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Immobilization of Sodium Channel Gating Charge in Crayfish Giant Axons by the Insecticide Fenvalerate

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SUMMARY

The type II pyrethroid fenvalerate is known to depolarize nerve membranes by keeping sodium channels in a very stable modified open state. We have performed experiments on crayfish giant axons to determine whether the asymmetric charge movement was affected in parallel with changes in sodium current. When all sodium channels were modified by repetitive stimulation in the presence of fenvalerate, on gating charge movement was reduced by 78%. When on gating currents were fractionated into three exponentially decaying components, it was found that fenvalerate selectively depressed the intermediate and slow components, leaving the fast component unchanged. Off gating currents could be fractionated into two exponentially decaying components. The fast component of off charge movement (τ = 50 μsec at −160 mV) was abolished by fenvalerate, whereas the slow component was suppressed by 50%. These results are consistent with previous conclusions that a large fraction of the intermediate and slow on and slow off components and essentially all of the fast off components are related to sodium channel gating. We conclude that fenvalerate traps the gating charges of sodium channels in the open state.

It is well established that pyrethroid insecticides act primarily on the voltage-activated sodium channel to modulate its gating kinetics (1–3). Pyrethroid insecticides have been classified into two major groups on the basis of poisoning symptoms and effects on nerves. Type I pyrethroids, such as tetramethrin and allethrin, cause repetitive firing of nerve fibers, whereas type II pyrethroids, such as deltamethrin and fenvalerate, cause depolarization of nerve membranes (4, 5). Although not chemically related to pyrethroids, the organochlorine insecticide DDT acts in many respects like a type I pyrethroid (4). All pyrethroids act by slowing the closing and inactivation of open sodium channels, but type II pyrethroids slow down these processes much more than type I pyrethroids do. For example, the time constant for closing of sodium channels in crayfish giant axons is slowed from 30 μsec to about 200 msec by the type I pyrethroid tetramethrin (6) and to about 1 min by the type II pyrethroid fenvalerate (7).

Gating current, which is asymmetric charge movement generated by voltage-dependent channels as they change conformation in response to changes in membrane potential, is a very important tool for the study of voltage-gated channels because it can give more direct information about the conformational changes of channels than can ionic current. The prolonged sodium current in pyrethroid-poisoned axons shows that the conducting pore of the channel is held open, but it does not tell us whether the gating charges themselves are held in the open position. It is, in fact, conceivable that the so-called gates that close the channel pore become uncoupled from their voltage sensors in the presence of the pyrethroid, so that, although the channel pore itself cannot close, the gating charges remain free to move in accordance with the applied electric field. However, the voltage dependence of channel closing does not appear to be affected by pyrethroid modification (6, 8, 9), suggesting that this is not the case and therefore that the gating charges of sodium channels would be effectively immobilized in the open state.

Dubois and Bergman (10) found that DDT specifically decreased the off gating current as expected, causing 50% of the off charge to return more slowly than normal after a single activating pulse. However, the effect has never been shown for a pyrethroid. Vijverberg (11) found that on and off gating currents were equally suppressed in squid axons by allethrin and in frog nodes of Ranvier by cismethrin, and Yeh and Narahashi[1] have made similar observations using tetramethrin on squid axons.

The studies mentioned above compared on and off gating charge movements in the presence of DDT or a pyrethroid. This method is inherently insensitive for two reasons. First, only a fraction (up to 50%) of the sodium channels are expected to be modified by the pyrethroid during a single pulse, and the

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[1] J. Z. Yeh and T. Narahashi, unpublished observations.

ABBREVIATIONS: DDT, 1,1′-(2,2,2-trichloroethylene)bis[4-chlorobenzene]; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

small size of the gating currents might make it difficult to see the expected effect. Second, under some conditions the expected immobilization of the gating charge by the pyrethroid may not be distinguishable from the natural immobilization associated with inactivation (12). For these reasons, we used an alternative approach, whereby gating currents were measured after modification of essentially 100% of the channels by repetitive stimulation in the presence of the type II pyrethroid fenvalerate. With this procedure, we were able to show that the major components of the gating current associated with sodium channel activation were completely abolished when all channels were modified by fenvalerate.

Materials and Methods

Giant axons (175–250 μm in diameter) isolated from the circumoesophageal connectives of the crayfish (*Procambarus clarkii*) were cannulated and internally perfused as described by Lund and Narahashi (6). The double-sucrose gap voltage-clamp technique originally developed by Julian *et al.* (13, 14) was used. The external solution contained 205 mM NaCl and 10 mM $CaCl_2$, buffered at pH 7.5 with 3 mM HEPES. The internal perfusate contained 170 mM cesium glutamate, 50 mM CsF, and 15 mM NaCl, buffered at pH 7.35 with 5 mM HEPES. The presence of 220 mM cesium in the internal solution effectively eliminated current through potassium channels. For gating current measurements, 1 μM tetrodotoxin or saxitoxin was added to the external solution to block the sodium current. Fenvalerate [(*S*)-α-cyano-3-phenoxybenzyl (*S*)-2-(*p*-chlorophenyl-3-methylbutyrate)] was added to the internal perfusate at a concentration of 10 μM, and at least 45 min of exposure were allowed before measurements were taken, because of the extremely slow onset of its effects (7). The fenvalerate sample, provided by the Sumitomo Chemical Company, was 98.3% chemically pure, with an *S*:*R* ratio in the acid moiety of 98.1:1.9. The experiments were carried out at 10.0 ± 0.2°.

Voltage-clamp pulsing and data acquisition were done with a PDP-11 computer. Coarse subtraction of linear leak and capacitive currents was made with an active transient subtraction circuit, and a modification of the P/4 pulse protocol of Bezanilla and Armstrong (15) was used to remove all traces of linear current components from both ionic and asymmetry current measurements (except for records photographed from the oscilloscope). A holding potential of −120 mV was used for these experiments, and for all gating current measurements except those shown in Fig. 7B both the control (P/4) and test (P) pulses were given from a hyperpolarized level of −160 mV that was established 20 msec before the beginning of the test pulse (see protocol in Fig. 2). Although the usual practice is to give only the control pulses from the hyperpolarized level, we also gave the test pulse from this level because there was charge movement negative to −120 mV in the presence of fenvalerate (see Fig. 8). The sampling interval was 10 μsec. Fitting of exponentials to data was done using a Levenberg-Marquardt least squares minimization program.

Results

Effects of fenvalerate on the sodium current. The effects of fenvalerate on the sodium current are described in detail elsewhere (7); only those aspects relevant to the gating current experiments will be presented here, to support our premise that repetitive stimulation in the presence of fenvalerate puts essentially all of the sodium channels into a state or states from which they can be neither activated nor deactivated on the normal millisecond time scale.

The control sodium current decayed to only 5% of its peak level after 12 msec at −10 mV, and there was a very rapidly decaying tail current after repolarization to −120 mV (Fig. 1A). After exposure to fenvalerate the peak amplitude was reduced and inactivation became incomplete. Furthermore, after termination of the pulse the sodium tail current remained large. The tail current in the presence of fenvalerate had an initial rising phase similar to that seen in pyrethroid-treated frog axons and thought to be due to direct reopening of modified inactivated channels (16). It is also noteworthy that the tail current at −120 mV was smaller than the current at −10 mV. This is due to a large decrease of sodium channel conductance at negative potentials as a result of block by calcium ions (17, 18). The majority of the tail current decayed very slowly, with a time constant of 45 sec (Fig. 1B).

Fig. 1C shows sodium currents recorded on moving film as evoked by repetitive stimulation at a frequency of 1 Hz in a fenvalerate-poisoned axon. Each consecutive overlapping trace had a short base line at the beginning of the sweep, followed by the sodium current during the pulse, and then by the tail current after the pulse. These parts are labeled 1, 2, and 3, respectively, for the first and last traces of the record. During the first pulse, the sodium current was activated normally but was inactivated only about 40%, as in Fig. 1A. The form of the tail current was also similar to that in Fig. 1A, where the rising phase was 14% of the final tail current amplitude. In the last pulse of the train, the current trace was considerably different. The short base-line segment before the depolarization was at the same amplitude as the previous tail current, because there was no significant decay of the tail current (τ = 45 sec) during the 1-sec interval between pulses. Furthermore, when these base-line segments were traced back they showed that by the last pulse the number of modified channels had reached a steady state, with essentially all of the channels modified. During the depolarizing pulse there was negligible activation, because all channels were modified, but there was some apparent inactivation. The rising phase of the tail current, about 20% of the total tail current, is due to recovery from this inactivation. It is not clear whether this apparent inactivation is due to inactivation of a fraction of the modified channels or to movement of all modified channels into a low conductance open state. However, it seems that after prolonged repetitive stimulation all channels are modified and cannot deactivate between pulses. Therefore, no activation occurs during a subsequent pulse, providing ideal conditions to observe an effect on activation gating current, which is thought to be the major component of the nonlinear dielectric displacement current.

Control on asymmetry currents. Fig. 2, *left*, shows control sodium and on asymmetry currents at various potentials. The sodium current was first recorded using a P/4 protocol with no hyperpolarizing shift. Then 1 μM tetrodotoxin was added to the external solution, and the control asymmetry currents were recorded using a hyperpolarizing shift to −160 mV for both test and control pulses. As will be shown below, this large shift was required to eliminate charge movement during the control pulses with fenvalerate. The asymmetry currents in Fig. 2, *right*, were recorded after exposure to 10 μM fenvalerate for 90 min. To ensure that all channels were modified by fenvalerate, the axon was depolarized to 0 mV for 10 msec 50 times, at a frequency of 2 Hz, immediately before the series of asymmetry current records was begun and once after each asymmetry current test pulse (this was also done for the control). Test pulses were given at 2 Hz, and 32 sweeps were averaged at each

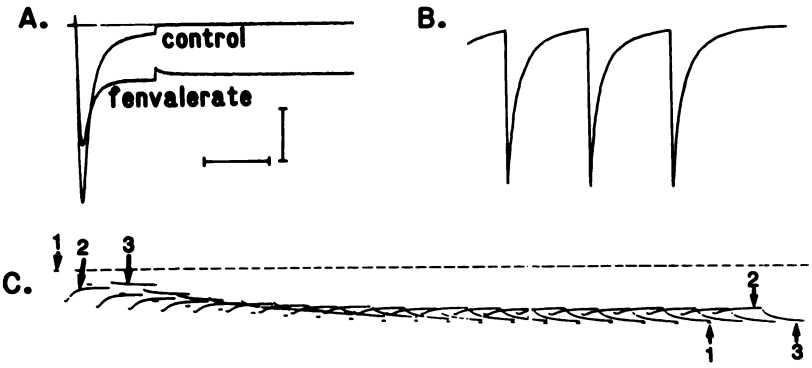

Fig. 1. A, Sodium current evoked by a 12-msec depolarizing pulse to −10 mV from a holding potential of −120 mV in control and after exposure to 10 μM fenvalerate for 70 min. With fenvalerate, the peak current was reduced by one third, and only 55% of the channels inactivated during the pulse. The tail current associated with step repolarization had a small rising phase, but it did not decay significantly during the 30 msec shown here. B, Chart recorder tracing (note slow time scale), showing the slow decay of the tail currents after depolarizing pulses to 0 mV, lasting 5 msec, from a holding potential of −120 mV in an axon that had been exposed to 10 μM fenvalerate for 45 min. C, Sodium currents in response to 21 consecutive 5-msec pulses to −20 mV from a holding potential of −120 mV, at 1 Hz, with 10 μM fenvalerate. Each *trace* shows a 0.5-msec segment before the pulse (*1*), the current during the pulse (*2*), and a 4.6-msec segment of the tail current (*3*). For clarity, each consecutive sweep was shifted to the right by moving the film between sweeps. Calibrations: A, 10 msec, 1 mA/cm²; B, 2 min, 0.6 mA/cm²; C, 6.4 msec, 5 mA/cm².

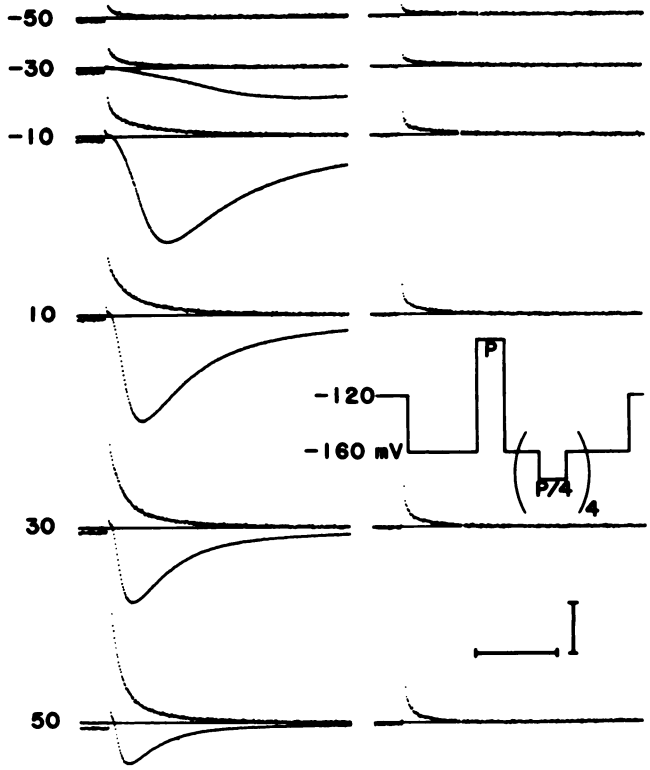

Fig. 2. *Left*, control sodium ionic and on asymmetry currents evoked by 3-msec pulses to the potentials (mV) indicated on the *left*. The P/4 protocol shown in the *inset* was used for asymmetry current measurements, and the same protocol without the hyperpolarizing shift to −160 mV was used for the ionic currents. Thirty-two sweeps were averaged for the asymmetry currents. *Right*, asymmetry currents taken in the same manner after exposure to 10 μM fenvalerate for 90 min. To ensure that all channels were modified by fenvalerate, the axon was stimulated repetitively before and during each family of asymmetry currents, as described in the text. The base line for the asymmetry currents is taken as the steady state level during the pulse. Calibrations: *horizontal*, 1 msec; *vertical*, 2 mA/cm² for ionic and 100 μA/cm² for asymmetry currents.

potential. Asymmetry current was not subtracted from the ionic current records.

Conventional kinetic models for sodium channel gating predict that on gating currents should be the sum of a number of exponentially decaying components (19, 20). Usually, three components are required to fit the currents adequately, but it is evident from most studies that more than three components are present. We were able to fit three exponentials to the asymmetry transients for all potentials between −60 and +30 mV. From the hyperpolarized level of −160 mV, steps to potentials as negative as −80 mV induced a perceptible asymmetry current. This was largely the "fast" component, which became faster and larger in magnitude as the test potential was made more positive, up to −50 mV, without significant activation of the sodium current. The fast component could still be seen for the depolarizations to −30 and −10 mV on top of a second slower phase that became perceptible at −30 mV (Fig. 2). The fast component was present at potentials at which there was not appreciable activation of the sodium current, and where there was activation the fast component was over well before activation even began (−30 and −10 mV in Fig. 2). This has led to the suggestion that the fast component of asymmetric charge movement either is due to early steps in the activation process that precede opening of the channel (21) or is not involved in sodium channel gating at all (20).

The slower phase of charge movement at −30 and −10 mV coincided more closely in time with sodium current activation than did the fast component. The slower phase was resolved into two exponentially decaying components, called intermediate and slow.

On asymmetry currents with fenvalerate. After modification of essentially all of the sodium channels by repetitive stimulation in the presence of fenvalerate, the on asymmetry current was drastically reduced at all potentials where there was significant activation of the control sodium current (Fig. 2, *right*). This can be seen from the records in Fig. 2 and from the charge-voltage (Q-V) relation in Fig. 3 for the same experiment, where the integrated charge movement before and after application of fenvalerate is compared with the peak conductance-voltage relationship. Fenvalerate changed not only the amplitude but also the kinetics of the on asymmetry current. Fig. 4 shows the asymmetry current for the step to +30 mV in Fig. 2, superimposing the records obtained before and after exposure to fenvalerate. Clearly, charge movement later in the pulse was suppressed much more than that occurring immediately after the step to +30 mV (time zero). Therefore, we sought to investigate the effects of fenvalerate on the fast, intermediate, and slow components of the gating currents.

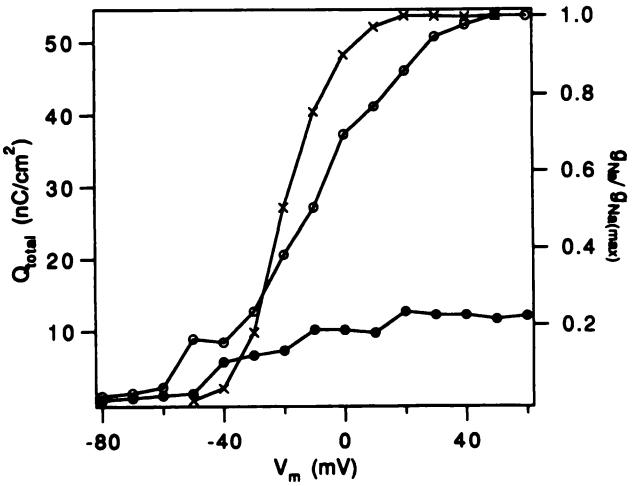

Fig. 3. The on asymmetry charge (Q_{total}) for control (○) and with 10 μM fenvalerate (●), as a function of pulse potential. The normalized peak sodium conductance [$g_{Na}/g_{Na(max)}$] (×) is also shown. Note the large reduction of on asymmetry charge in the presence of fenvalerate.

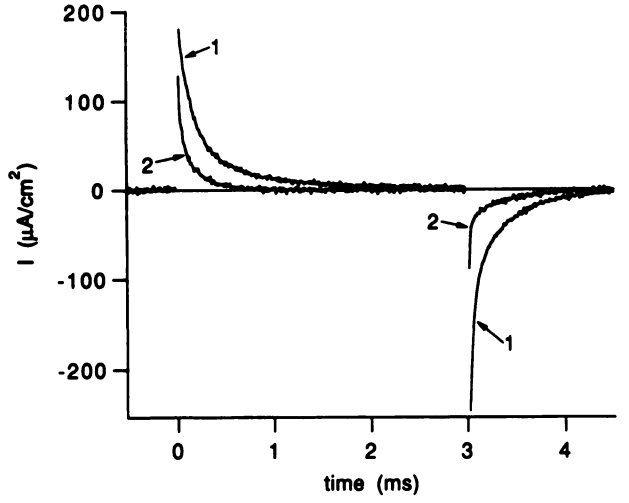

Fig. 4. A, On and off asymmetry currents for a 3-msec depolarizing pulse to +30 mV from a hyperpolarized level of −160 mV before (*trace 1*) and after (*trace 2*) exposure to 10 μM fenvalerate. These are the same records as those at +30 mV in Fig. 2 but show the off asymmetry current as well. A nonlinear leak current of 8 μA/cm^2 was subtracted from *trace 1* during the pulse.

The fast component could not be reliably separated from the intermediate component at +30 mV; at −30 mV, where the separation was excellent, fenvalerate had no appreciable effect on either the time constant of or the charge movement in the fast component, whereas it decreased the time constants and reduced the charge movement in both the intermediate and slow components by 50% (Table 1). The reduction of intermediate and slow charge movement was even greater at +30 mV.

The time constants of and charge movement in the intermediate and slow components for the experiment in Fig. 2 are plotted against membrane potential in Fig. 5. As potential increased in the control, the intermediate charge movement, Q_m, reached a plateau at −30 mV, increasing only slightly from −30 to 0 mV. Q_m then increased almost 5-fold between 0 and +30 mV. Between 0 and +10 mV, the intermediate time constant, τ_m, increased sharply from 92 to 130 μsec. This discontinuity in both of its parameters suggests that the intermediate

TABLE 1

Time constants (τ) and gating charge (Q) for fast, intermediate, and slow gating current components for the experiment in Fig. 2, at −30 and +30 mV

The fast component could not be reliably quantitated at +30 mV.

	τ_f	Q_f	τ_m	Q_m	τ_s	Q_s
	μsec	nC/cm^2	μsec	nC/cm^2	μsec	nC/cm^2
−30 mV						
Control	7.0	1.2	86.4	3.1	700	10.8
Treated	5.8	1.3	35.3	1.5	336	5.8
Treated/control		1.1		0.48		0.54
+30 mV						
Control			135	18	580	33
Treated			44	2.9	196	9.9
Treated/control				0.16		0.3

component contains at least two subcomponents, a faster one that predominates at potentials more negative than 0 mV and a slower one that becomes significant at more positive potentials. In keeping with the notation of Starkus and Rayner (19), who previously found two components within the intermediate component in crayfish axons, these will be called fast intermediate current (I_{mf}) and slow intermediate current (I_{ms}), respectively. In the presence of fenvalerate, Q_m was most strongly suppressed at potentials more positive than 0 mV, where no contribution from Q_{ms} was evident after fenvalerate treatment (Fig. 5). The jump in τ_m above 0 mV was also abolished by fenvalerate, providing more evidence that fenvalerate abolishes I_{ms}, leaving I_{mf} to dominate the intermediate component. Fenvalerate also decreased Q_m at potentials between −40 and 0 mV, suggesting that it also depressed charge movement in I_{mf}, although to a lesser extent than I_{ms}. The time constant of I_m was also decreased by fenvalerate at potentials where it should have been dominated by I_{mf}.

The slow component, I_s, accounted for most of the gating charge movement and occurred over the same potential range as activation (Figs. 3 and 5B). The charge moving in the slow component was very strongly suppressed by fenvalerate (Fig. 5B). The time constant of the slow component in the control increased from 423 μsec at −50 mV to 700 μsec at −30 mV and then decreased gradually as potential was made more positive. The τ_s-V relation retained its biphasic shape after treatment with fenvalerate, but the time constants were decreased substantially at all potentials.

Thus, it is clear that fenvalerate selectively suppressed the slow and slow intermediate components of the asymmetry current, which are thought to be associated with sodium channel activation. It also had a weaker effect on the fast intermediate component. How much of the fenvalerate-insensitive charge movement is actually associated with sodium channel gating is not known with certainty.

Effect of fenvalerate on the off gating current. Fig. 4 shows on and off asymmetry current transients for a step to +30 mV from −160 mV, before and after fenvalerate treatment. As expected, fenvalerate reduced the on and off asymmetry currents proportionately; Q_{off}/Q_{on} was 1.03 for the control and 0.985 after fenvalerate treatment. The control off transient could be fit by two exponential functions, a fast one with a time constant of about 50 μsec and a slow one with a time constant of about 400 μsec (Fig. 6). When measured at the repolarization potential of −160 mV, the time constant of the slow component of the off asymmetry current showed some dependence on the activating pulse potential (Fig. 6; Table 2).

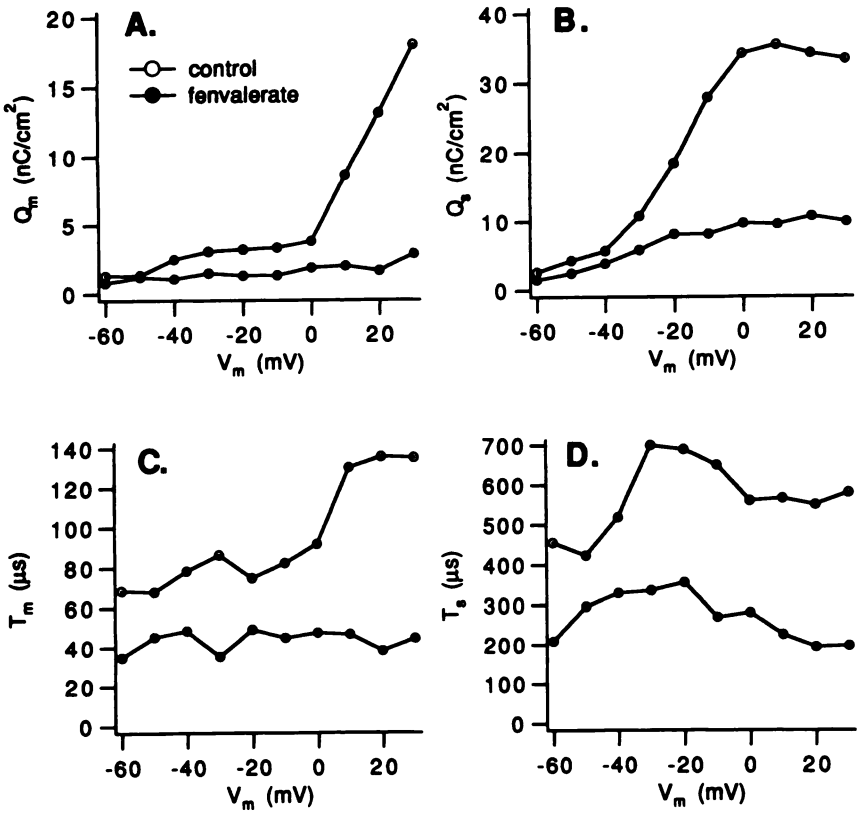

Fig. 5. Magnitude and time constant of charge movement in the intermediate (A and C) and slow (B and D) components of the on asymmetry currents for the experiment in Fig. 2, before (○) and after (●) fenvalerate treatment.

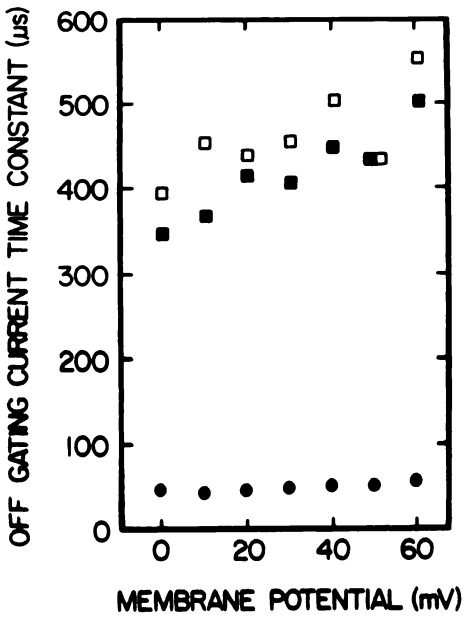

Fig. 6. Time constants of the fast (●) and slow (■) components of the off asymmetry current after a 3-msec pulse, as a function of pulse potential in control. Although all currents were measured at −160 mV, the decay time constant of the slow component is seen to depend on the pulse potential. The off asymmetry current with fenvalerate retains only the slow component (□), with a time constant that was not significantly different from the control. The data are from Table 2.

TABLE 2

Components of off gating currents in control and with fenvalerate at −160 mV after a 3-msec pulse to the indicated potential

V_m	Control					Fenvalerate, slow	
	Fast		Slow		Q_{total}	Q	τ
	Q	τ	Q	τ			
mV	nC/cm^2	μsec	nC/cm^2	μsec	nC/cm^2	nC/cm^2	μsec
0	13.0	45.6	33	345	46.0	11.8	393
10	13.5	43.9	37.3	366	50.8	12.0	454
20	13.3	47.0	40.6	413	53.9	12.2	439
30	12.8	49.7	39.5	407	52.3	12.0	455
40	13.3	51.7	42.2	449	55.5	13.3	502
50	12.9	52.6	39.9	434	52.8	11.1	434
60	12.8	58.3	44.9	504	57.7	14.3	554

A similar effect has been observed in squid axons (22), but its meaning is not clear.

Repetitive stimulation in the presence of fenvalerate completely eliminated the fast off component and decreased the slow off component of charge movement to 31 ± 2% (mean ± standard deviation) of control for this axon for pulses between 0 and +60 mV. The average suppression of the slow off component in three axons was 32 ± 3% (mean ± standard deviation). The slow off time constant in fenvalerate was similar to the control in both magnitude and dependence on activating pulse potential between 0 and +60 mV, but was significantly slower by 10% (Fig. 6).

Close inspection of the off asymmetry current in fenvalerate (Fig. 4) revealed a small, very fast component of off charge movement. This was a consistent observation, but we were not able to study this component because it was too fast to be clearly resolved at our 10-μsec sampling rate. This fast component may also be present in the control, but it would be obscured by the 50-μsec component. The fast (50-μsec) component of the control off asymmetry current is associated with deactivation of the sodium channels, and the complete elimination of this component by fenvalerate is consistent with the

fact that fenvalerate eliminates the main component of on asymmetry current associated with activation. The slow off component probably has several components. A large part of it probably represents return of immobilized sodium gating charge (12), but it may also contain components related to return of charge associated with sodium inactivation and potassium activation. It seems that fenvalerate eliminates about 68% of the slow off charge movement.

Reduction of asymmetry current during repetitive stimulation in the presence of fenvalerate. We have seen above how major components of the asymmetry currents are reduced when all channels are modified by repetitive stimulation in the presence of fenvalerate. If reduction of the movable asymmetry charge during repetitive stimulation in the presence of fenvalerate is indeed due to retention of sodium channels and associated gating charge in an open configuration, we should be able to observe partial reversibility, because the charge should become free to move again after a long rest as channels return to the closed state. Furthermore, the time course of reduction of asymmetry current should mirror the increase in slow sodium tail current as channels become modified during repetitive stimulation.

To study asymmetry currents during repetitive stimulation, we cannot use signal averaging, because we must observe the current for each trace. Thus, to reduce the noise to a usable level, we used a 31.5-kHz four-pole Bessel active filter (Ithaco model 4302).

Fig. 7A shows the effect of repetitive stimulation in the presence of fenvalerate on the on asymmetry currents for 3-msec pulses to +10 mV. At +10 mV the control on transient (Fig. 7A, *trace 1*) contained mainly the intermediate and slow components. At rest, with fenvalerate (Fig. 7A, *trace 2*), the on transient was significantly depressed. The reduction of the movable gating charge at rest was associated with reduction of the number of fast activatable channels by fenvalerate and even larger reduction of the number of inactivating channels (Fig. 1A). Repetitive stimulation had an additional effect on the on transient. Fig. 7A, *trace 2*, was the first and Fig. 7A, *trace 3*, the last in a train of 20 pulses at 1 Hz after a long rest with fenvalerate. Clearly, repetitive stimulation further decreased the transient, particularly in the early part of the traces.

Even with heavy filtering, the single-sweep P/4 records in Fig. 7 are too noisy for quantitative measurements. The P/4 procedure used for these records increased the noise of the signal by a factor of $\sqrt{5}$. Thus, to study the time course of reduction of asymmetry current during repetitive stimulation, we used asymmetry currents obtained without computer subtraction. These were photographed directly from the oscilloscope on moving film. The example in Fig. 8A shows asymmetry currents evoked by 4-msec pulses to +10 mV given every 2 sec. The fast and intermediate components showed up very faintly on the film, and only the peaks could be printed. Again, as in Fig. 7, the intermediate component was specifically reduced during repetitive stimulation, whereas the slower component did not seem to be affected. The peak of the on asymmetry current, normalized to the first pulse, is plotted in Fig. 8B for pulses at 1-, 2-, and 5-sec stimulus intervals. As with growth of the slow tail current (7), the reduction of on asymmetry current was dependent upon the number of pulses rather than stimulus rate. Furthermore, the time course of reduction of asymmetry current was similar to the time course for increase in slow tail current (Fig. 8B), as expected if entry into the modified open state leads to immobilization of the gating charge. The correspondence between these two axons was not exact, because the rate of build-up of modified channels with pulse number was highly dependent on the degree of exposure to fenvalerate, which was difficult to control. These results directly demonstrate the relationship between the retention of channels in the modified open state by fenvalerate and reduction of the intermediate component of the on asymmetry current.

Fig. 7B shows the off asymmetry currents at the end of each of the pulses in Fig. 7A. As described above, the control off transient (Fig. 7B, *trace 1*) had two components. Because the fast off component represents return of charge carried by the intermediate on component, it is not surprising that it behaved like the latter; it was about 50% reduced at rest and was nearly eliminated by repetitive stimulation with fenvalerate.

The slow off component was reduced at rest with fenvalerate and was further reduced by repetitive stimulation. However, the reduction of the slow off component during repetitive stimulation was greater in the earlier part of the trace and decreased at later times. This suggests that the slow off component has multiple components that are not quantitatively separable, a faster one that was reduced by repetitive stimulation in the presence of fenvalerate and a slower one that was not affected. Because it was reduced during repetitive stimulation, the faster component of the slow off current probably corresponds to recovery of immobilized activation gating charge. The slower component might reflect return of the slow component of on charge movement that was not affected by fenvalerate and may or may not be associated with sodium channels.

Induction by fenvalerate of charge movement negative to −120 mV. Fig. 9 compares asymmetry currents recorded after rest (Fig. 9, *trace 1*) and after repetitive stimulation (Fig. 9, *trace 2*) in a fenvalerate-treated axon with (Fig. 9A) and without (Fig. 9B) a shift of the holding potential to −160 mV during test and control pulses. The apparent reduction of the on asymmetry current during repetitive stimulation was much greater without the hyperpolarizing shift (Fig. 9B), but

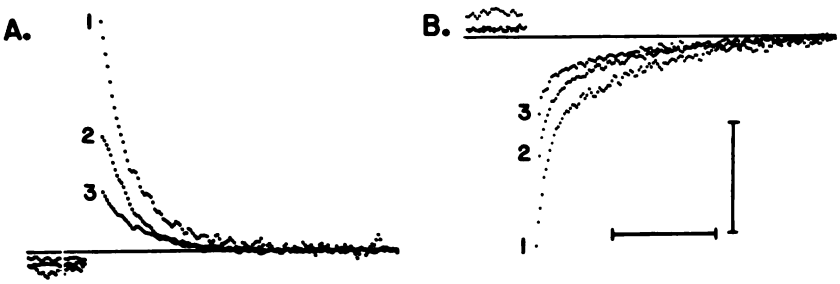

Fig. 7. Single P/4-sweep on (A) and off (B) asymmetry currents, to show the effect of repetitive stimulation as described in the text. A, On asymmetry currents at +10 mV in control (*trace 1*), the first in a train of 20 pulses at 1 Hz after a long rest with 10 μM fenvalerate (*trace 2*), and the last in the train (*trace 3*). B, Off asymmetry currents at the end of each of the pulses in A. Calibration: 500 μsec, 100 μA/cm^2.

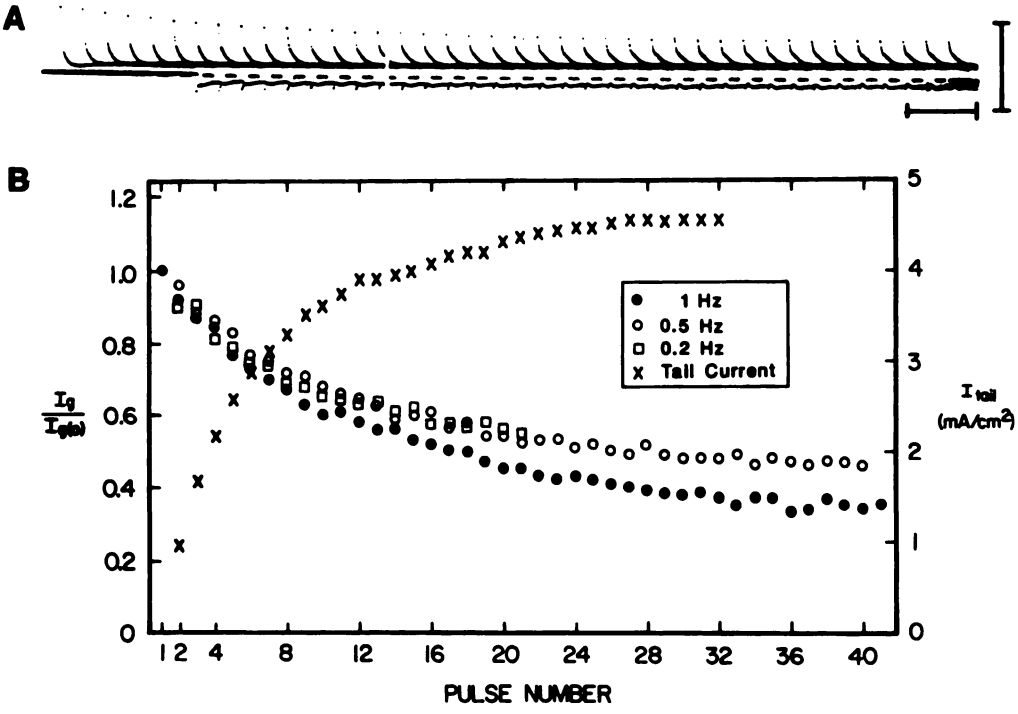

Fig. 8. A, Single-sweep asymmetry currents in response to 40 consecutive 4-msec pulses to +10 mV at 1 Hz after a long rest with 10 μM fenvalerate. The records were photographed from the oscilloscope, with only analog leakage subtraction, with each consecutive sweep being shifted to the right by movement of the film between pulses. Calibration: 2 msec, 200 μA/cm². B, The peak amplitude of the on asymmetry current (●), normalized to the first pulse, is plotted against pulse number. Similar results for pulsing at 0.5 Hz (○) and 0.2 Hz (□) are also shown, which show that the block of on asymmetry current depends on the number of pulses rather than the pulse rate. ×, Magnitude of the slow tail current after each pulse from an experiment like that in Fig. 1C, for stimulation at 1 Hz. The pulse dependence of the tail current is similar to that of the on asymmetry current.

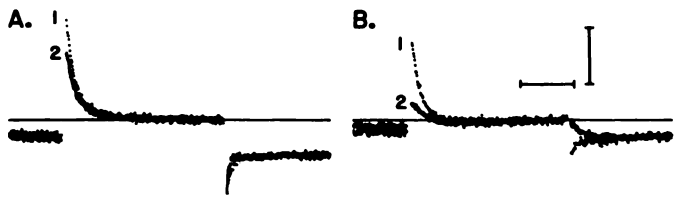

Fig. 9. Asymmetry currents for the first (*trace 1*) and last (*trace 2*) in a train of fifteen 3-msec depolarizing pulses to +20 mV, after a long rest with 10 μM fenvalerate. In A the control and test pulses were given from the hyperpolarized level of −160 mV (see protocol in Fig. 2), whereas in B the pulses were given from the holding potential at −120 mV. Note the greater reduction of on asymmetry current and the outward direction of the off asymmetry current after stimulation in B. Calibration: 1 msec, 100 μA/cm².

the outward direction of the off transient shows that in this case there was charge moving during the control pulses upon step depolarization from −155 to −120 mV. The much greater apparent reduction of the on transient also suggests that there was charge movement during the control pulse upon step repolarization from −120 to −155 mV. Because of this anomalous charge movement, we hyperpolarized the axon to −160 or −180 mV during test and control pulses for all other experiments. When this was done, no charge movement was observed for steps to potentials more negative than −60 mV (Fig. 3), indicating that this anomalous charge became immobile after several milliseconds at −160 mV.

Discussion

This is the first reported study of gating currents measured with the sucrose gap method. In general, the gating currents observed here are similar to those measured with the axial wire method in crayfish axons (19, 23). Starkus and Rayner (19) found a time constant of decay of I_s of 832 ± 348 μsec at 0 mV, which is not significantly different from the value of 689 μsec found in the present study (Fig. 5D). Also, Swenson (23) reported an average value of τ_s of 615 μsec at 0 mV. Starkus and Rayner (19) obtained a value of 174 ± 7 μsec for τ_m at 0 mV, whereas we obtained values of 91 μsec at 0 and 130 μsec at +10 mV. Our value at +10 mV should be compared with theirs at 0 mV, because it includes the effects of τ_{ms}, which was also included in their measurement. Then, the difference between 130 and 174 μsec could be due to the fact that the temperature in our study was 2–4° warmer, and the intermediate components were found to be highly temperature dependent, with Q_{10} values of 2.2–2.5 (20). There is a significant discrepancy between our values for the fast components, however. Whereas Starkus and Rayner (19) reported a value of 15.2 ± 1.6 μsec for τ_f at 0 mV and 23 μsec at −20 mV, we obtained a significantly lower value of 7 μsec at −30 mV. In the squid giant axon, τ_f can be as fast as 5 μsec (20).

Fenvalerate did not affect all gating current components equally. The slower components of the on gating current were preferentially reduced. I_s and I_{ms} were the most strongly affected; it appeared that I_{ms} was completely abolished, whereas I_s was also greatly suppressed and its time constant was decreased. I_f was not affected by fenvalerate. The components of gating current that were depressed by fenvalerate are the same ones that are depressed by depolarizing prepulses that inactivate sodium channels (19), confirming that fenvalerate blocks movement of the charges that are known to be associated with sodium channel gating.

The fast component of the on asymmetry current was not affected by fenvalerate. Whereas Armstrong and Gilly (21) suggested that the fast on component is due to early preopening steps of sodium activation, there is no direct evidence that this component is even associated with sodium channel gating. In fact, if, as seems to be the case, fenvalerate does immobilize the gating charge in the open configuration, the channel would not be expected to pass through any early preopening states after modification, suggesting that this component is not associated with preopening steps of sodium activation or with

sodium channel gating at all, as was also concluded by Keynes *et al.* (20).

I_{mf} and a portion of I_s were also not depressed by fenvalerate. I_{mf} is not immobilizable by prepulses in crayfish axons (19) and is comparable to the nonimmobilizable I_{g2} and I_{g3a} in squid axons (20). These components are part of the nonimmobilizable gating current, I_{gn}, which was not affected by local anesthetics (24). Although it is insensitive to prepulse immobilization, I_{gn} was immobilized in parallel with I_{gi} at decreased holding potentials, suggesting that it does undergo slow inactivation (19, 24). Furthermore, the ratio of I_{gi} to I_{gn} is the same in unmyelinated squid and crayfish and myelinated frog axons (19), further indicating that I_{gn} does arise from sodium channel gating. Bekkers *et al.* (24) concluded that displacement of Q_n is independent of whether Q_i charges are fully mobile or are blocked by sodium inactivation or local anesthetics. The present study also indicates that movement of Q_{mf} charge, a component of I_{gn} (19), is independent of whether Q_i charge movement is blocked by pyrethroids. Q_s was decreased 70% by fenvalerate (Table 1). The remaining slow component decayed significantly more quickly than did control I_s. The slow component was completely eliminated by prepulse inactivation in crayfish axons (19). The remaining Q_s that was not eliminated by fenvalerate may be involved in the gating that remains even when all channels are modified by the pyrethroid (Fig. 1C).

The off asymmetry current is simpler than the on transient, being readily resolved into two components. This makes the effects of fenvalerate more striking, because we can clearly see the complete abolition of the fast off component in Figs. 4 and 7. The fast component is thought to be due to return of activation gating charge, so its abolition by fenvalerate is expected.

The effect of fenvalerate on the slow off gating component was not so straightforward. The entire slow component was about 30% reduced at rest with fenvalerate, and repetitive stimulation selectively reduced its early part even further (Fig. 7). This explains why the slow off time constant in the presence of fenvalerate was slightly longer than in the control (Fig. 6), and it suggests that entry of channels into the fenvalerate-modified open state selectively eliminates some component of the slow off charge movement. As pointed out earlier, this is most probably the return of activation gating charge immobilized by the inactivation process, because this charge is known to return in the slow off component (12), and it would be expected to be immobilized by fenvalerate, which prevents channels from being deactivated. The slower off component, which is not further reduced by repetitive stimulation, has an amplitude and time constant similar to those of potassium channel gating current (25), which would not be expected to be suppressed by fenvalerate.

The anomalous charge movement induced by fenvalerate at potentials negative to −120 mV, as shown in Fig. 8, deserves comment. The origin of the anomalous charge movement is not clear, but it seems reasonable to assume that it is associated with fenvalerate-modified sodium channels. It may, in fact, underlie the ability of some modified channels to shuttle back and forth between open and inactivated or two open states (Fig. 1C), and it would be interesting to see whether this latter property of modified channels is also eliminated by hyperpolarization to −160 mV. This anomalous charge movement may also be due to movement of gating charges associated with preopening state transitions of fenvalerate-modified channels, and more careful studies of this phenomenon might lead to important information about the interaction of fenvalerate with these states.

The overall conclusion from this work is that when sodium channels are in the fenvalerate-modified open state their activation gating charges are immobilized. This is predicted from the sodium current experiments with crayfish and squid giant axons exposed to tetramethrin (6, 8), crayfish giant axons exposed to fenvalerate (7), and mouse neuroblastoma cells exposed to a range of pyrethroids (9). The stabilization of a variety of channel states caused by deltamethrin, a type II pyrethroid, was also proposed as a result of analyses of the behavior of single sodium channels in neuroblastoma cells (26). Based on this property, fenvalerate and other pyrethroids could be useful in a more quantitative separation of the asymmetry current into its components.

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