

Gating of Na Channels

Inactivation Modifiers Discriminate among Models

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ABSTRACT Macroscopic Na currents were recorded from N18 neuroblastoma cells by the whole-cell voltage-clamp technique. Inactivation of the Na currents was removed by intracellular application of proteolytic enzymes, trypsin, α -chymotrypsin, papain, or ficin, or bath application of *N*-bromoacetamide. Unlike what has been reported in squid giant axons and frog skeletal muscle fibers, these treatments often increased Na currents at all test pulse potentials. In addition, removal of inactivation gating shifted the midpoint of the peak Na conductance-voltage curve in the negative direction by 26 mV on average and greatly prolonged the rising phase of Na currents for small depolarizations. Polypeptide toxins from *Leiurus quinquestriatus* scorpion and *Goniopora* coral, which slow inactivation in adult nerve and muscle cells, also increase the peak Na conductance and shift the peak conductance curve in the negative direction by 7–10 mV in neuroblastoma cells. Control experiments argue against ascribing the shifts to series resistance artifacts or to spontaneous changes of the voltage dependence of Na channel kinetics. The negative shift of the peak conductance curve, the increase of peak Na currents, and the prolongation of the rise at small depolarization after removal of inactivation are consistent with gating kinetic models for neuroblastoma cell Na channels, where inactivation follows nearly irreversible activation with a relatively high, voltage-independent rate constant and Na channels open only once in a depolarization. As the same kind of experiment does not give apparent shifting of activation and prolongation of the rising phase of Na currents in adult axon and muscle membranes, the Na channels of these other membranes probably open more than once in a depolarization.

INTRODUCTION

During a step depolarization, voltage-gated Na channels of excitable cells open transiently. In the Hodgkin-Huxley model (Hodgkin and Huxley, 1952), parallel

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and independent activation and inactivation gating processes were assumed to give the rise and fall of Na conductance. Inactivation was a slow, first-order process that put Na channels into a refractory state during a depolarization lasting a few milliseconds. Subsequent voltage-clamp studies revealed that the kinetics of Na channel inactivation are more complex and required that inactivation proceed more slowly in the beginning of a depolarization than later (reviews by Hille, 1976; Goldman, 1976; Khodorov, 1981). Then new evidence from gating-current and patch-clamp experiments showed a definite connection between the rate or extent of inactivation and the extent of activation (Armstrong and Bezanilla, 1977; Nonner, 1980; Armstrong, 1981; Keynes, 1983; Aldrich et al., 1983; Aldrich and Stevens, 1984). Hence, in newer gating schemes, activation and inactivation are interdependent. Despite these significant advances, there remain apparent qualitative disagreements in the observations, and the models in the literature disagree on such basic facts as whether the microscopic steps of inactivation are faster or slower than the microscopic steps of activation (compare Aldrich et al., 1983, with Vandenberg and Horn, 1984).

One approach is to attempt to eliminate inactivation so that the kinetics of activation can be seen in isolation. In a coupled system, this could be difficult to do since even the distinction between the two processes might be blurry. Nevertheless, some treatments, such as internal perfusion with pronase, *N*-bromoacetamide, or chloramine-T, prolong the macroscopic Na current (Armstrong et al., 1973; Oxford et al., 1978; Wang et al., 1985) and lengthen the microscopic open time of single channels (Patlak and Horn, 1982). In the squid axon, they have no effects on the voltage-dependent rate or extent of channel opening, and they are commonly said to "eliminate inactivation" without changing activation.

One prediction of almost any gating model is that the Na currents would become larger when inactivation has been eliminated. However, in studies of squid giant axons and other adult tissues, the Na currents almost always became smaller, and it has generally been supposed that the chemical treatments "destroyed" some Na channels in addition to affecting gating. A similar study from this laboratory obtained the same result in frog skeletal muscle fibers (Nonner et al., 1980). Our interest in the problem was reawakened when we found that a scorpion peptide toxin that slows inactivation increases Na currents and alters their voltage dependence in neuroblastoma cells (Gonoi et al., 1984). This article examines the effects of a broader range of inactivation modifiers and draws conclusions about the relative rates of Na channel activation and inactivation in neuroblastoma cells.

METHODS

Chemicals

Papain (type IV, two times crystallized), α -chymotrypsin (type II, three times crystallized), trypsin (type IX, crystallized), ficin (two times crystallized), and *N*-bromoacetamide (NBA) were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. *Leiurus quinquestriatus* scorpion toxin was purified as described previously (Catterall, 1976a) from venom purchased from Sigma Chemical Co. The purified peptide toxin of *Goniopora* spp. (Hashimoto and Ashida, 1973) was kindly provided by Drs. Katsuro Ashida and Motohatsu Fujiwara (Kyoto). Other materials were obtained from

the following sources: tetrodotoxin (TTX) was from Calbiochem-Behring Corp. (San Diego, CA); fetal calf serum and Dulbecco-Vogt modified Eagle's minimum essential medium (DMEM) were from Gibco Laboratories (Chagrin Falls, OH); and the formaldehyde solution was from MCB Manufacturing Chemists (Cincinnati, OH).

Cell Culture

Neuroblastoma N18 cells were cultured in 100-mm-diam plastic petri dishes (Falcon Labware, Oxnard, CA) for 5–8 d until the cells became confluent in the dish. Details of the culture techniques were described previously (Catterall, 1975). The culture medium consisted of 5% fetal calf serum and 95% DMEM. In preparation for recordings, cells were harvested without enzyme treatment by trituration in Ca^{2+} - and Mg^{2+} -free Dulbecco's phosphate-buffered saline and then reseeded in 35-mm-diam plastic dishes (Falcon Labware) containing 1 ml of the culture medium at a cell density of 2×10^5 cells/ml. Recordings were made from cells attached to the bottom of the dishes 2–6 h after reseeding. The reseeding procedure enabled us to record from isolated, rounded cells as required for voltage-clamp measurements.

Voltage-Clamp Recording

At the beginning of the experiment, the culture medium was replaced with 1 ml of mammalian Ringer's solution (150 mM NaCl, 5 mM KCl, 1.5 mM CaCl_2 , 1.0 mM MgCl_2 , 5 mM glucose, 5 mM Na HEPES, pH 7.4) and the plastic dish was mounted on the cooled stage (15°C) of an inverted phase-contrast microscope. When formaldehyde was to be added to the bath medium, 5 mM Na phosphate, pH 7.4, was used instead of Na HEPES as the buffer. The voltage clamp was based on the one-pipette-gigaseal, whole-cell recording technique (Hamill et al., 1981). Glass microtubes (1.5 mm o.d., Curtin Matheson Scientific Inc., Houston, TX) were pulled in two steps and fire-polished without extra coating. Tip resistances after fire polishing were between 200 and 400 k Ω in mammalian Ringer's. Immediately after the pipette formed a gigohm seal on the cell membrane, the membrane was ruptured with a pulse of suction to create whole-cell recording conditions with continuity between the pipette-filling solution and the cell cytoplasm. The pipette was filled with either (a) 90 mM CsF, 60 mM CsCl, and 10 mM NaF solution or (b) 85 mM CsF, 60 mM CsCl, 10 mM NaF, and 5 mM EGTA solution. The pH of the latter solution was adjusted to 7.2 with CsOH solution. With either internal solution, K currents were blocked. Since Na currents seemed to be identical with these pipette solutions, the observations on them are combined in the Results. In a series of experiments, we tested various Cs salts in the pipette solution to test which anions allow the most stable Na current recording. Fluoride was superior to other anions including aspartate, chloride, sulfate, phosphate, citrate, oxalate, methanesulfonate, EGTA, and EDTA.

A List EPC-7 patch-clamp system (List Electronic, Darmstadt, Federal Republic of Germany) was used for whole-cell voltage-clamping without salt bridges between the electrodes and the recording solutions. Series resistance of the pipette was compensated by an internal feedback circuit. Linear leakage and capacitive currents were subtracted both with an analog subtractor and with a computer. Currents were digitized and stored on magnetic tape with an LM² computer (Kehl et al., 1975). The rapidly changing part of the record was sampled every 25 μs after analog filtering with a four-pole low-pass Bessel filter cutting off (–3 dB) at 4 kHz. Further details of the recordings from the neuroblastoma N18 cells have been described previously (Gonoi et al., 1984).

Recordings were made from round cells that had diameters between 18 and 25 μm and no processes. Except in the experiment described in Fig. 1, the cells were maintained at a holding potential of –80 mV. For measurements of voltage-dependent activation of Na current, the membrane was hyperpolarized to –120 mV for 100 ms to remove inactivation

of Na channels, and depolarized with test pulse potentials from -40 or -60 to typically $+90$ mV in intervals of 10 mV. For measurement of the voltage dependence of Na channel inactivation, the prepulse potential was varied from -140 to -20 or 0 mV for 100 ms, followed by a test pulse to $+10$ mV for 8.75 ms to elicit the Na current. The recording conditions for Fig. 1 are described in the text.

When applying papain, α -chymotrypsin, trypsin, or ficin to the inside of the cells, we dissolved the enzymes in the internal solutions at a concentration of between 0.1 and 0.5 mg/ml. Ficin was obtained as a suspension containing 23 mg protein/ml. The suspension was diluted 200 times with the internal pipette solution containing 5 mM EGTA to obtain a clear solution. The internal pipette medium was then filtered through $0.2\text{-}\mu\text{m}$ filters before filling the micropipettes. The enzymes enter the cytoplasm by diffusion from the pipette soon after the patch membrane was ruptured, as was seen by the ensuing changes in Na current inactivation. The other agents were added in the bath medium at more than 30 min after making a seal, when the Na current was stable.

Na conductance was calculated by the relation

$$g_{\text{Na}} = I_{\text{Na}} / (E - E_{\text{Na}}),$$

where E is the membrane potential and E_{Na} is the reversal potential for current in Na channels as measured in each experiment.

RESULTS

In control experiments, in which cells were not treated with enzymes or other agents, whole-cell Na currents of N18 neuroblastoma cells were stably recorded without deterioration for as long as 3 h. In the first few minutes after the pipette sealed onto a cell, the Na current increased in amplitude typically 50–100%. This increase presumably reflects a reversal of resting slow inactivation when the membrane is hyperpolarized from a typical resting potential of about -40 mV (Catterall, 1976*b*) to a holding potential of -80 mV. After the initial increase, the Na current amplitude and other properties were usually stable in time (for instance, see Fig. 8). In the rest of the article, when we discuss changes in peak Na current, we are referring to changes that occur after the initial few minutes.

Trypsin, α -Chymotrypsin, Papain, and NBA Remove Inactivation

Including a proteolytic enzyme, such as trypsin, α -chymotrypsin, or papain, in the micropipette solution progressively removed the fast inactivation gating of the Na current. The changes were similar for each enzyme with only minor exceptions. Fig. 1 shows Na currents at different times after a seal was made with a micropipette containing trypsin at a final concentration of 0.5 mg/ml. The cell was stimulated with a depolarizing test pulse to 0 mV every 1.5 min, and the resulting Na currents at 0 mV are superimposed in the figure. At first, the evoked Na current is transient, activating and inactivating in <2 ms. Partial removal of the Na channel inactivation became apparent 15 min after making the seal, and inactivation appeared to be removed in 33 min. At this time, the Na current decayed $<5\%$ during the 10-ms test pulse (Fig. 1). In seven experiments with 0.1–0.5 mg/ml trypsin and in two experiments with 0.25 mg/ml α -chymotrypsin, it generally took 10–25 min to recognize the first small loss of inactivation, and in five experiments with 0.25 mg/ml papain, it took 25–35 min. The time required to observe the first signs and the time to complete the removal of inactivation seemed to depend upon the enzyme concentration.

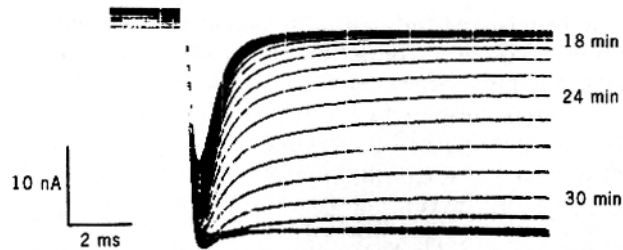


FIGURE 1. Progressive removal of inactivation and increase of the whole-cell Na current by intracellular application of trypsin. The recording pipette contained 0.5 mg/ml trypsin in the standard intracellular solution. After the seal was made, Na currents were elicited once every 1.5 min by a test pulse to 0 mV for 10 ms after a prepulse to -105 mV for 90 ms. Holding potential, -75 mV. Na currents were superimposed on a storage oscilloscope screen and photographed. Times after making a seal on the cell are indicated on the right side. Little happens for the first 15 min.

When inactivation was halfway removed (at 24 min after making the seal in Fig. 1), the decaying phase of the Na currents could be divided into two components (Fig. 2). The rapidly decaying phase with a decay time constant of 0.76 ms corresponds to the normal rapid inactivation of Na current in control cells. The slowly decaying phase had a decay time constant of 46 ms in this recording at a test pulse of 0 mV. Whatever the type of proteolytic enzyme used, the two phases of decay with fast and very slow time constants were a common characteristic of the Na currents at this stage of modification, as if some channels had been modified and some were still gating normally.

Concomitant with the removal of fast inactivation, the peak Na current

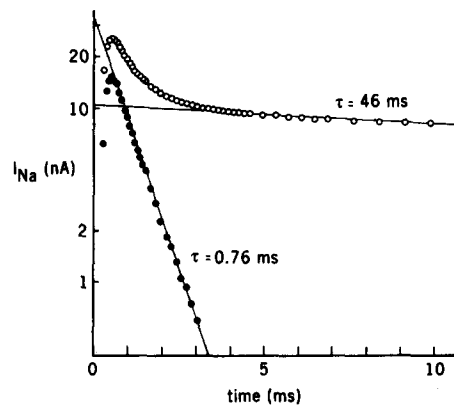


FIGURE 2. Decay of macroscopic Na current when inactivation was partially removed by intracellular trypsin (0.5 mg/ml). The inward Na current during a depolarizing test pulse to 0 mV is plotted on a semilog scale at 24 min after making the seal (open circles). Then a slow component of the current decay, which has a time constant of 46.1 ms, was subtracted and the remaining fast component was plotted (filled circles). The decay time constant of the fast component, 0.76 ms in this record, corresponds well to unmodified Na current. The lines were drawn by eye.

increased in 10 out of 14 experiments with trypsin, α -chymotrypsin, and papain (see Fig. 1). The time course with α -chymotrypsin is shown in Fig. 3. Starting 12 min after the seal was made, the peak Na current at a test pulse potential of +10 mV (open circles) began to increase until it had grown 2.5 times as large by 39 min. The extent of removal of inactivation was judged by the size of current remaining at 3 ms after the peak (filled circles). It was practically zero in control experiments or soon after making a seal with a pipette containing the enzyme (see Figs. 1, 3, and 4) and increased along a similar time course (scaled smooth curve) as the peak Na current until inactivation was almost totally removed at 39 min. In control experiments without the enzyme treatment, the peak Na current at a test pulse of +10 mV measured at 40 min after making the seals was

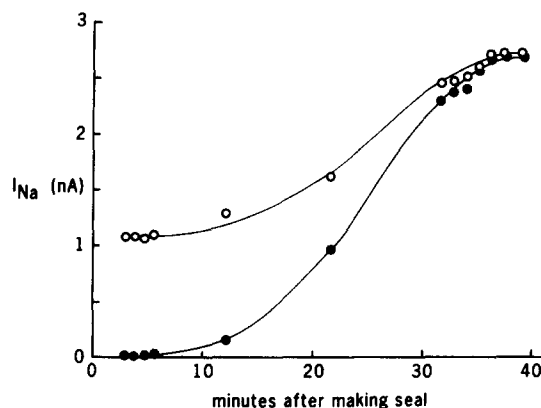


FIGURE 3. Removal of macroscopic Na inactivation and increase of peak current amplitude with intracellularly applied α -chymotrypsin. Open circles: peak Na current at 10 mV test pulse plotted against the time after making a seal between the cell and a recording pipette containing 0.25 mg/ml α -chymotrypsin. Filled circles: Na current remaining 3 ms after reaching the peak of the current is plotted as a measure of the removal of macroscopic Na current inactivation. The curve for the peak was drawn by eye, and the same curve minus the lower asymptote was scaled and drawn through the 3-ms points.

1.17 ± 0.13 (SD, $N = 4$) times that measured at 10 min. Hence, spontaneous changes of the peak current amplitude during the experimental period are small when no enzymes are used.

Fig. 4 shows families of Na currents before (*A*) and after (*B*) the removal of inactivation by papain. In *A*, the Na currents activate and inactivate within a few milliseconds during each depolarization. In *B*, the Na channels no longer inactivate, even at the end of the 39-ms test pulse. Similar sustained Na currents during the test pulse period were observed after the removal of inactivation by trypsin or α -chymotrypsin. In some recordings, Na currents were no longer decaying, even at the end of a test pulse of up to 95 ms in duration (not shown).

We were surprised to discover that after inactivation was removed, the Na current became activated at more negative test pulse potentials than before (Fig. 4). Thus, before modification by papain, no Na current was elicited by a -50 -mV test pulse and very little was elicited by -40 mV, whereas afterward a

significant sustained current appeared (Fig. 4B) at these potentials. The change is more easily seen in Fig. 5A by comparing superimposed traces before (dashed line) and after (solid line) enzyme action in another papain-treated cell. The figure shows the changes of current for three depolarizing steps. At -40 mV, the unmodified current was too small to be distinguished from the baseline, and at -20 mV, it appeared as a small inward transient finished in <10 ms. After papain had acted for 57 min, the slowly rising, sustained currents at -40 and -20 mV were far larger than the unmodified peak currents had been. At $+10$ mV, the sustained current was also larger than the original peak current, but the fractional increase was nowhere near as large as for the small depolarizations. Similar results were obtained in every experiment with papain, trypsin, and α -

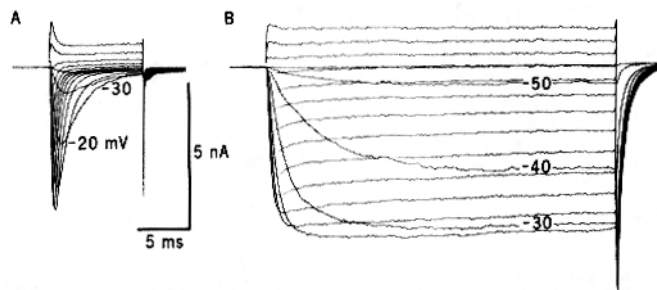


FIGURE 4. Families of Na currents before and after action of intracellular papain. The recording pipette contained 0.25 mg/ml papain. (A) 10 min after making the seal, Na currents still look normal in their activation and inactivation. Small nonlinear leak currents were observed at test pulses more positive than $+60$ mV. (B) 60 min after making the seal, inactivation was 70–80% removed. Test pulses applied once every second in A and once every 3 s in B were preceded by a prepulse to -120 mV for 100 ms. Holding potential, -80 mV. Numbers on the current traces indicate the test pulse potentials. Note that at small depolarizations, Na currents became considerably larger after removing the inactivation than those elicited by the same test pulse potential before removing the inactivation.

chymotrypsin. One example with α -chymotrypsin is shown in Fig. 5B. Again, Na currents were enhanced more at small depolarizations than at large depolarizations.

Fig. 6 compares the peak Na conductance (g_{Na})-voltage relationship before (open circles) and after (filled circles) the removal of inactivation in another cell treated with papain. The midpoint of the g_{Na} -voltage curve was shifted 24 mV in the negative direction by the action of papain. The g_{Na} -voltage curve also became a little steeper at the midpoint. The shifts observed with trypsin, α -chymotrypsin, and papain are summarized in Table I. The mean negative shift of the g_{Na} -voltage curve obtained by combining the results with the three enzymes was 26.3 ± 4.5 mV (SD, $N = 14$), whereas no shift of the midpoint was observed in control experiments (see Fig. 8). The g_{Na} -voltage curve usually became a little steeper after the effects of the enzymes, but the change was not always statistically significant (Table I).

While the peak g_{Na} -voltage curve suggests that the voltage dependence of

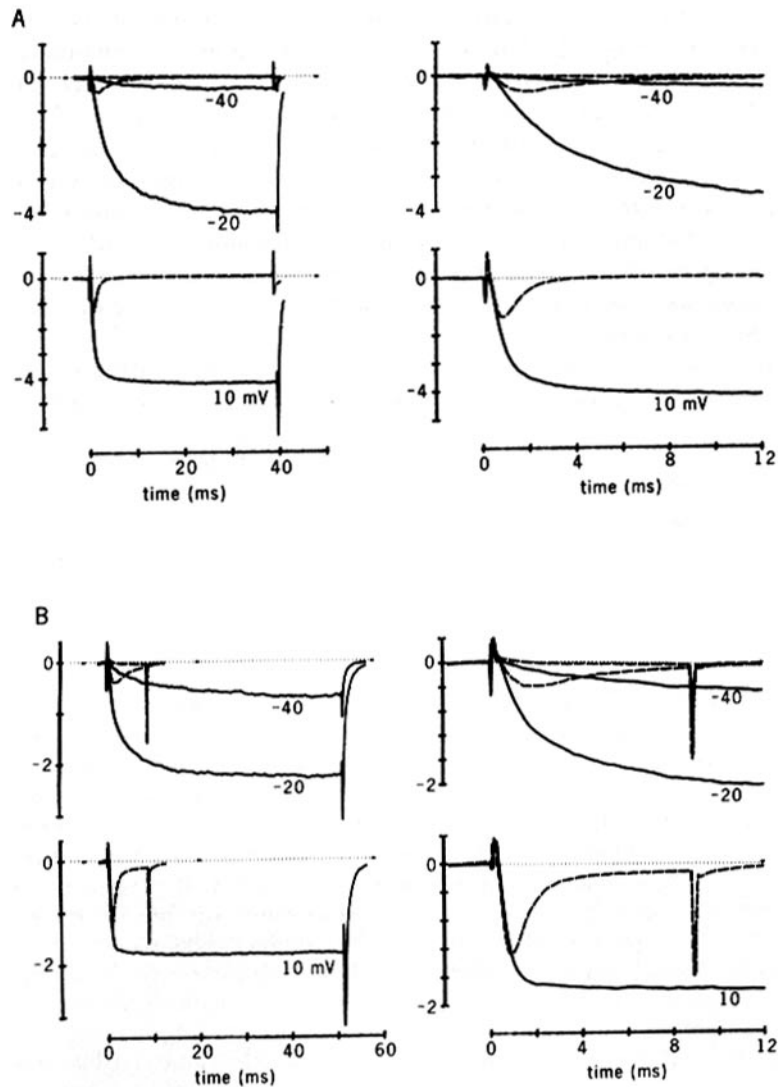


FIGURE 5. Comparison of Na current time courses before (dashed lines) and after (solid lines) significant modification of inactivation by intracellular proteolytic enzymes. The same records are shown at the left and right but on different time bases. Currents are measured with test pulses to the indicated membrane potentials preceded by 100-ms prepulses to -120 mV. Holding potential, -80 mV. (A) Currents recorded at 5 and 56 min after making a seal with a pipette containing 0.25 mg/ml papain. A different cell from that in Fig. 4 was used. Here and in B, the control trace at -40 mV has been drawn as a dashed line but is so small that it is obscured by the baseline. (B) Currents recorded at 5 and 65 min after making a seal with a pipette containing 0.25 mg/ml α -chymotrypsin. Even after 5 min, the enzyme appears to have exerted some effect. The test pulse duration for the "control" records is much shorter than for those after 65 min of treatment.

activation is shifted by proteolytic enzymes, the time course of the Na currents suggests another type of change. After modification, Na channels take an unexpectedly long time to reach maximal activation in the voltage range from -50 to -20 mV. New channels continue to join the conducting pool for >20 ms during depolarizations to -40 or -20 mV (Fig. 5). At potentials greater than -30 mV, the initial rate of appearance of conducting channels was about the same before and after modification, but after modification, channels continued to join the conducting pool even after most normal channels would be inactivated. Had the effect of enzyme been only to shift the voltage dependence of activation to more negative potentials, the rate of activation would instead have become faster at these potentials.

NBA treatments showed some similarities to and some differences from the enzyme effects. NBA was applied in the bathing medium. We found that NBA

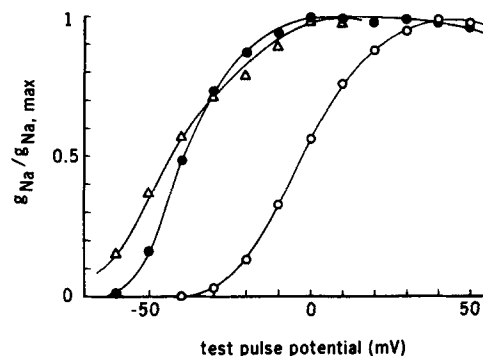


FIGURE 6. Voltage dependence of peak Na conductance before and after the inactivation is modified by intracellularly applied papain. Na conductance was calculated as described in the Methods and normalized to the maximal values. Open circles: at 10 min after making the seal, inactivation was not yet removed. Filled circles: at 60 min after making the seal, inactivation was almost completely removed. Open triangles: a few minutes later, the maximal Na current has been reduced from -3.8 to -0.6 nA by mixing Na-free Ringer's into the bathing solution.

at a final concentration of between 100 and 500 μ M removed fast inactivation gating almost completely, as reported previously for squid giant axon, frog muscle fiber, and GH₃ cells (Oxford et al., 1978; Nonner et al., 1980; Horn et al., 1984). Concomitant with the loss of inactivation, the peak g_{Na} -voltage curve was shifted in the negative direction, as we found with proteolytic enzymes. The amount of the negative shift was 19 mV at the midpoint of the curve (Table I). However, in contrast to the effects seen in most of the enzyme experiments, the Na current decreased progressively in time and the g_{Na} -voltage curve became a little less steep after the application of NBA (Table I).

Ficin Removes Inactivation at Least Partially

Intracellular ficin, tested on four cells, was not as easy to use as the other enzymes. Two cells were lost when Na inactivation was only partially removed,

and in the other cells it took 2 h for inactivation to be halfway removed. Again, a -50 -mV test pulse elicited no current soon after the seal was made and a significant amount of current after partial removal of inactivation. The currents in test pulses to -40 , -30 , and -20 mV were significantly increased, while the maximum current increased only a little. Analysis of the decay phase of the partially modified current revealed two exponential components, as was seen for trypsin. After inactivation was modified, the midpoint of the peak g_{Na} curve shifted in the negative direction by ~ 10 mV, and the Na current was not totally inactivated, even after a 100-ms prepulse to -20 mV. The results from the four cells are summarized in Table I.

TABLE I
Na Channel Parameters after Modification

Agent	N	Changes of g_{Na} at +30 mV*	Shift of $E(g_{Na} = 0.5)^\ddagger$		E_{Na}
			E mV	slope factor § mV	
Control [¶]	31	1.00	0.0	8.0 \pm 1.0	69.8 \pm 6.0
Papain	5	1.21 \pm 0.49	-27.3 \pm 4.7	6.8 \pm 1.2	68.7 \pm 11.4
α -Chymotrypsin	2	1.86 \pm 1.20	-29.5 \pm 4.2	5.1 \pm 1.0	61.8 \pm 2.5
Trypsin	7	1.35 \pm 0.32	-21.5 \pm 3.2	7.2 \pm 2.7	49.3 \pm 11.7
NBA	2	0.86 \pm 0.05	-19.1 \pm 2.8	12.2 \pm 0.8	38.7 \pm 8.8
Ficin	4	0.96 \pm 0.36	-10.2 \pm 4.2	7.6 \pm 1.2	46.5 \pm 1.8 [¶]
<i>Leiurus</i> scorpion toxin	2	1.37 \pm 0.09	-7.1 \pm 1.0	6.7 \pm 2.1	70.7 \pm 2.3
<i>Goniopora</i> coral toxin**	3	1.42 \pm 0.13	-9.8 \pm 2.1	5.9 \pm 0.4	70.1 \pm 2.9
Formaldehyde	6	0.39 \pm 0.14	+24.9 \pm 4.4	15.1 \pm 1.4	60.7 \pm 13.6

* Ratio of g_{Na} before the action of enzymes became apparent or just before adding a toxin or agent in the bath to maximal g_{Na} after the removal of the inactivation was almost complete.

‡ Midpoint of the g_{Na} - E curve (see Fig. 6).

§ Calculated by a least-squares method using the equation

$$g_{Na} = 1 - 1/\{1 + \exp[(E - E_{g_{Na}=0.5})/k]\},$$

where E is test pulse potential and k is the slope factor.

[¶] Control values are the "before" measurements for the 31 test cells, except that E_{Na} values for the four ficin test cells are omitted.

[¶] The Na reversal potential with ficin was lower than the other series (control = 46.3 ± 1.6) because the ficin suspension was obtained in 2 M NaCl and the final Na ion concentration in the pipette medium was 20 mM instead of 10 mM.

** Data adapted from Gonoï et al. (1985).

Means \pm SD.

Polypeptide Toxins Slow Inactivation

In the many excitable cells in which they have been studied, polypeptide toxins such as *Leiurus* scorpion toxin, sea anemone toxin, and *Goniopora* coral toxin slow the fast inactivation of Na channels instead of completely removing it. Some of our studies with *Leiurus* scorpion toxin and *Goniopora* coral toxin on the macroscopic Na current of N18 neuroblastoma cells have been published (Gonoï et al., 1984, 1986). The results from new experiments and others adapted from our previous reports are summarized in Table I. Scorpion toxin and coral toxin shifted the g_{Na} -voltage curve in the negative direction by ~ 7 and 10 mV,

respectively. Thus, with *Leiurus* toxin, depolarization to -40 mV elicited a clear Na current (Fig. 7) where there was almost none before. With every cell examined, *Leiurus* or *Goniopora* toxin increased the size of Na currents throughout their time course. Particularly with larger depolarizations (see Fig. 7, -20 and $+10$ mV), the modified current began rising almost in parallel with the control trace but then it continued well beyond the control by the peak time. The time to peak was always delayed by the toxins, but not by the large factor seen with proteolytic enzymes, and the rate of decay of Na currents was slowed. The slowing of macroscopic inactivation was most dramatic for large depolarizations (Fig. 7). The peak g_{Na} -voltage curve became a little steeper with the application of either toxin (Table I).

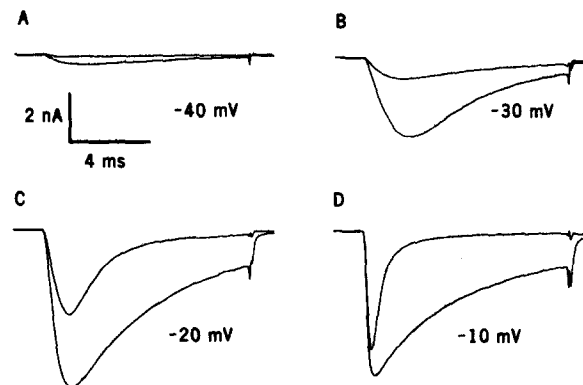


FIGURE 7. Comparison of Na currents at each test pulse potential before and after the slowing of the inactivation by scorpion toxin. At 60 min after the seal was made, *Leiurus* toxin was added to the bathing medium, giving a final concentration of 200 nM. Na currents were elicited by test pulses preceded by 100-ms prepulses to -120 mV. Holding potential, -80 mV. The currents before and after the application of the toxin are superimposed for comparison. The control current is always smaller than that with toxin. Test pulse potentials are indicated on the traces.

Inactivation Removal by Formaldehyde

Formaldehyde also removes the inactivation of vertebrate Na channels. We applied it to the bath at a final concentration of between 0.12 and 60 mM. Formaldehyde depressed the Na current to one-third to one-fifth of the original peak size, and over a 1-h period, inactivation became extensively removed as reported in frog muscle (Nonner et al., 1980). Soon after the removal of inactivation started with a low formaldehyde concentration, a negative shift of the g_{Na} -voltage curve of up to 5 mV was sometimes observed. However, as removal of inactivation progressed further, the g_{Na} -voltage curve became significantly less steep and the midpoint eventually shifted in the positive direction by 25 mV (Table I). At depolarizations eliciting a small Na current, the rising phase of the current was considerably prolonged compared with the control, as had been seen with enzymes. Hence, while formaldehyde has some actions like those of the other agents tested, it also has additional actions.

Two Possible Artifacts Do Not Explain the Shift

When the g_{Na} -voltage curve is shifted in the negative direction, one has to consider two well-known sources of artifact. One is a spontaneous negative shift that develops in many cell types after whole-cell clamping is begun. The other arises from a voltage drop in the recording electrode that would give an apparent negative shift whenever inward Na currents are augmented—the series resistance artifact.

We consider series resistance effects first. The error arises because all applied membrane current passes through the resistance of the pipette, so the voltage at the recording tip deviates from that seen at the amplifier-end of the pipette. If inward Na currents are augmented, the artifact causes the cell to be clamped at a more positive membrane potential than commanded and all voltage-dependent parameters will appear to have a negative shift on the voltage axis. The error may be reduced by electronic compensation, by reducing the pipette resistance, and by reducing the Na current.

We always applied the series resistance compensation available on the List patch-clamp amplifier, and we used micropipettes that had large tip openings and very low resistances (200–400 k Ω in Ringer's). Since our peak current is on the order of 5 nA and the actual increase of current was only part of that, the apparent potential shift without any compensation would be smaller than 2.5 mV, assuming a 500-k Ω series resistance after making a seal. This is too small to explain the shifts we observed. Occasionally, we failed to see an increase of current in the experiments with enzymes or NBA, but we still observed a similar amount of negative shift in these experiments. In addition, after inactivation was removed by the enzymes, we decreased the Na current deliberately to see whether the g_{Na} -voltage curve could be shifted back in the positive direction. In these experiments, inactivation was first removed with papain or α -chymotrypsin and the g_{Na} -voltage curve became shifted. Then the maximum Na currents were reduced to between one-fifth and one-eighth of their previous amplitude by adding some Na-free choline Ringer's solution. In all five such experiments, the g_{Na} -voltage curve did not shift back in the positive direction, staying at almost the same position as after the removal of inactivation (Fig. 6, triangles). The mean additional shift of the curve after reducing the Na current was -0.8 ± 2.5 mV (SD, $N = 5$). The g_{Na} -voltage curve became a little less steep after the current was reduced. The possibility of the series resistance artifact causing the negative shift of the g_{Na} -voltage curve was previously tested on scorpion toxin-modified Na currents in N18 cells and was ruled out on similar grounds (Gonoi et al., 1984).

The second possible artifact is a spontaneous shift with time of the g_{Na} -voltage relationship after making a seal. Such gradual shifting has already been reported in GH₃ rat clonal pituitary cells (Fernandez et al., 1984) and rat myoballs in culture (Gonoi et al., 1985). We tested whether a spontaneous shift of the g_{Na} -voltage relationship occurred in four N18 cells without any treatment with the agents but found none during 60 min of recording (Fig. 8, filled symbols). There was also no apparent change in the steepness of the g_{Na} -voltage curve (data not shown). Under the same recording conditions, we could demonstrate a -10 - and

-30-mV shift of the g_{Na} -voltage curve for two GH_3 cells. Hence, we conclude that the shift of the g_{Na} -voltage curve seen after modifying inactivation can be explained neither by the series resistance effect nor by a spontaneous shift in time. We discuss the origin of the negative shift in the Discussion.

Comparison of Other Properties after the Modifying Treatments

Some of the properties of the Na current before and after modification of Na inactivation are summarized in Table I.

Peak g_{Na} with a +30-mV test pulse was measured as an index of the maximal g_{Na} of the cell (see Fig. 6). The enzyme-induced changes varied from one cell to

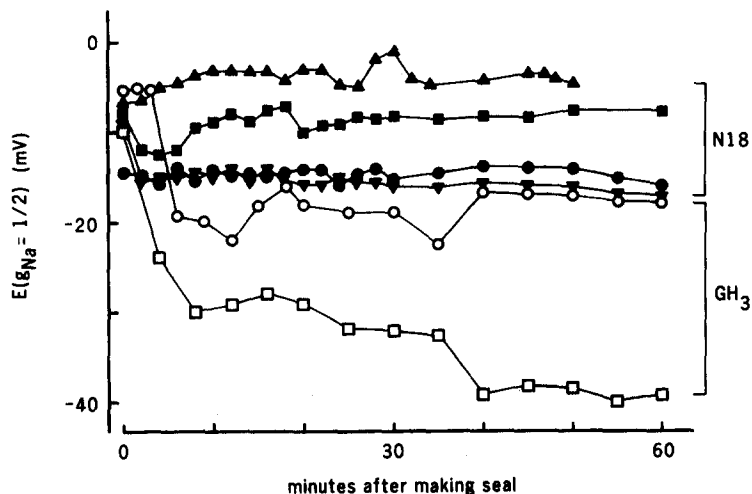


FIGURE 8. Spontaneous shifts of the half-activation potential of the Na conductance after making a seal between a micropipette and an N18 or GH_3 cell. The recording pipettes contained no enzymes. The half-activation potential of the peak Na conductance was calculated by a least-squares method and plotted on the ordinate for four different N18 cells (filled symbols) and two different GH_3 cells (open symbols). Abscissa: time after making a seal between cell and pipette.

another. In two papain-treated cells, the maximal g_{Na} increased to 1.7 and 1.9 times, and in two others it actually decreased. With α -chymotrypsin treatment, g_{Na} increased to 2.7 times the original (see Fig. 2), and with trypsin treatment, the maximal change obtained was an increase to 1.7 times. The maximal g_{Na} tended to begin decreasing again within 5 or 10 min after inactivation had been nearly completely removed in the enzyme treatment experiments. In the experiments with polypeptide toxins, the maximal g_{Na} was always increased to 1.3–1.5 times over that just before applying the toxins. With NBA or formaldehyde, the maximal g_{Na} always decreased. With ficin, a small transient increase of the maximal g_{Na} was occasionally observed before it decreased.

We looked for changes of E_{Na} to check for possible changes of the ionic selectivity of the Na channels or changes of the Na ion concentration of the cell

through increasing leak. With papain, ficin, and the two polypeptide toxins, E_{Na} was stable (Table I). With α -chymotrypsin and formaldehyde, it decreased moderately. With trypsin and NBA, it decreased drastically, which suggests damage to the cell.

DISCUSSION

Comparison with Previous Work

We report here three consistent new findings with some inactivation-removing agents applied to N18 cells: (*a*) an increase of peak g_{Na} , (*b*) an accompanying negative shift of the peak g_{Na} -voltage curve, and (*c*) particularly for enzymes, a great prolongation of the growth of the Na current at small depolarizations. Proteolytic enzymes, scorpion and coelenterate peptide toxins, formaldehyde, and NBA have been used in many previous macroscopic voltage-clamp studies to remove or slow Na inactivation (Koppenhöfer and Schmidt, 1968; Armstrong et al., 1973; Sevcik and Narahashi, 1975; Shrager, 1975; Bergman et al., 1976; Bezanilla and Armstrong, 1977; Oxford et al., 1978; Catterall, 1979; Nonner et al., 1980; Oxford, 1981; Stimers et al., 1985; Wang and Strichartz, 1985; reviewed by Catterall, 1980, and Hille, 1984). Most of the work was done with squid and crayfish giant axons, frog axons, and frog muscle fibers. It was clearly shown in these and many other reliable studies that the activation of Na currents was relatively unchanged in voltage dependence and time course by removal of inactivation. No shift greater than ± 3 mV has been reported. Furthermore, peak g_{Na} usually decreased and sometimes remained unchanged. In a few studies, a small increase of g_{Na} was observed (Warashina and Fujita, 1983; Zubov, 1980; Rojas and Rudy, 1976; Stimers et al., 1985; Starkus and Shrager, 1978).

Despite the pronounced differences in observations (particularly findings *b* and *c* above), we consider that there is no conflict between previous work and ours. Instead we hypothesize that the Na channels of N18 neuroblastoma cells differ from those in the other cells and that the inactivation-removing agents are a diagnostic test for this difference.

Ours may be the first published voltage-clamp description of the effects of papain, an -SH-requiring enzyme of broad specificity, and of ficin, an -SH-requiring enzyme cleaving at lysyl and argenyl bonds. However, other studies have suggested that these enzymes should act on inactivation (Tasaki and Takenaka, 1964; see Rojas and Rudy, 1976). Of the four enzymes we tested, papain seemed the least injurious to the cell. The other enzymes shortened the lifetime of the preparation and raised the leak conductance. Trypsin was the most injurious.

Implications for Gating Models

We now consider possible interpretations of the Na current changes induced in neuroblastoma cells by removing or slowing inactivation. The discussion hinges on kinetic models for the gating of Na channels. The conclusion will be that the changes in neuroblastoma cells are more readily explained by the gating models suggested by Bezanilla and Armstrong (1977) and Aldrich et al. (1983) than by

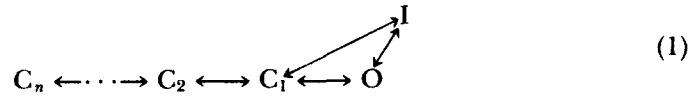
the models of Hodgkin and Huxley (1952), Gilly and Armstrong (1982), or Vandenberg and Horn (1984). In this discussion, we need to distinguish between macroscopic gating kinetics, which are the observed whole-cell kinetics from the population of channels present, and microscopic gating kinetics, the postulated elementary steps underlying the macroscopic result.

No model has difficulty explaining the increase of g_{Na} after inactivation is slowed or removed. Whether inactivation is independent of activation or is coupled to it, the elimination of inactivation has to permit more Na channels to be open at any time. Therefore, in virtually all previous studies, where g_{Na} was not found to increase, the chemical treatment has been assumed to "destroy" some Na channels while modifying inactivation in others. Alternative hypotheses would be that the single channel conductance is consistently reduced when inactivation is modified in these cells or that modification also increases the equilibrium fraction of channels passing in and out of some form of dormant states analogous to slow Na inactivation or the state called hibernation by Horn et al. (1984). The latter seems a likely possibility since it has been demonstrated directly in GH_3 cells. For some reason, neuroblastoma Na channels are more resistant to this change than all the other Na channels studied.

In Hodgkin-Huxley (HH) models, the predicted increase of maximum g_{Na} is typically a factor of ~ 2 . Fig. 9A shows the time course of channel opening in an HH model for small and large depolarizations (dashed curves) and also shows the effect of a fivefold slowing and a complete inhibition (solid curves) of inactivation gating in the model. Because the time constants of activation and inactivation speed up about equally with depolarization in an HH model, inactivation at the time of the peak current reaches a similar value for each depolarization and removal of inactivation would produce roughly the same increment in g_{Na} at each potential, as can be seen in the calculation. Hence, the predicted peak g_{Na} rises but its voltage dependence is little changed when inactivation is slowed. The removal of inactivation would not induce large currents at small depolarizations, where they are absent in normal channels. Furthermore, the slowing or removal of inactivation would prolong Na currents a little but would not cause them to keep rising for tens of milliseconds, as was seen with papain or α -chymotrypsin. Therefore, within the framework of HH models, we would have to suppose that enzymes and peptide toxins have three separate actions on neuroblastoma Na channels: they slow or prevent the closure of the inactivation gate, they bias the voltage sensor of the activation process so that activation gates open with less depolarization, and they prolong the activation process so that it takes tens of milliseconds at small depolarizations.

In another class of gating models, a single kinetic modification can give an increase of peak g_{Na} , an apparent negative shift of activation gating, and an apparent prolongation of activation. These are coupled models where the steps of microscopic inactivation are fast and far less voltage dependent than the steps of microscopic activation. The idea is a simple one inspired independently by macroscopic and gating current measurements in squid giant axons (Bezanilla and Armstrong, 1977) and by single channel measurements in N1E115 neuroblastoma cells (Aldrich et al., 1983). Assume that Na channels have three major

classes of conformational states, resting-closed (C), open (O), and inactivated (I), and that most of their permitted transitions are sequential:



Unlike the HH model, in this one it is possible to fit many macroscopic and microscopic measurements by assuming that some of the microscopic steps of

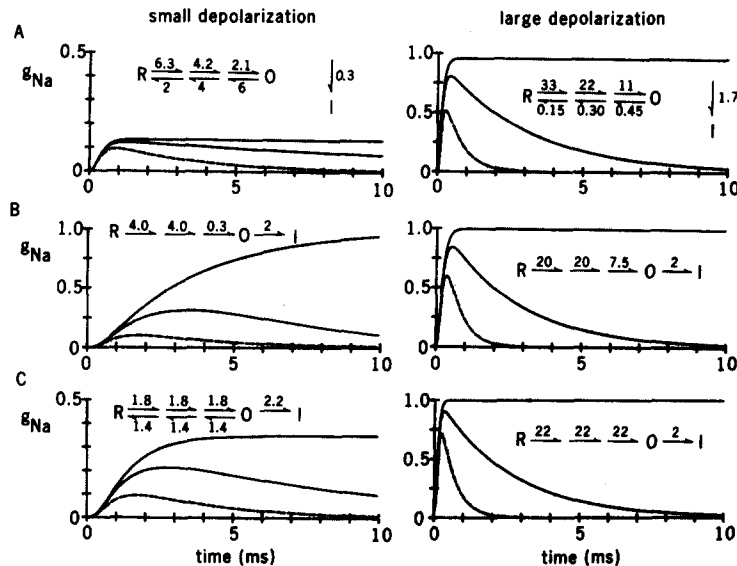
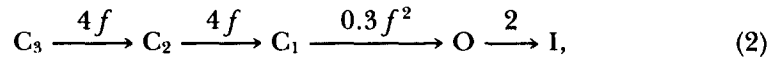


FIGURE 9. Simulations of Na conductance kinetics using three different kinetic models. Each frame shows normal kinetics (dashed line), using the rate constants shown, as well as the modified kinetics (solid lines) when microscopic inactivation is slowed fivefold or blocked altogether. The left-hand column shows small depolarizations that normally elicit a peak g_{Na} of <10% of the maximum. The right-hand column shows large depolarizations that elicit $\geq 50\%$ of the maximum g_{Na} . (A) Hodgkin-Huxley model for frog nodes of Ranvier (Hille, 1971) with rate constants divided by 2.2 to simulate lowering the temperature from 22 to 15°C. The values used correspond to depolarization to -40 mV (left) and $+15$ mV (right) and the state diagram with independent activation and inactivation corresponds to m^3h kinetics. (B) The irreversible linear model (diagram 2 in the text) with $f = 1$ (left) and $f = 5$ (right). (C) The Bezanilla-Armstrong (1977) model for squid giant axons using rate constants from their Table IV multiplied by 2.4 to simulate raising the temperature from 2 to 10°C. The values are interpolated for depolarizations to -35 mV (left) and $+80$ mV (right).

activation (from C_n to O) are slower than the microscopic steps of inactivation (particularly from O to I) at most voltages and by having the rate of inactivation from O to I be nearly voltage independent (Bezanilla and Armstrong, 1977; Armstrong and Gilly, 1979; Gilly and Armstrong, 1982; Aldrich et al., 1983).

Consider, for example, a simplified, irreversible (at depolarized potentials)

kinetic scheme with the following rate constants:



where f is a voltage-dependent factor that makes the microscopic steps of activation speed up with depolarization, and the units of the rate constants are reciprocal milliseconds. In this irreversible scheme, the single channel open time, determined only by the microscopic rate constant for inactivation, is always 0.5 ms. Fig. 9B shows the macroscopic time course of channel opening in such a model (dashed curves) for a small depolarization ($f=1$) and a large depolarization ($f=5$). The macroscopic kinetics for unmodified channels resemble those of the HH model but the underlying events are different.

At low depolarization, channels continue to activate for 5 or 6 ms. Eventually, all of them pass through the open state, but since they inactivate on average 0.5 ms after opening, fewer than 10% of the channels are simultaneously open, even at the peak. At high depolarization, channels activate rapidly and more synchronously, so that 60% are open at the peak. Again they stay open for only 0.5 ms. The solid lines show the effects of slowing the microscopic inactivation rate constant fivefold or infinitely. In this model, with irreversible activation, blocking inactivation causes 100% of the channels to accumulate in the open state.

Qualitatively, this sequential model has all the desired features. Complete block of inactivation would increase Na currents at all depolarizations. At small depolarizations, the rise of current would become greatly prolonged (Fig. 9B) and the current would greatly increase in size, as was seen with papain, trypsin, or α -chymotrypsin (Fig. 5). At large depolarizations, the prolongation of rise and increase in size would be far smaller. Hence, the peak g_{Na} -voltage curve would appear to be strongly shifted (see the fivefold slowing in Fig. 9B). For agents that slow but do not stop the microscopic inactivation step, the prolongation of rise and the increase in the size of currents at small depolarizations would be smaller and the apparent shift of the peak g_{Na} -voltage curve would also be smaller. Even when the microscopic inactivation step is slowed equally at all voltages, the macroscopic inactivation rate appears to be more slowed for large depolarizations than for small ones, as was seen with *Leiurus* toxin (Fig. 7).

While irreversible kinetic diagram 2 reproduces many features of our experiments, it is not a full description of gating. Unfortunately, at present, no model of Na channel gating is close to being complete, since each has focused on a narrow range of phenomena, as in this article. Many articles have concentrated on supplying rate constants for reversible schemes related to diagram 1 (Bezánilla and Armstrong, 1977; Armstrong and Gilly, 1979; Gilly and Armstrong, 1982; Aldrich et al., 1983; Aldrich and Stevens, 1984; Horn et al., 1984; Horn and Vandenberg, 1984; Vandenberg and Horn, 1984; Stimers et al., 1985). In all versions, microscopic activation is faster than microscopic inactivation at large depolarizations, so that all channels open synchronously and then inactivate irreversibly, and each channel will open only once before becoming nearly inactivated. The articles differ, however, in the assignment of rate constants at small depolarizations, where the peak g_{Na} stays well below its maximum. At one extreme are models like our diagram 2 or the simpler one of Aldrich et al.

(1983), where even at small depolarizations, channels open only once. Here microscopic activation is slow and nearly irreversible, and g_{Na} never rises very high because a fast microscopic inactivation removes channels from the conducting pool soon after they arrive. At the other extreme are coupled models, where activation is fast and in dynamic equilibrium with the conducting pool (Nonner, 1980). Here channels open multiple times before inactivating by a slower microscopic inactivation, and g_{Na} never rises very high because the equilibrium of the activation steps favors the closed states. Most models fall between these extremes, combining incomplete activation (finite rates of deactivation) with rapid inactivation to keep g_{Na} at low depolarizations.

Models all along the spectrum between these extremes may generate similar macroscopic Na currents for normal channels, but the predictions differ once inactivation is blocked. As we have seen, the irreversible extreme, with a single opening per Na channel, predicts a shifted peak g_{Na} -voltage curve and considerable prolongation of the rising phase of currents at small depolarizations. Models at the opposite extreme, with activation reaching equilibrium and giving repeated reopenings of Na channels, predict results more like those seen in all previous experiments—no apparent change in the voltage or time dependence of channel opening. The rate constants chosen by Bezanilla and Armstrong (1977) fall in between. Fig. 9C shows simulations with this model, including the effect of slowing and blocking microscopic inactivation. There is a clear prolongation of the rise time and selective enhancement of size at the small depolarization where, on average, a channel will open only 1.5 times before inactivating. These effects, though smaller than with the irreversible model, show that the original Bezanilla-Armstrong rate constants would not appropriately describe pronase experiments in squid giant axons, where no shift or prolongation occurs. Rate constants given subsequently by Gilly and Armstrong (1982) and Stimers et al. (1985) reproduce the pronase effect in squid axons well. With them, channels open on average more than three times before inactivating and about half the inactivation occurs from a closed state, properties that are reminiscent of the classical HH model.

What do our observations imply? We have found that slowing or removing Na inactivation in N18 neuroblastoma cells alters the amplitude, time course, and voltage dependence of Na current in a way that is not seen when the same experiment is done with squid giant axons, crayfish axons, frog nodes of Ranvier, or frog muscle. Thus, despite the similarity between normal macroscopic Na currents of neuroblastoma cells and those in traditional adult preparations, there is a microscopic difference. The several changes of neuroblastoma currents can be described as a pure block of microscopic inactivation in the class of kinetic models where Na channels open only once before inactivating. Single channel studies have shown that this is how cell-attached patches of N1E115 neuroblastoma cells behave (Aldrich et al., 1983). The more irreversibly the model sends resting channels through the open state and on to the inactivated state at low depolarizations, the more pronounced will be the effects of removing inactivation. Since macroscopic currents of the many adult preparations studied do not show the changes seen in neuroblastoma cells, we would predict that their Na channels open multiple times at low depolarizations so the macroscopic peak g_{Na} -voltage curve is controlled more by the voltage-dependent equilibrium of micro-

scopic activation than by depletion of channels through rapid microscopic inactivation. Single channel studies with GH₃ cells show that this is how outside-out membrane patches excised from these cells behave (Horn and Vandenberg, 1984; Vandenberg and Horn, 1984). Studies on adult axon preparations are still lacking, but Na channels of adult muscle do show these properties (Patlak and Ortiz, 1986).

Our analysis is based on several simplistic assumptions. The major one is that pharmacological and chemical modifications of a single macromolecule, the Na channel, can selectively modify a single microscopic step in the complex conformational changes of gating. During the last 20 years of research, the ease with which inactivation can be modified by a wide variety of agents acting through many different chemical mechanisms on both sides of the membrane has always been in surprising contrast to the difficulty of changing activation. We are impressed here by the similar shifts of the peak $g_{Na}-E$ curve seen with each of these agents applied to neuroblastoma cells and by the absence of such effects in other studies. Whether our working hypothesis is correct cannot be determined definitively until the complex gating of Na channels is better understood.

We end at a purely speculative level. It is now a commonplace that multiple forms of each class of ionic channel can be detected even in a single membrane. For Na channels, differences in gating kinetics, pharmacology, antigenic determinants, phosphorylation, and messenger RNA have been observed, each within one cell type. Thus, the concepts of interconversion by covalent modifications at the protein level or by simple changes of environment (temperature, salts) and of diversity arising at the genetic level all seem valid. In this context, we cannot say yet at what structural level the differences between neuroblastoma and typical adult, peripheral axon-muscle Na channels occur. Whatever it is, we can imagine that the neuroblastoma form of channels could coexist with the others in all cells in varying proportions. Further, since inactivation is so easily modified by many kinds of applied agents, we can imagine that the cell does this too in its daily life, either by covalent modification or by transcribing from a different gene. Such modification of neuroblastoma-type channels would lead to apparently slowly activating, noninactivating, low-threshold channels, whereas such modification of the other type of channels would lead to rapidly activating, noninactivating channels of normal threshold. These channels seen in various cells could contribute to phenomena now known as "sleepy channels," "threshold channels," "window current," and "subthreshold persistent Na current," as well as to the presence of Na channels of longer open time late in a depolarizing pulse (Matteson and Armstrong, 1982; Gilly and Armstrong, 1984; Attwell et al., 1979; Stimers et al., 1985; Sigworth, 1981; Patlak and Ortiz, 1986).

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