was usually about 20 μs , but it varied somewhat depending on the properties of the axon being examined. The other two amplifiers in the series multiplied the signal by an adjustable factor of 20–2,000. These amplifiers were filtered with a time constant adjustable between 5 and 12 μs .

Our initial experiments were performed with a Waveform Educator (Princeton Applied Research Corp., Princeton, N. J.), which served to sum and average the membrane current. This machine has serious disadvantages for our application, and later experiments were performed with a digital averager of new design, which will be described in detail elsewhere. Together with a sample and hold amplifier and a 12-bit A/D converter, the digital averager could store and average 256 data points, each point representing an interval 5–100 μs . After averaging, the data were reconverted to analog form, and stored on magnetic tape (FM), photographed, and transcribed at a slow rate on an inkwriter.

RESULTS

The trace in Fig. 3 is the sum of the membrane current produced by 10 positive and 10 negative steps of exactly matched amplitude. The axon was in 5% Na sea water (SW), internally perfused with 290 CsF. In these solutions, the sodium current (I_{Na}) is quite small, but normal in behavior: it peaks and inactivates in the usual way. I_{Na} is preceded by a transient outward current (I_g) that is too small to be seen in solutions containing the usual concentration of Na ion. This outward current is what we shall refer to as “turn-on” gating current, meaning that it is associated with the turn-on of g_{Na} (the sodium conductance). When the pulse is terminated (after 800 μs) there is a tail of inward current that is composed, as will be shown momentarily, of

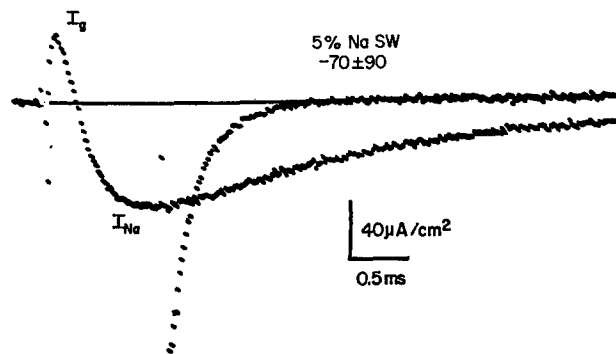


FIGURE 3. Gating current (I_g) and sodium current (I_{Na}) recorded in 5% NaSW //290 CsF. Two traces have been superimposed. In one the pulse (90-mV amplitude) was interrupted just after the peak of I_{Na} , and in the other it continued beyond the end of the trace. Each trace is the sum of current from five positive and five negative steps, 2°C.

both sodium current and an inward “turn-off” gating current, that is associated with the turning-off of the pores.

Gating current can be seen without interference from I_{Na} by performing the same experiments in the absence of external Na ion, and with tetrodotoxin (TTX) in the external medium (Tris SW TTX//290 CsF). In this solution gating current is outward (Fig. 4 *a*) and has a distinct rising phase that is quite unlike the sigmoid rising phase of I_{Na} recorded from the same axon in artificial sea water (ASW) (Fig. 4 *b*). The origin of the rising phase of turn-on I_g is examined in a later section. I_g peaks about 100 μ s after the onset of the pulses and then decays, and is virtually gone shortly after the peak of the I_{Na} transient. The peak amplitude of I_g recorded in this way is roughly $50 \times$ less than the amplitude of I_{Na} in normal sodium sea water, so that in Fig. 4 *b* gating current is almost totally obscured by I_{Na} .

When the pulses end (that is, on return of the membrane potential to -70 mV, from 0 and -140 mV), gating current is inward, and it decays approximately exponentially (Fig. 4 *c*). I_g at turn-off is largest immediately after the voltage change, and it decays monotonically, with a time-course similar to that of I_{Na} at turn-off (Fig. 4 *d*).

Gating Current is an Intramembranous Charge Movement

To demonstrate that the current we have observed and labeled as gating current is produced by intramembranous charge movement, it is first necessary to show that it is not ionic current. One argument is that gating current has a time-course unlike that of any known ionic current. This is not a conclusive argument, and it might be said that we are observing a newly discovered outward ionic current through channels that activate very rapidly, and then inactivate; i.e., a conductance similar to g_{Na} , but smaller and with a faster

time-course. If this were the case, the inward current tails at pulse-end should become smaller with time as inactivation progresses, and should be proportional in amplitude to the outward current. Fig. 5 shows clearly that this does not happen. The last tail in the figure, which occurs when the outward

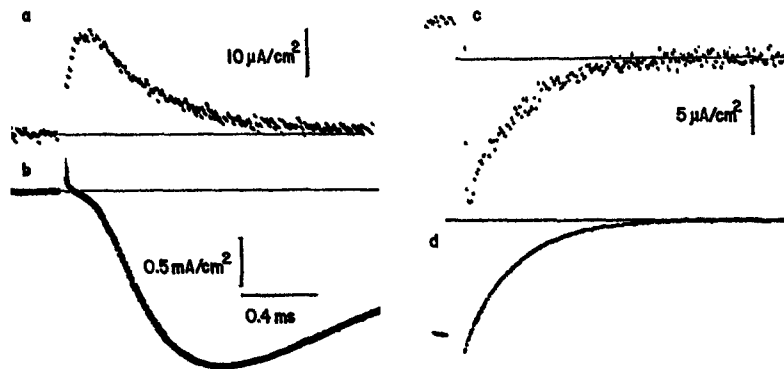


FIGURE 4. Gating current and I_{Na} during turn-on and turn-off of g_{Na} . The gating current traces are the sum of current during (a) and immediately after (c) 50 positive and 50 negative steps of 70-mV amplitude, from a holding potential of -70 mV. Tris SW TTX // 185 Cs 100 K, 2°C . (b) I_{Na} after a step from -100 to 0 mV. ASW // 95 Cs 248 K, 2°C . (d) I_{Na} on return to -70 mV, after a $700 \mu\text{s}$ pulse to 0 mV. ASW 10 K // 275 K 15 TEA, 2°C . a and b from one axon, c and d from another.

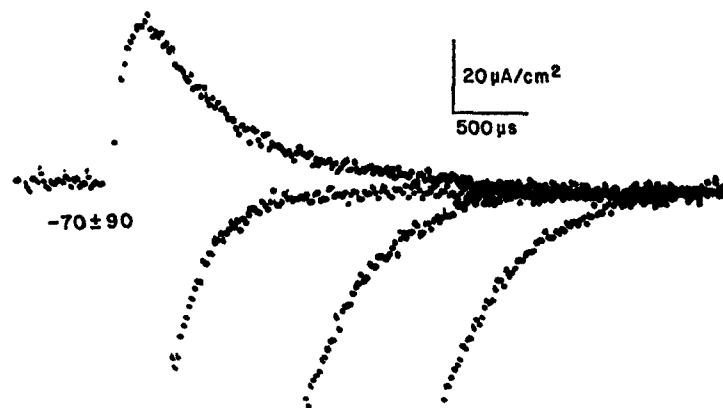


FIGURE 5. Gating current during turn-on of g_{Na} (outward current) and during turn-off after pulses of various durations (inward tails of current). 120 Ca Tris SW TTX // 290 Cs, 2°C . 50 positive and 50 negative steps.

current is almost zero, is about the same in amplitude as the first tail, which is at the time of nearly maximal outward current. Thus a single ionic conductance cannot explain the observed pattern.

Another possibility is that the falling phase of the observed (outward) current is the sum of a steady outward current and a relatively slowly de-

veloping inward current. If this were so the current tails at pulse-end would grow larger as the inward current develops, and this does not occur. A third possibility is that the current is ionic, and the outward current decays not because of a permeability change, but because of accumulation or depletion of the permeant ion in a confined space near the membrane. If this were true, the tails should grow larger as depletion or accumulation progresses, and this does not happen as can be seen in Fig. 5, where the first tail is about the same size as the third.

From these considerations we conclude that the observed currents are not ionic, but are due instead to movement of charge confined within the membrane. If this is true, the time integral of outward current during turn-on should be exactly equal to the integral of current during turn-off. (The *observed* charge movement is not necessarily equal: a component of inward charge movement, for example, might be slow and consequently too small to measure.) Total charge movement during turn-on and during turn-off were determined by integrating (with a planimeter) gating current as a function of time, and the on and off movement are plotted against each other in Fig. 6. The points are derived from experiments on six axons, which were subjected to pulses of various amplitudes, and durations from 0.2 to 2 ms. In all cases turn-on and turn-off areas were very nearly equal and there is no systematic deviation from the line which represents exact equality. This provides strong additional evidence that what we have called gating current is caused by movement of charges fixed within the membrane.

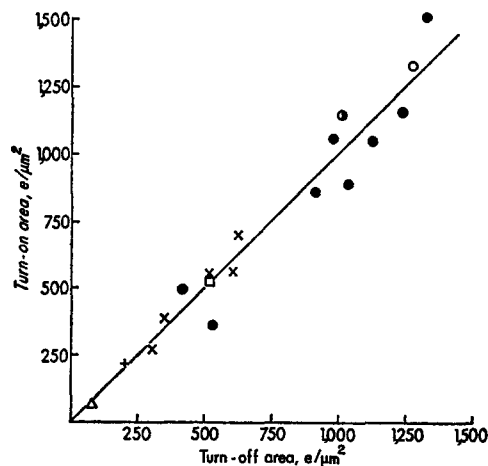


FIGURE 6. Outward charge movement during the steps is plotted against the inward current at step-end. All points fall near the line, which represents equality of inward and outward charge movement. This is good evidence that the moving charges are fixed within the membrane. The points are taken from six experiments, each of which is represented by a different symbol.

Gating Current Is Associated with Na Activation

The preceding sections have shown that gating current is caused by movement of intramembranous charge, and that it has the time-course expected for events associated with the opening of the Na activation gates. A much stronger argument for identifying gating current with the Na pores is that three procedures which reversibly decrease I_{Na} also decrease gating current, reversibly. The first of these procedures involves internal perfusion with 10 mM $ZnCl_2$, which has been shown by Begenisich and Lynch (1974) to reversibly eliminate I_{Na} . The abolition of gating current by $ZnCl_2$ is shown in Fig. 7, lower trace. After removal of Zn^{2+} recovery was virtually complete.

In the second procedure, the membrane was depolarized continuously to +56 mV for 2 min, and holding potential was then returned to its usual value (-70 mV). Fig. 8 shows gating current and I_{Na} (the axon was in 5% Na SW//290 Cs) recorded before the period of depolarization (upper left trace), immediately after it (0 s) and at two later times during recovery. In the trace taken at 0 s there is a fast inward tail of current that we cannot identify, but no turn-on gating current and no I_{Na} . I_{Na} and gating current recover together as the later traces show, and after 420 s recovery is complete.

Gating current and I_{Na} are both decreased by a positive prepulse that inactivates the sodium conductance, g_{Na} . As in the case of prolonged depolarization just described, this procedure reduces gating current by the same factor that I_{Na} is reduced. After destruction of Na inactivation by Pronase (Calbiochem, La Jolla, Calif.), neither gating current (which is not grossly altered by Pronase) nor I_{Na} are affected by a prepulse. These experiments regarding inactivation and gating current have been briefly described in a preceding paper (Bezanilla and Armstrong, 1974), and will be presented in detail in a later report.

A final point is that gating current cannot be recorded from axons that are incapable of producing sodium currents when in ASW. Fig. 9 illustrates

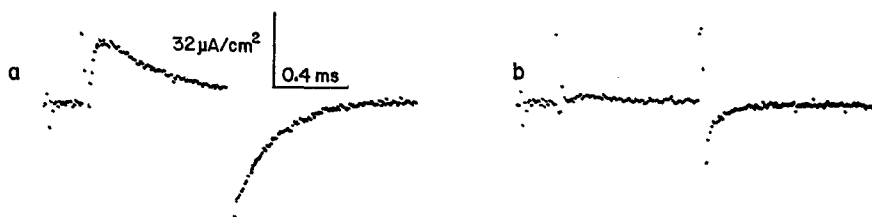


FIGURE 7. Abolition of gating current by internal perfusion with 10 mM $ZnCl_2$ (b), which also abolishes I_{Na} (not illustrated). Recovery was almost complete after Zn^{2+} was removed. (a) Tris SW TTX//185 Cs 100 K, 2°C. (b) Same axon, Tris SW TTX//185 Cs 100 K 10 Zn, 2°C. 50 positive and 50 negative steps.

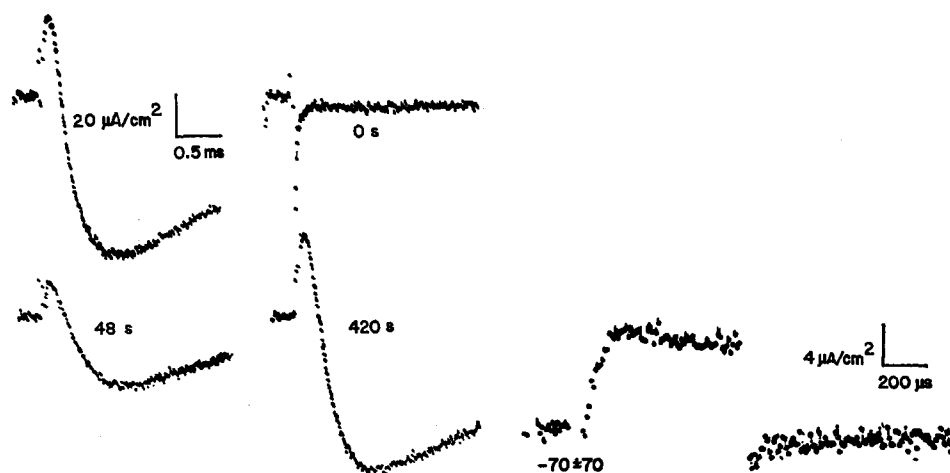


FIGURE 8

FIGURE 9

FIGURE 8. Depression of I_{Na} and gating current by prolonged depolarization. After recording the control trace (upper left), V_m was held continuously at +56 mV for 2 min. Gating current was then determined at the indicated times after returning the holding potential to -70 mV. 5% Na SW // 290 Cs, 2°C. Five positive and five negative steps.

FIGURE 9. The dead axon pattern: the averaged current from an axon no longer capable of yielding gating current. Tris SW TTX // 100 Cs 180 K, 1.5°C. 50 positive and 50 negative steps.

what we call the “dead axon” pattern, recorded from such an axon. The main feature of the pattern is the almost complete absence of a transient outward current, and its replacement by a maintained outward current, which is probably ionic, produced by nonlinearity of the leakage current. (There is a very small transient current at turn-on and turn-off that resembles gating current: the axon was not completely dead). The reason for believing that the bulk of the outward current in Fig. 9 is ionic is that total outward charge movement during the pulse is much larger than inward movement after the pulse. Axons that yielded the dead axon pattern were never capable of producing I_{Na} on return to ASW. (In a previous report Armstrong and Bezanilla, 1973, the dead axon pattern was interpreted as the sum of a maintained outward step of ionic current added to a quick inward current tail, which is responsible for the rising phase in Fig. 9.)

TTX Does Not Affect Gating Current

Two experiments that illustrate this point are shown in Fig. 10. For the left record in part *a* of the figure the fiber was in Ca SW // 290 Cs, with no TTX present. Trace I shows an initial outward gating current that is followed and partially obscured by a small inward current that we presume to be Ca current through the Na channels (cf. Baker et al., 1971). When 3×10^{-7} M

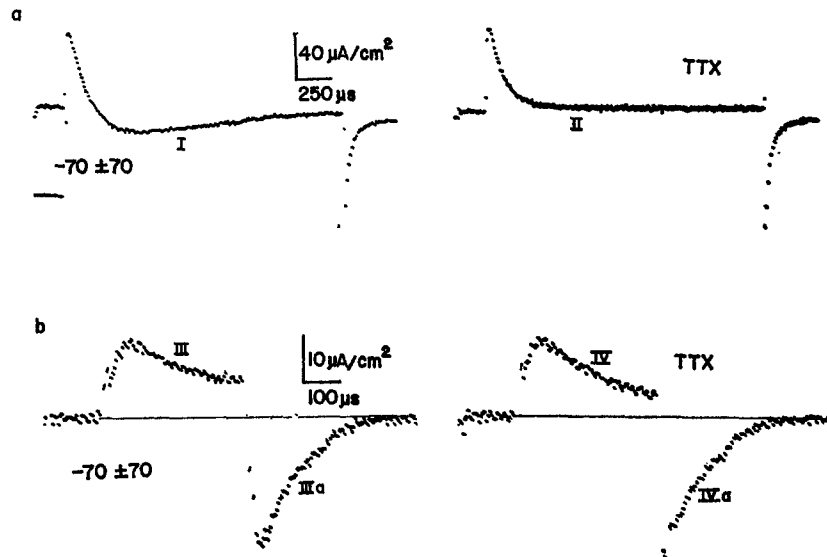


FIGURE 10. TTX does not affect gating current. (a) Averaged current from an axon in Ca SW // 290 Cs (trace I), and in Ca SW TTX // 290 Cs, at 2°C. The small inward current in trace I is Ca^{++} current through the Na pores. 100 positive and 100 negative steps. (b) Current from an axon in Tris SW // 275 Cs (trace III) and in Tris SW TTX // 290 Cs, at 2°C. 50 positive and 50 negative steps.

TTX was added to the external medium, the inward current was abolished (trace II) but gating current was unaffected.

Trace III (Fig. 10 b) was recorded from a fiber in Tris SW // 290 Cs, and IV was recorded after adding $3 \times 10^{-7}\text{M}$ TTX. The currents were not much affected, but TTX did seem to eliminate a small outward component of current during the step, making trace IV lower than III. This outward component was probably Cs^+ current through the Na channels (cf. Meves and Vogel, 1973). At pulse-end turn-off gating current was almost unaffected. IV *a* is slightly lower in amplitude than III *a*, perhaps because the latter contains a small component of inward Ca^{2+} current through the Na channels.

Origin of the Rising Phase of Turn-on Gating Current

The current shown in Fig. 3 a is a sum, composed of an outward current during the positive steps and an inward current during the negative steps. The outward current presumably is associated with a transition of the gates from "closed" to "open" conformation; and the inward current with a transition closed to "more fully closed" conformation (see Discussion). The more interesting component is the outward one, associated with gate opening. Can the two components be separated, making it possible to measure the outward component in isolation? We have approached this problem in three ways. The first two ways rely on the expectation that over a sufficiently

negative voltage range there should be no gating charge movement, because all of the gating structures are in "fully closed" conformation throughout the range.

GATING CURRENT AS A FUNCTION OF HOLDING POTENTIAL One way to fully close the gating structures might be to make the holding potential (V_H) very negative, and a series of records at steadily more negative holding potentials is shown in Fig. 11. At $V_H = -50$ mV, there is a large initial inward tail of current, which is followed by a small outward current. At $V_H = -80$ mV, there is no inward tail, but there is a distinct rising phase which is largely absent in the record at $V_H = -100$ mV. These records suggest that an inward current that flows during the negative step and is responsible for the initial tail at $V_H = -50$ and the rising phase at $V_H = -80$, gets smaller as V_H is made more negative, while an outward component that flows during the positive step gets larger. At $V_H = -100$ mV there remains (in some experiments) an indication of a rising phase, which might disappear for V_H still more negative. Unfortunately, the membrane shows evidence of breakdown at very negative voltages, and Fig. 11 *c* shows this to be a problem even when V_H is no more negative than -100 mV. After an initial outward peak of gating current, the trace crosses the base line in the figure, and there follows a steadily increasing inward current that is almost certainly ionic, and related to membrane breakdown during the negative pulse, which took V_m to -200 mV. To circumvent this problem of breakdown, we devised the procedure described in the next paragraph.

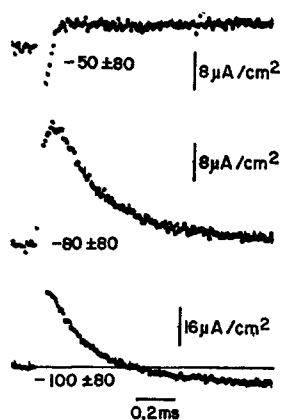


FIGURE 11

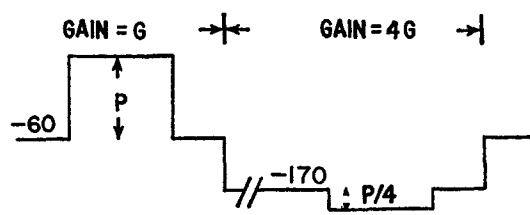


FIGURE 12

FIGURE 11. Gating current form and amplitude depend on the holding potential. Each trace is the sum of current from 50 positive and 50 negative steps of 80-mV amplitude, from the holding potential indicated. Tris SW TTX //290 Cs, 2°C.

FIGURE 12. Voltage diagram for the divided pulse procedure. The current during the positive step was multiplied by G , while the current from the negative step, which was one-fourth as large, was multiplied by $4G$ before being added to the positive step current.

DIVIDED PULSE PROCEDURE The pulse sequence for this procedure is shown in Fig. 12. For 200 ms before the positive step, V_m was held at -60 mV, and a step of P mV was then applied. The current during this step was amplified by G and fed to the averager. After the step, and a short period at -60 mV, V_m was changed to -170 mV and held there for 200 ms. A step of $-P/4$ was then applied, and current was amplified by $4G$ and fed to the averager. The apparatus was tested for artifact by applying steps of $+P$ and $-P/4$ to a test circuit composed of resistors and capacitors, and adjusting the pulses until no signal was recorded. The advantages of this procedure were several. (a) V_m stayed within safe limits: there was no evidence of membrane breakdown. (b) The negative step covered a voltage range where, judging from the results below, there was little gating charge movement. (c) It was thus possible to get a good idea of the amplitude and time-course of the gating charge movement during the positive step, with little contamination from the negative step current. A disadvantage of the method is that the signal to noise ratio during the negative step is reduced, and the records are therefore relatively noisy.

The results of this procedure are compared with the simple procedure in Fig. 13. Part *a* of the figure shows the current for a fiber held at -60 mV,

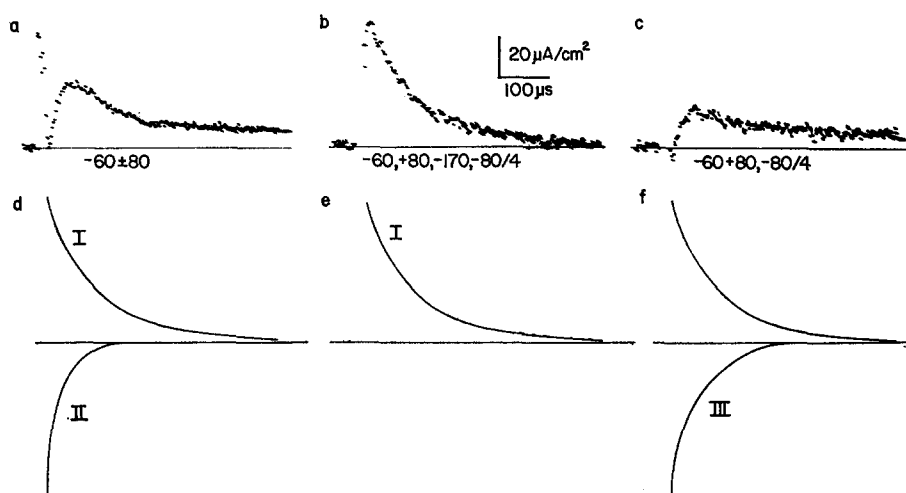


FIGURE 13. The origin of the rising phase of turn-on gating current. (a) The gating current recorded using positive and negative pulses of 80-mV amplitude from a holding potential of -60 mV has a prominent rising phase. (b) Current recorded from the same axon by the divided pulse procedure (Fig. 12) has almost no rising phase. (c) For this trace the divided pulse procedure was used, but V_m was -60 mV before both the positive and the negative step. The rising phase is more prominent than in part *a* of the figure. Tris SW TTX // 290 Cs, 2°C , 20 positive and 20 negative steps for each trace. (d-f) Reconstructions of the recorded traces. I is the curve of part *b* of the figure, which is postulated to be the current flowing during the positive step. II and III are the currents presumed to flow during the negative steps in *a* and *c* (see Discussion).

and pulsed alternately to +20 and -140 mV. There is a very definite rising phase, a peak, and the suggestion of a slow component on the falling phase (see below). Trace *b* employed the divided pulse procedure, with steps from -60 to +20 mV, and -170 to -190 mV. The outward current is larger than in trace *a*, and there is virtually no rising phase. We think that trace *b* is an almost pure record of the gating current flowing during the positive step. As a control we also tried the divided pulse procedure but with no change of holding potential; i.e., a positive step from -60 to +20 mV, and a negative step from -60 to -80 mV. The result, trace *c*, generally resembles trace *a*, but the rising phase is slightly more prominent and the outward current smaller. This is the result to be expected if gating charge movement is slower and larger over the range -60 to -80 mV than over the -170- to -190-mV range.

A possible objection to direct comparison of traces *a* and *b* is that average V_m is more negative during the divided pulse procedure, and this may somehow condition the membrane to yield larger gating currents. We tested this possibility by applying steps from -70 to 0 mV and -70 to -140 mV to a fiber held half the time at -70 mV and half the time at -140; i.e., average V_m was approximately -105 mV. The gating current was the same as for $V_m = -70$ mV: the more negative average potential made no difference.

DIRECT RECORDING OF GATING CURRENTS ON SINGLE SWEEPS In favorable cases we could see what we believe to be gating current in a single sweep at high amplification. Current traces for a single positive and a single negative step are shown in Fig. 14. The positive step is followed by an outward current of approximately the same time-course and amplitude as trace *b* of Fig. 13. There is a small rising phase of brief duration, which may be the result of the recovery from the slight overshoot evident in the voltage trace. The negative step is followed by an inward current that decays with a relatively rapid time-course. The flattening at the beginning of the current may, again, be

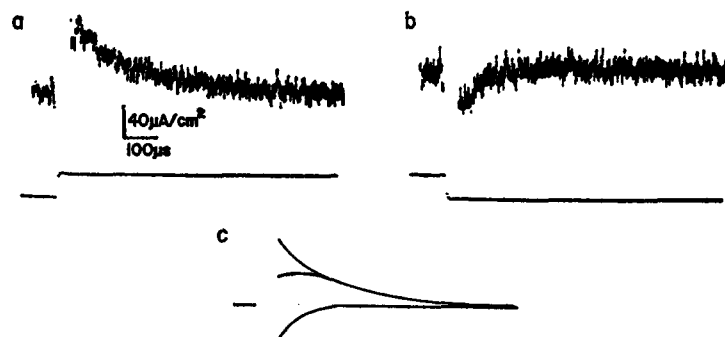


FIGURE 14. Gating current recorded on a single sweep, following a step of +70 mV (*a*) and -70 mV (*b*) from a holding potential of -70 mV. Tris SW TTX, 290 Cs, 2°C. (*c*) Tracings of the currents from *a* and *b*, and their sum.

due to recovery from a slight voltage overshoot. These two traces were added together graphically to yield the curve in Fig. 14 *c*. This curve has a general resemblance to, for example, trace *a* of Fig. 13, leading us to believe that the latter is indeed the sum of a negative and a positive component.

DISCUSSION

The results that have been presented show that gating current is a component of capacitative current, caused by the movement of intramembranous charge; and that it is closely associated with the opening and closing of the activation gates of the Na pores. Several reasons are given for thinking gating current is capacitative rather than ionic. Perhaps the most compelling argument is that outward charge movement as the gates open during turn-on is equal to inward movement during turn-off, as the gating structures return to closed conformation. Close association of gating current with the Na pores is clear from the experiments which show that internal perfusion with Zn^{2+} , inactivation of g_{Na} with a short, positive prepulse, and prolonged depolarization all decrease both I_{Na} and gating current, in proportion. The time-course of gating current leaves no doubt that it is associated with activation, rather than inactivation of the Na pores: it precedes the opening of the pores, and it has almost the same time-course as I_{Na} at turn-off (Fig. 4). Further, turn-on gating current is little if at all affected by internal perfusion with Pronase, which destroys Na inactivation (Armstrong et al., 1973).

For convenience we have referred to all of the current recorded with these procedures simply as gating current, but it may contain more than one component. In addition to the component described here, which is associated with Na activation, we have observed (as will be reported later in detail) a small, slow component of current which we speculate may be associated with K activation. One might anticipate that many membrane molecules not associated with gating have dipole moments and contribute to capacitative current. This cannot be completely discounted, but the experiments described show that the bulk of the recorded current closely parallels I_{Na} in behavior, for example in response to prolonged depolarization. This seems unlikely to be coincidence, and we think most of the recorded current is associated with Na activation. For the same reasons it seems unlikely that the recorded current results from changes of membrane thickness.

Schneider and Chandler (1973) have recorded a nonlinear component of capacitative current from muscle fibers that may well be related to excitation-contraction coupling. Their current is roughly a hundred times slower than the ones described here, far too slow to be associated with the action potential permeabilities.

A major goal in studying gating current is to understand its kinetic relation to g_{Na} (sodium conductance), and for this reason it is important to know

the precise form of the current produced by the opening of the gates. Specifically, it is important to know if this current has a rising phase (which would have interesting molecular implications) or if it is largest initially and decays monotonically. In any method which uses subtraction to eliminate the linear portion of capacitative current (and subtraction seems unavoidable), the current produced as the gates open is contaminated by an unknown amount of inward current that flows during the negative step. Use of the divided pulse procedure makes it possible to minimize the inward current, for the negative step can cover a very negative voltage range where there is little or no gating charge movement. Using this procedure, we recorded the trace of Fig. 13 *b*, which has almost no rising phase. When this trace is exponentially extrapolated back to the beginning of the step, the result is curve I (Fig. 13), which may be a good representation of the current associated with the opening of the pores.

Curve I can be used to reconstruct the current record of Fig. 13 *a*, as shown in Fig. 13 *d*. The observed trace in 13 *a* is considered to be the sum of curve I, the positive step current, and the negative step current, curve II, which is the difference between curve I and the observed trace in 13 *a*. Curve II is the current that flows during the negative step from -60 to -140 mV. The magnitude of this inward current is at first surprising, for g_{Na} is very low at both -60 and -140 mV: few gates actually open or close over this range. This charge movement presumably represents a change in the gating structures from one closed conformation to another, or, loosely, from closed to fully closed, if it is understood that conductance of a pore is zero in both states. In Fig. 13 *b*, the inward current is inappreciable, as shown in 13 *e*, for the negative step goes from -170 to -190 mV, and the gates are in fully closed conformation at both potentials. The current trace in Fig. 13 *c* is reconstructed in 13 *f*, by the same method, and there is again a substantial inward current. This was expected, for the negative step was from -60 to -80 mV, a voltage range in which many gates change from closed to more fully closed conformation. From Fig. 13 *c* it is clear that the pulse division procedure in itself is not obscuring the rising phase, and the crucial point is that the negative step be from one very negative voltage to another.

Additional evidence that the reconstructions in Fig. 13 *d-f* are valid, comes from the single sweep recording of gating current in Fig. 14. Except for a short period at the beginning of the traces, both the outward (Fig. 14 *a*) and the inward (Fig. 14 *b*) current closely resemble the currents used in the reconstructions of Fig. 13. In summary, we believe that curve I in Fig. 13 gives the form of the current associated with the opening of the Na activation gates for a step from -60 to $+20$ mV. This current is largest immediately after the step, and resembles the current expected from reorientation of dipolar molecules in a suddenly altered field. Though there seems no doubt

that most of the rising phase originates from inward current during the negative step, a trace of it remains in most experiments in spite of the divided pulse procedure, perhaps because of imperfect time resolution, or because V_m was not sufficiently negative to fully close all of the gates. Whether or not this remaining trace of rising phase is a genuine feature of the positive step current remains an open question.

The change from closed to fully closed conformation when V_m is made more negative seems to have consequences that are detectable in recordings of I_{Na} : the turn-on of this current is greatly delayed if V_m is very negative before a depolarizing step, as shown in Fig. 15. Two current traces are shown, one for a step from -70 to -10 mV, and the other for a step from -130 to

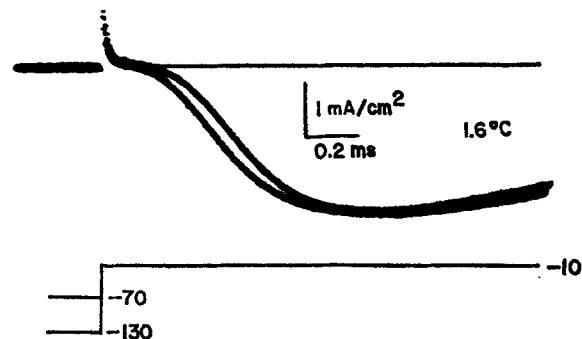


FIGURE 15. Hyperpolarization to -130 mV before a step delays the turn-on of g_{Na} . ASW //275 K, 1.6°C .

-10 mV. For the step from -130 , there is a pronounced lag before I_{Na} rises, for the gating structures start from fully closed (or almost fully closed) conformation. The lag is much less following the step from -70 mV: the gating structures at -70 are closed rather than fully closed, and less molecular rearrangement is required to attain open conformation. A similar phenomenon involving I_K has been reported by Cole and Moore (1960). An increase in duration of the lag is predicted by the Hodgkin and Huxley equations for both currents but the predicted effects are much smaller than the observed ones.

The Hodgkin and Huxley equations predict gating currents that are qualitatively similar to the observed ones, if it is assumed that the m particles are charged, and their movement generates a current that is proportional to dm/dt . In particular, dm/dt is largest immediately after a step, so the predicted current is largest initially and decays exponentially, qualitatively resembling curve I in Fig. 13. There are, however, two significant differences between the gating currents predicted by their equations and the observed ones. The first is that the predicted turn-off currents have a time constant three times longer than the time constant for decay of I_{Na} ; while, as seen in

Fig. 4, the time constant of the observed turn-off current is only slightly longer than that of I_{Na} . The second difference is that gating current inactivates together with I_{Na} , while the Hodgkin and Huxley equations predict that gating current is unaffected by inactivation. These differences will be described in more detail in a later paper.

The gating currents that are shown in this paper are substantially larger than the ones first reported (Armstrong and Bezanilla, 1973). The reason for the difference is mainly that the pulse frequency in the present experiments ($\frac{1}{200}$ ms, 400 ms between positive pulses) is lower than in the original experiments ($\frac{1}{12}$ ms, 24 ms between positive pulses). It has been shown that gating currents inactivate together with I_{Na} , and at the higher frequency there was not enough time between pulses for full recovery. Even with 400 ms between positive pulses current amplitude is somewhat depressed, and rough tests show that amplitude might be 10–15% higher if frequency were further reduced. Gating currents of similar amplitude have been recorded by Keynes and Rojas (1973).

The capacitance of squid axon membrane ($1 \mu\text{F}/\text{cm}^2$; Cole, 1968) is about three times as high as would be predicted on the basis of a 75-Å membrane with an average dielectric constant of 3 (which is a reasonable figure for the dielectric constant of nonpolar substances like those that must constitute the bulk of the membrane). It now appears that gating charge movement may contribute as much as $0.25\text{--}0.5 \mu\text{F}/\text{cm}^2$ to low frequency capacitance in the voltage range where gating occurs.

The absence of a TTX effect on the gating currents tells a good deal about the drug's mechanism of action. Previous studies have shown clearly that TTX does not affect the kinetics of g_{Na} (Hille, 1966; Takata et al., 1966), and this fact has three possible interpretations. Either TTX blocks the Na pores without affecting the gates, or it seals the gates in closed position, or it blocks the pores with the gates sealed in open position. Our results show that the gating structures still move in response to V_m changes, so TTX must act at a locus removed from the gates. TTX blocks I_{Na} only when applied outside (Narahashi et al., 1967), suggesting that the Na activation gate is at the inner end of the pore. There is already some evidence that the K activation gate (Armstrong and Hille, 1972) and the Na inactivation gate (Armstrong et al., 1973) are inside.

Calcium ion has often been speculated to play a direct part in gating but the present results do not support these speculations: gating current is capacitive, not ionic. It might still be argued that Ca^{2+} fixed in the membrane is important to gating, but this seems unlikely to us, for single muscle fibers exposed to EGTA-containing medium with a Ca^{2+} concentration of approximately 10^{-9}M can give action potentials for 20 min or more (Armstrong et al., 1972). Ultimately, action potentials cease not because the action potential machinery fails, but because resting potential amplitude falls from 80–90

mV to about 40 mV. If membrane-bound Ca^{2+} plays a part in the action potential, it must be bound very firmly indeed.

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