0026-895X/94/040716-10$03.00/0

MOLECULAR PHARMACOLOGY, 46:716-725, 1994

Slow Binding of Phenytoin to Inactivated Sodium Channels in Rat Hippocampal Neurons

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Received March 16, 1994; Accepted June 12, 1994

SUMMARY

The anticonvulsant phenytoin inhibited Na^+ currents in rat hippocampal neurons with a potency that increased dramatically at depolarized holding potentials, suggesting weak binding to resting Na^+ channels but tight binding to open or inactivated channels. Four different experimental measurements, i.e., steady block at different holding potentials, on and off kinetics at depolarized holding potentials, shifts in the inactivation curve, and dose-dependent slowing of recovery from inactivation, yielded an estimated K_d of ~7 μM for phenytoin binding to inactivated channels. Prolonged depolarizations of at least several seconds were necessary for significant block by therapeutic concentrations of phenytoin. The slow development of block does not reflect selective binding of phenytoin to slow inactivated states of the channel, because block developed faster and required less depolarized voltages than did slow inactivation. Instead, it appears that phenytoin binds tightly but slowly (~10^4 M^{-1} sec^{-1}) to fast inactivated states of the Na^+ channels. This tight but slow binding may underlie the ability of phenytoin to disrupt epileptic discharges with minimal effects on normal firing patterns.

The anticonvulsant phenytoin is widely used in the treatment of generalized tonic-clonic seizures and partial seizures (1). The effectiveness of phenytoin as an anticonvulsant is probably related to its ability to inhibit high-frequency action potential firing while having little effect on lower frequency firing (2–4). At therapeutic concentrations of 4–8 μM in cerebrospinal fluid (5, 6), voltage-dependent sodium channels are likely the primary target of the drug (3, 7–9).

Studies in a variety of preparations have shown that phenytoin block of Na^+ channels is much more potent at depolarized holding potentials than at hyperpolarized membrane potentials. The voltage dependence of the action of phenytoin is most clear from voltage-clamp experiments (10–16) but is also evident in phenytoin block of action potentials, which is more pronounced at more depolarized voltages (3, 17). Such voltage dependence is a characteristic of many drugs that block Na^+ channels and can be understood by the modulated receptor hypothesis (18, 19), which proposes that drug binding to open or inactivated channels is tighter than to resting states. Consistent with the ability of phenytoin to more effectively block high frequency trains of action potentials, voltage-clamp experiments show greater block with higher frequencies of depolarization, a property that can be explained by the ability of phenytoin to slow recovery from inactivation (10, 12, 13, 16, 20).

Compared with other drugs that inhibit Na^+ channels in a voltage-dependent and use-dependent manner, a distinctive feature of the action of phenytoin is a requirement for relatively long depolarizations of at least 1 sec or so. In many preparations, phenytoin has little effect on the voltage dependence of Na^+ channel availability when prepulses of 100 msec or less are used (10, 21), but prepulses of several seconds always show dramatic voltage dependence (10, 11). Similarly, when studied with trains of action potentials, block by phenytoin is more pronounced late in the train (2–4).

Why are long depolarizations needed for potent block by phenytoin? One especially interesting possibility is that phenytoin binds selectively to slow inactivated states of the Na^+ channel. Long depolarizations drive Na^+ channels into slow inactivated states, from which recovery takes seconds rather than milliseconds (22–26). The development of slow inactivation and the development of phenytoin block occur on similar time scales. Consistent with phenytoin binding to slow inactivated states, Schauf (12) and Quandt (26) found that, when fast inactivation is removed by enzymes, slow inactivation remains and phenytoin is still capable of producing potent block. However, these experiments leave open the question of whether phenytoin binds selectively to slow inactivated states rather than fast inactivated states.

We studied phenytoin block of Na^+ channels in acutely

This work was supported by National Institutes of Health Research Grant HL35034.

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ABBREVIATIONS: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

dissociated rat hippocampal neurons with two primary goals, to obtain quantitative estimates for the strength of phenytoin binding to inactivated Na^+ channels and to determine whether phenytoin binds selectively to the slow inactivated state of the Na^+ channel. We found that phenytoin does not selectively bind to the slow inactivated state; in fact, phenytoin binding to the slow inactivated state may be slightly weaker than that to the fast inactivated state. Instead, the requirement for long depolarizations results because phenytoin binding to fast inactivated channels is slow. A variety of pulse protocols yielded an estimated K_d value of ~7 μM for phenytoin binding to inactivated channels, whereas binding to resting channels was at least 100 times weaker.

Materials and Methods

Cell preparation. Coronal slices of the whole brain were prepared from 7–14-day-old Long-Evans rats. CA1 regions were dissected from the slices, cut into small chunks, and enzymatically treated for 10 min at 37° with 3 mg/ml protease XXIII (Sigma Chemical Co., St. Louis, MO) in dissociation medium (82 mM Na_2SO_4, 30 mM K_2SO_4, 3 mM $MgCl_2$, 2 mM HEPES, 0.001% phenol red indicator, pH 7.4). Tissue chunks were then transferred to a solution containing 1 mg/ml bovine serum albumin (Sigma) and 1 mg/ml trypsin inhibitor (Sigma) in dissociation medium. As cells were needed, the enzymatically treated chunks were triturated in dissociation medium to release single cells. The cells were put into the recording chamber containing Tyrode's solution (150 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, pH 7.4). Cells were used within 8 hr of preparation.

Whole-cell recording. Whole-cell voltage-clamp recordings (27) were obtained using pipettes pulled from 100-μl Boralex micropipettes (Dynalab, Rochester, NY) that had been coated with Sylgard (Dow-Corning, Midland, MI) and fire-polished. Pipette resistances were 1.5–2 MΩ when the pipettes were filled with internal solution containing (in mM) 75 CsCl, 75 CsF, 2.5 $MgCl_2$, 5 HEPES, 2.5 EGTA, 14 creatine phosphate-Tris (Sigma), 4 Mg-ATP (Sigma), and 0.3 GTP-Tris (Sigma), pH adjusted to 7.4 with CsOH. Seals were formed and the whole-cell configuration was obtained in Tyrode's solution. The cell was then lifted from the bottom of the chamber and moved in front of an array of flow pipes (Drummond Microcaps, 2 μl, 64-mm length) emitting either control or drug-containing external recording solutions (Tyrode's solution with or without 10–100 μM phenytoin). Phenytoin was dissolved in dimethylsulfoxide to make a 100 mM stock solution, which was diluted into Tyrode's solution for final concentrations of 10–100 μM. The final concentration of dimethylsulfoxide was 0.1% or less and was found to have no detectable effect on Na^+ currents. It was not possible to dissolve phenytoin in Tyrode's solution (pH 7.4, 25°) at concentrations significantly greater than 100 μM, consistent with the reported solubility of phenytoin as 22 μg/ml (~90 μM) in buffer solution of pH 7.5 at 25° (28). Currents were recorded at room temperature (~25°) with an Axoclamp 200A amplifier, filtered at 5 kHz with a four-pole Bessel filter, digitized at 20–50-μsec intervals, and stored using a BASIC-FASTLAB analog/digital interface and software (Indec Systems, Sunnyvale, CA). Residual series resistance generally ranged from 0.4 to 0.8 MΩ after partial compensation (typically >90%), and the product of residual series resistance and cell capacitance was generally <15 μsec. All statistics are given as mean ± standard error.

Results

The effect of phenytoin is stronger at depolarized holding potentials. The blocking effect of phenytoin on Na^+ currents was highly sensitive to the holding potential, as previously found for a variety of cell types (10, 11) including hippocampal neurons (15). At a holding potential of −100 mV, where there was virtually no resting inactivation (see Fig. 2B), up to 100 μM phenytoin had almost no effect on Na^+ current (Fig. 1A). At more depolarized holding potentials, where there was some inactivation of Na^+ current, 10–100 μM phenytoin had a significant blocking action (Fig. 1B). When studied at depolarized holding potentials, phenytoin blocked currents elicited by different test potentials by equal amounts and had no effect on the shape of the peak current-voltage relation, consistent with previous reports (10, 11, 13, 16).

Fig. 1C shows dose-response curves obtained for phenytoin applied at different holding potentials. The block by various concentrations of phenytoin could be reasonably fit by a simple one-to-one binding curve at each holding potential. The highest affinity block was at a holding potential of −50 mV, where Na^+ current was inactivated by >95% and phenytoin inhibited the current with an apparent K_d of 7 μM. The weakest block was at a holding potential of −90 mV, where 100 μM phenytoin blocked current by only ~10%. The fitted K_d was 600 μM at −90 mV, but this is a rough estimate because there was so little block by 100 μM phenytoin, which is near the limit of solubility. Because inactivation may not be completely removed at −90

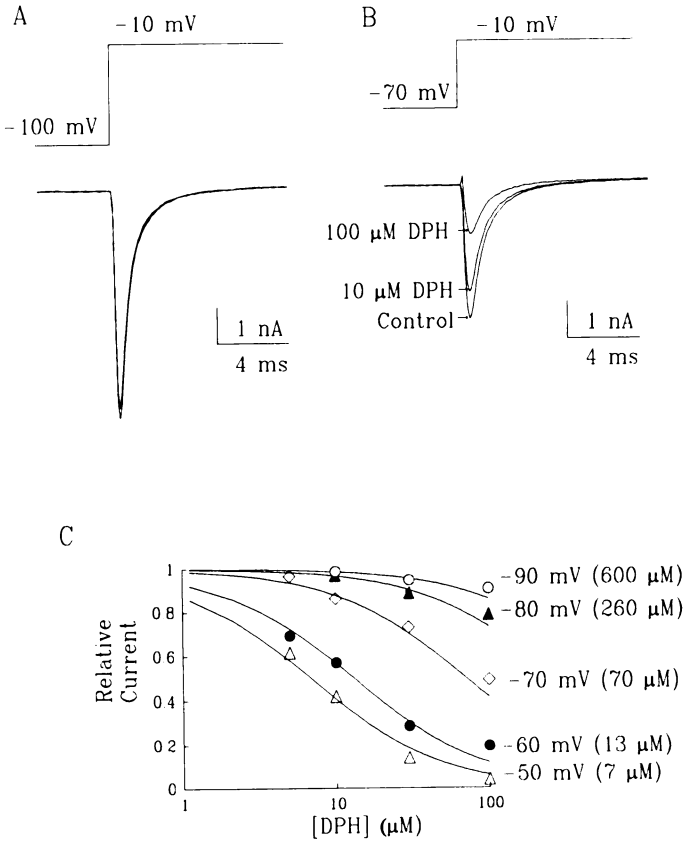


Fig. 1. Inhibition of Na^+ current by phenytoin (*DPH*) at different holding potentials. A, Currents with control or 10 or 100 μM phenytoin-containing solutions in a cell held at −100 mV and stepped to −10 mV for 15 msec every 2 sec. The currents shown in this and other figures are steady state currents after at least 1 min in control or drug-containing solutions. B, Inhibition of currents elicited from a holding potential of −70 mV in the same cell as in A. C, Dose-response curves for inhibition of Na^+ current by 5–100 μM phenytoin at different holding potentials. All data are from one cell (different from that in A). The cell was held at −50 to −90 mV and stepped to 0 mV for 5 msec every 1 sec. The *lines* were drawn according to the equation $1/(1 + ([DPH]/K_d))$, where [DPH] is the phenytoin concentration and K_d is the apparent dissociation constant. The K_d values for the fitted curves are shown in parentheses to the *right of the curves*.

mV (see Fig. 3, A and B), the K_d might be even greater than 600 μM at very negative holding potentials.

Correlation of the affinity of phenytoin with inactivation. The powerful effect of holding potential is consistent with the hypothesis that phenytoin binds much more tightly to the inactivated state than to the resting state of the channel (10, 11, 21). As the cell is held at more positive potentials, there is more steady state inactivation of Na^+ channels and therefore more binding and more block. We tested this hypothesis in a quantitative manner, based on the following simplified state diagram:

$$\begin{array}{ccccc} & & \overset{(V)}{R} & \longleftrightarrow & I \\ DPH & \uparrow\downarrow & & & \uparrow\downarrow & DPH \\ & & \underset{(V)}{RD} & \longleftrightarrow & ID \end{array} \qquad \text{(scheme 1)}$$

where R and I are the resting and inactivated states of the channel, DPH is the phenytoin (or diphenylhydantoin) molecule, and RD and ID are the phenytoin-bound (and inhibited) resting and inactivated states. The horizontal transitions are voltage dependent but the binding and unbinding steps are not. With this scheme, the fraction of channels in R would be reduced by drug with the form expected for simple 1:1 binding, with an apparent dissociation constant (K_{app}) given by

$$K_{app} = \frac{1}{(h/K_R + (1 - h)/K_I)}$$

where h is the fraction of channels in state R in the absence of drug and K_R and K_I are the dissociation constants for the resting and inactivated states, respectively (29). Because $K_R \gg K_I$,

$$K_{app} \approx \frac{K_I}{(1 - h)}$$

or

$$\frac{1}{K_{app}} \approx \frac{(1 - h)}{K_I}$$

Thus, the apparent K_d at a particular holding potential is predicted to be equal to K_I divided by the degree of inactivation $(1 - h)$, and if the reciprocal of K_{app} is plotted versus holding potential then it should have the same voltage dependence as the degree of steady state inactivation (the mirror image of the conventional inactivation curve). Fig. 2 shows that this prediction is borne out by the data. At depolarized potentials, where $1 - h$ approaches 1, K_{app} should approach K_I. From Fig. 2A, K_I is thus estimated as 1/0.16 or ~6 μM.

Shifts in the inactivation curve. K_I can be estimated by another approach based on scheme 1. In control, the inactivation curve (the fraction of channels in state R) can be approximated well by a Boltzmann distribution, $1/(1 + \exp[(V - V_h)/k])$, where V is the membrane potential, V_h is the half-inactivated potential, and k is the slope factor. With drug added, the shape of the curve should remain the same, with the midpoint shifted by ΔV, where $\exp(\Delta V/k)$ is equal to $[1 + (D/K_I)]/[1 + (D/K_R)]$ (see Ref. 30). For $K_R \gg K_I$ and drug concentrations low relative to K_R, $\exp(\Delta V/k) \cong [1 + (D/K_I)]$.

With phenytoin present at 10–100 μM, there was no change in the inactivation curve measured using 100-msec prepulses

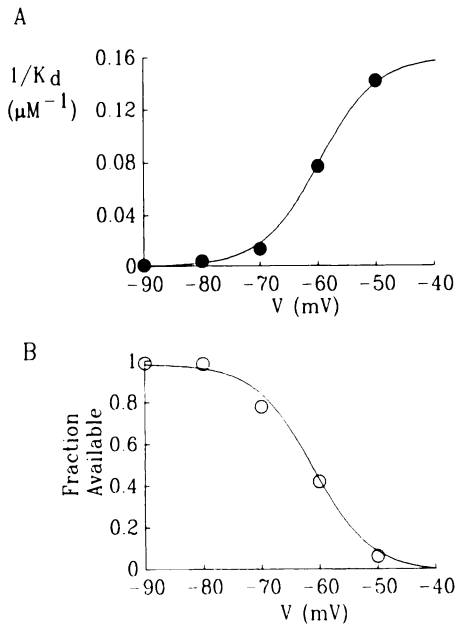


Fig. 2. Correlation of phenytoin affinity with fast inactivation of Na^+ channels. A, Affinity of phenytoin, expressed as $1/K_d$, plotted against the holding potential. K_d values are from the experiment shown in Fig. 1C. The *line* is a Boltzmann function, $1/K_d = 0.16/[1 + \exp(-(V + 61)/5)]$, where V is the holding potential. B, Inactivation curve determined for the same cell as used for the determination of the dose-response data in Fig. 1C and in A. The cell was held at −110 mV and given a 100-msec inactivating prepulse to potentials from −100 to −40 mV, followed by a 10-msec test pulse to 0 mV. The test pulse currents were normalized to that with a −100-mV prepulse (8 nA). The *line* is a Boltzmann function that is a mirror image of that in A, $1/[1 + \exp((V + 61)/5)]$.

to different voltages (Fig. 3A). However, when the inactivation curve was measured using 16-sec prepulses, it was shifted in the hyperpolarizing direction, as predicted (Fig. 3B). Both in controls and with phenytoin, the inactivation curves could be well fit by simple Boltzmann functions, with a slope factor (~5 mV) that was not altered in the presence of drug (Fig. 3C). The shift in the midpoint voltage increased with drug concentration and reached an average of 15 ± 2 mV at 100 μM phenytoin (Fig. 3D). Fig. 3E tests the quantitative predictions of the simple model. As predicted, $\exp(\Delta V/k)$ is related linearly to phenytoin concentration over the range of 10–100 μM. The line fitting the data points has a y-intercept of 1 and a slope of 0.14 μM^{-1}. The K_I estimated from this fit (the reciprocal of the slope) is 7 μM.

Slow inactivation of Na^+ channels in hippocampal neurons. There are two possible explanations for the requirement for long depolarizing prepulses to produce a shift in the inactivation curve. One is that phenytoin binds selectively to a slow inactivated state that Na^+ channels enter after long depolarizations. The second is that phenytoin binds slowly to "fast" inactivated channels. Subsequent experiments were designed to distinguish between these possibilities.

First, we characterized slow inactivation in these neurons. The presence of channels in the slow inactivated state was assayed using pulse protocols in which a test pulse to −10 mV followed 15 msec at −110 mV, long enough to allow complete recovery from normal fast inactivation, which occurs with a time constant of ~2 msec at −110 mV (31). Fig. 4, A and B, shows the induction of slow inactivation at different voltages. There was no slow inactivation at −70 mV; all Na^+ channels

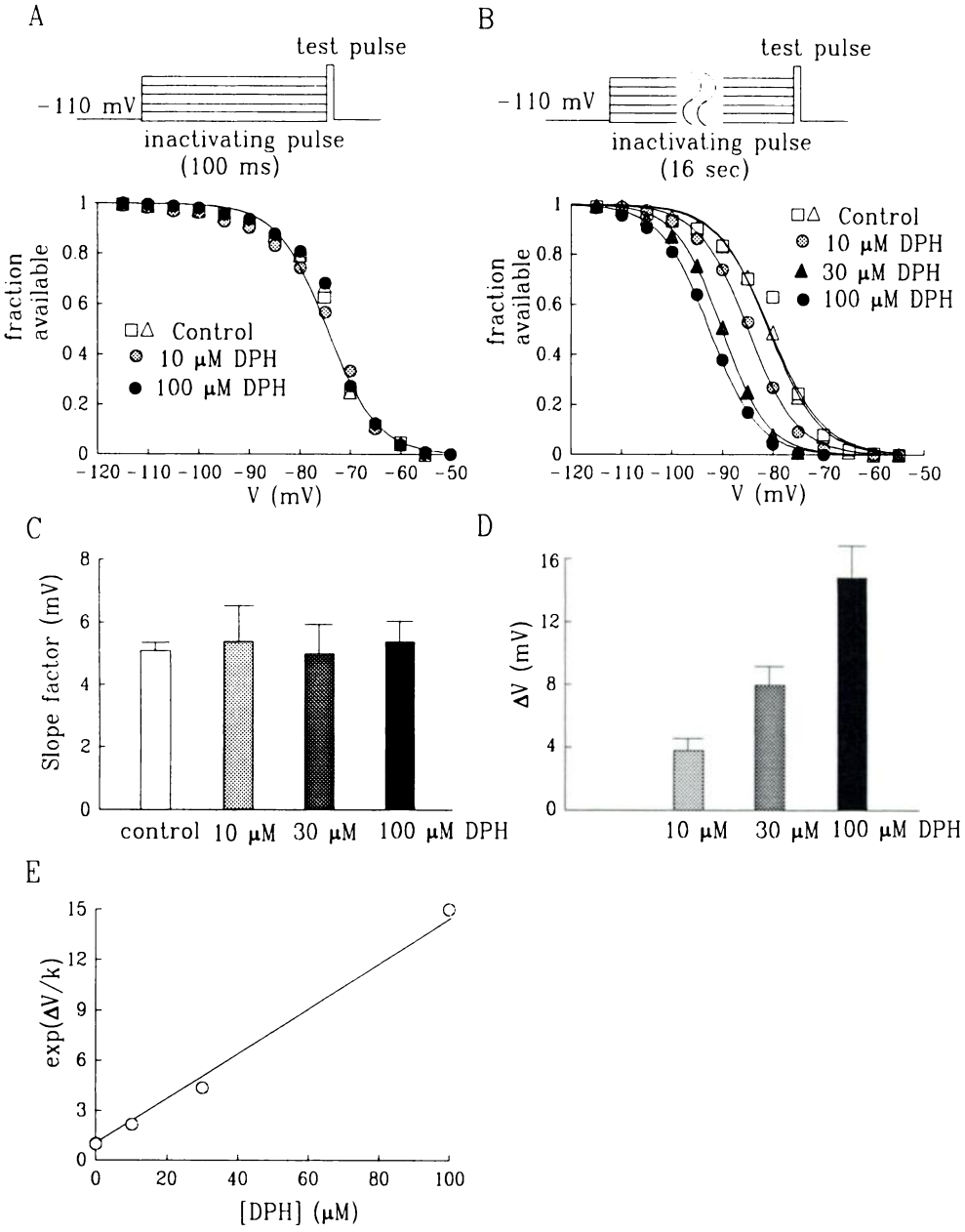
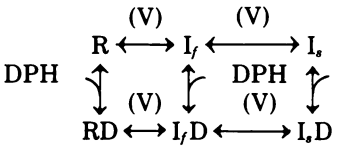


Fig. 3. Shift by phenytoin (*DPH*) of inactivation curves for long but not short inactivating prepulses. A, Inactivation curves determined with prepulse durations of 100 msec. There was no effect of 10 or 100 μM phenytoin. Control data were obtained both before and after the two sets of pulses with phenytoin, to check for any voltage drift. The curve is a Boltzmann function, $1/[1 + \exp((V + 74)/5)]$. B, Same experiment in a different cell but with prepulses of 16 sec; prepulse-test pulse combinations were delivered every 25 sec. Two sets of control data were obtained before and after the three sets of pulses with 10, 30, and 100 μM phenytoin and demonstrated no voltage drift during this long experiment. The *lines* are fits (using least-squares fitting) to the Boltzmann function $1/[1 + \exp((V - V_h)/k)]$, with V_h values of −81, −81, −85, −90, and −93 mV for control before phenytoin, control after phenytoin, and 10, 30, and 100 μM phenytoin and k values of 5.3, 5.1, 4.9, 4.5, and 4.9 mV, respectively. C, Lack of change of slope factor k by phenytoin. Values of k averaged from different cells for control and 10, 30, and 100 μM phenytoin were 5.1 ± 0.4 (n = 7), 5.4 ± 1.6 (n = 4), 5.0 ± 1.1 (n = 4), and 5.4 ± 0.7 mV (n = 7), respectively. D, Dose-dependent shift of the midpoint of the inactivation curve. The shift (ΔV) was determined in each cell as the difference between V_h values in control solutions and with various phenytoin concentrations. The ΔV values were 4 ± 1 (n = 4), 8 ± 1 (n = 4), and 15 ± 2 (n = 7) for 10, 30, and 100 μM phenytoin, respectively. E, Plot of $\exp(\Delta V/k)$ versus phenytoin concentration (data from the means in D). The data points were fit well by a straight line, $\exp(\Delta V/k) = 1 + 0.14 \cdot [DPH]$.

recovered from inactivation during the 15-msec gap even after a 16-sec prepulse to −70 mV. As the inactivating pulse was made more positive, the occupancy of the slow inactivated state increased and finally saturated at ~80% with a 16-sec pulse at potentials greater than +10 mV. At any given voltage, the development of slow inactivation could be well fit by a single-exponential function. The time constant (~4–5 sec) showed little voltage dependence from −50 to +70 mV.

The rate of recovery from slow inactivation was voltage dependent (Fig. 4C). Recovery from slow inactivation was complete in about 1 sec at −130 mV but took about 7 sec at −70 mV. There was always a very fast initial component of recovery (~20–30%), suggesting a maximal occupancy of the slow inactivated state of about 80% even with the longest and largest depolarizations (Fig. 4B).

Fig. 4D shows the voltage dependence of inactivation measured with three different protocols, i.e., with 100-msec prepulses, with 16-sec prepulses, and with 16-sec prepulses followed by a 15-msec period at −110 mV to allow recovery from fast inactivation. The results can be interpreted by a simple scheme that assumes two kinds of inactivated state, a conventional fast inactivated state (I_f), which is reached and recovered from within millseconds, and a slow inactivated state (I_s), for which both entry and recovery take seconds.



(scheme 2)

The curve for inactivation measured with a 16-sec prepulse (V_h of −69 ± 9 mV and slope factor of 5.1 ± 0.4 mV, n = 7) was only slightly different from that measured with 100-msec prepulses (V_h of −64 ± 9 mV and slope factor of 5.6 ± 0.8 mV, n = 11), suggesting that I_s is significantly occupied only at voltages where occupancy of I_f is substantially complete. The protocol with a 15-msec return to −110 mV measures the

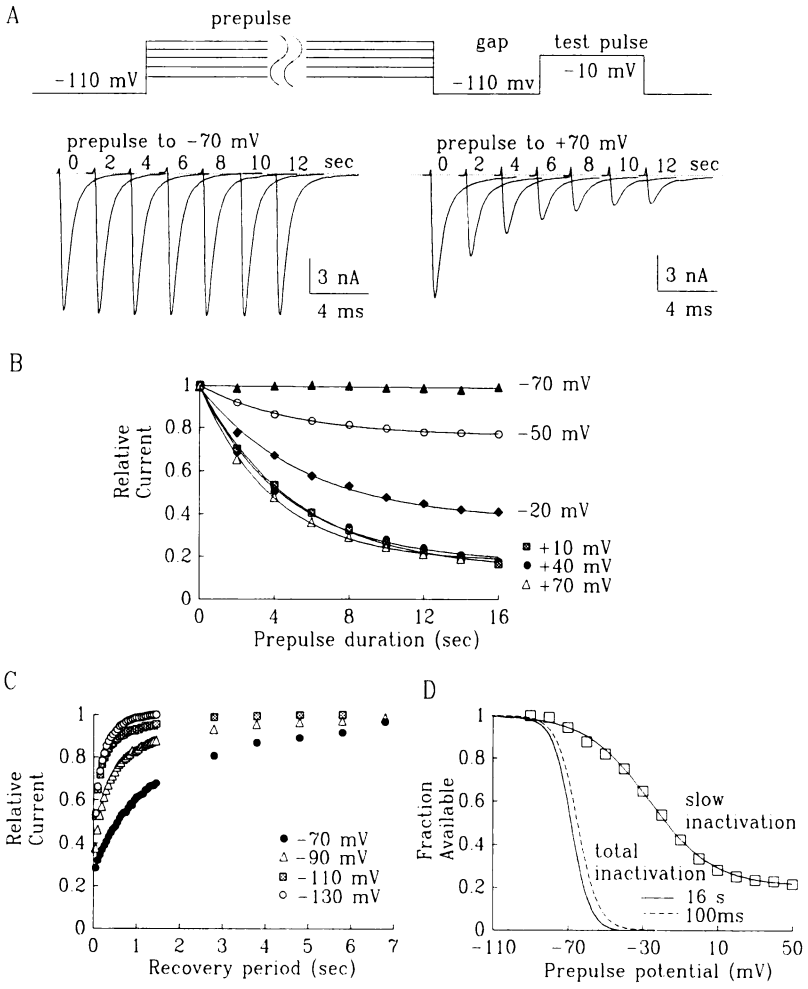


Fig. 4. Slow inactivation of Na^+ channels in hippocampal neurons. A, Pulse protocol and sample currents. Cells were held at −110 mV, and the pulse protocol was repeated every 25 sec. An inactivating prepulse of variable voltage and length (from −70 to +70 mV and from 0 to 16 sec) was followed by a gap potential at −110 mV for 15 msec (to allow recovery from fast inactivation), after which the fraction of available channels was measured by a test pulse to −10 mV. When the inactivating pulse was −70 mV, all of the channels recovered from inactivation within 15 msec. When the inactivating pulse was +70 mV, channels were slowly driven into the slow inactivation state and could not recover during the 15-msec gap. B, Time course of development of slow inactivation, with the same cell and pulse protocol as in A. The currents were normalized to the first current in each series when there was no inactivating pulse. The *lines* are monoexponential functions, i.e., $0.76 + 0.24 \cdot \exp(-t/4.8)$ at −50 mV, $0.37 + 0.63 \cdot \exp(-t/5.3)$ at −20 mV, $0.18 + 0.82 \cdot \exp(-t/4.6)$ at +10 mV, $0.14 + 0.86 \cdot \exp(-t/5.1)$ at +40 mV, and $0.17 + 0.83 \cdot \exp(-t/4.1)$ at +70 mV, with t in seconds. C, Time course of recovery from slow inactivation. Cells were held at −110 mV. Slow inactivation was induced by a 16-sec pulse to +50 mV, followed by a variable recovery period and then a test pulse to −10 mV. Test pulse current was normalized to that with a 20-sec recovery period. The recovery time courses could not be fit by simple exponential functions (even disregarding the initial fast phase). Times for 50% to 90% recovery were 5.2, 1.3, 0.6, and 0.4 sec at −70, −90, −110, and −130 mV, respectively. D, Voltage dependence of slow inactivation (□). The pulse protocol was as in A, with 16-sec prepulses to different voltages. The test pulse current was normalized to that with a prepulse to −110 mV. The *right solid line* is a Boltzmann distribution, $0.21 + 0.79/[1 + \exp((V + 27)/17)]$, where V is the prepulse potential; for comparison, the *left solid line* shows the voltage dependence of fast inactivation measured with 16-sec prepulses (mean midpoint and slope values from seven cells) $[1/[1 + \exp((V + 69)/5.1)]]$ and the *dashed line* shows the voltage dependence of fast inactivation measured with 100-msec prepulses (mean midpoint and slope values from 11 cells) $[1/[1 + \exp((V + 64)/5.6)]]$.

occupancy of I_s. The points derived from this measurement in a particular cell (Fig. 4D) could be fit by a Boltzmann function with a midpoint of −27 mV, a slope factor of 17, and a maximal occupancy of ~80%. For collected results, the average midpoint was −31 ± 4 mV (n = 7) and the slope was 17 ± 1 (n = 7). The relatively shallow voltage dependence of this curve is consistent with the movement of ~1.5 equivalent charges between I_f and I_s (see Ref. 19, pp. 55–56).

Lack of selective binding of phenytoin to the slow inactivated state. Fig. 5A shows the development of block by 100 μM phenytoin at different depolarized voltages. The block by phenytoin can be compared directly with the rate and voltage dependence of slow inactivation shown in Fig. 4, A and B, because the pulse protocols are identical and the experiments were done in the same cell. At each depolarized voltage, the kinetics of block by phenytoin were substantially faster than the development of slow inactivation. For example, at −50 mV phenytoin block developed with a time constant of 1.6 sec, whereas slow inactivation developed with a time constant of 4.8 sec; with a step to +40 mV, phenytoin blocked with a time constant of 0.6 sec, whereas slow inactivation developed with a time constant of 5.1 sec. The results with a prepulse to −70 mV are of particular interest. There was no slow inactivation even with a 16-sec pulse, but there was substantial (~40%) block by phenytoin.

These results argue against the possibility that phenytoin binds exclusively to the slow inactivated state. If it did, the development of block by phenytoin could never be faster than the development of slow inactivation, in contrast to the experimental results. We can conclude that phenytoin must also be able to bind to the fast inactivated state.

Assuming the simple model in scheme 2, the kinetics of phenytoin binding to the fast inactivated state can be estimated from the results in Figs. 4 and 5. If phenytoin binds to fast inactivated channels in a 1:1 manner, the rate of entry into states from which recovery is slow (i.e., the slow inactivated state or the phenytoin-blocked state) should be a constant (the rate of slow inactivation) plus a linear function of phenytoin. Fig. 5B shows that this prediction is satisfied. At high phenytoin concentrations, the time constant in Fig. 5A would be determined primarily by the rate constant of phenytoin binding to fast inactivated channels, which would be approximated by the slope of the lines in Fig. 5B. From these slopes, the rate constant for phenytoin binding to the fast inactivated state of the channel is ~10,000 M^{-1} sec^{-1}.

Kinetics of phenytoin block with rapid solution changes. The kinetics of phenytoin block were examined in a different way by moving the cell abruptly into and out of phenytoin-containing solution. Both the development and re-

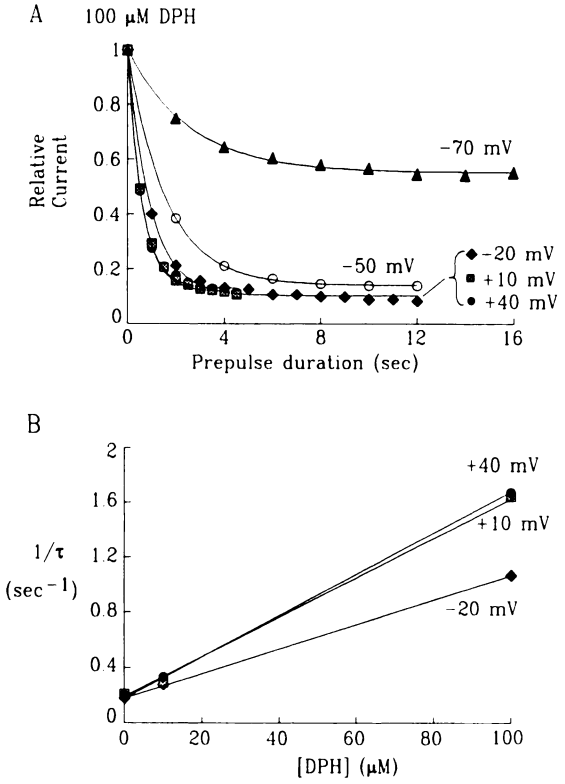


Fig. 5. Voltage and time dependence of phenytoin (*DPH*) block during prepulses. A, Time course of block by 100 μM phenytoin with various prepulses. The *lines* are monoexponential functions with time constants (steady state values in parentheses) of 2.6 sec (0.55) at −70 mV, 1.6 sec (0.14) at −50 mV, 0.9 sec (0.10) at −20 mV, 0.6 sec (0.12) at +10 mV, and 0.6 sec (0.13) at +40 mV. B, Rate of phenytoin block versus phenytoin concentration. The reciprocals of the time constants for entry into the slowly recovering phase in control and 10 and 100 μM phenytoin-containing solutions are plotted against the phenytoin concentration. The *lines* are linear fits with intercepts and slopes of 0.18 sec^{-1} and 9,000 M^{-1} sec^{-1} at −20 mV, 0.19 sec^{-1} and 14,000 M^{-1} sec^{-1} at +10 mV, and 0.18 sec^{-1} and 14,000 M^{-1} sec^{-1} at +40 mV, respectively.

versal of block could be well fit by monoexponential functions (Fig. 6A). The development of block was faster with higher drug concentrations, whereas the time constant of recovery was 6–7 sec at all drug concentrations. The rate of block was a linear function of phenytoin concentration (Fig. 6, B and C). At a holding potential of −50 mV (where most channels would be in the fast inactivated state), the rate constant from the linear fit was ~7,000 M^{-1} sec^{-1}, similar to the estimate from the development of block when the cell was depolarized in the continuous presence of phenytoin. [The agreement suggests that the rate of block is little affected by the depolarizing test pulses (5 msec to 0 mV twice each second) used to monitor the Na^+ current in the experiment in Fig. 6; this is reasonable because they are so short, relative to the time constant for block near 0 mV (0.5 sec at 100 μM).] The unblocking rate constant was ~0.06 sec^{-1}, yielding a calculated dissociation constant of ~9 μM. If the development of block reflects mainly binding to the fast inactivated state, then the rate of block should be slower at more negative holding potentials, where fewer channels are inactivated. Consistent with this prediction, block developed more slowly at −70 mV than at −50 mV (Fig. 6B).

Affinity of binding to the slow inactivated state. Clearly, phenytoin does not bind exclusively to the slow inactivated state. Nevertheless, because a substantial fraction of Na^+ channels can enter the slow inactivated state with long depolarizations, it is interesting to ask whether there is a difference in the strength of phenytoin binding to the fast inactivated and slow inactivated states. We tried to answer this question by comparing the time courses of recovery when block was induced at different voltages, where there should be different fractions of fast inactivated and slow inactivated channels. Fig. 7A shows the time course of recovery with different concentrations of phenytoin after block was induced by a long pulse to −50 mV. Because fast inactivation is complete at −50 mV but there is only a small amount of slow inactivation (~20%) (Fig. 4D), the slow recovery from phenytoin block reflects mainly unbinding from fast inactivated channels, and the fraction of channels recovering slowly should be governed mostly by the K_d for binding to fast inactivated channels. The fraction of channels recovering slowly from phenytoin-bound states was estimated from the difference between the slow component of recovery in controls (reflecting ~20% occupation of the slow inactivated state) and the larger slow components of recovery with phenytoin (reflecting primarily phenytoin-bound fast inactivating channels as well as smaller fractions of slow inactivated channels and phenyotin-bound slow inactivated channels). Fig. 7B shows the dependence on phenytoin concentration of the additional, slowly recovering fraction, which was quantitated by integrating the difference between the time course of recovery with phenytoin and the time course of recovery in controls. This dose-response relation can be reasonably fit by a rectangular hyperbola corresponding to 1:1 binding, with an apparent K_d of 4 μM.

Fig. 7, C and D, shows the results of the same experiment but with a 16-sec prepulse to +50 mV, where the amount of slow inactivation is maximal (~60% in this cell) (Fig. 7C). In this case, the apparent K_d from the rectangular hyperbola fit to the additional, slowly recovering fraction was 14 μM (Fig. 7D). This estimate of the affinity of phenytoin for the slow inactivated state is not very precise, because the determination depends on calculating the difference from a rather large, slowly recovering fraction in controls and a substantial fraction of channels are in the fast inactivated state even at +50 mV (Fig. 4). Nevertheless, the comparison suggests a somewhat lower affinity for binding to the slow inactivated state than to the fast inactivated state.

Discussion

Comparison with other cell types. Probably the most important fact about phenytoin block of voltage-dependent Na^+ channels is that block is greatly enhanced by depolarizations lasting several seconds or more. Our results in hippocampal neurons confirm and extend previous observations in a variety of cell types (10–16). Because the active form of phenytoin is a neutral molecule (32), it is unlikely that the voltage dependence of phenytoin block reflects direct voltage dependence of binding to the channel. Rather, it has previously been proposed that the voltage dependence reflects the voltage dependence of Na^+ channel inactivation and tighter binding of phenytoin to the inactivated state of Na^+ channels than to the resting state (10, 11, 21). The results in Fig. 2 showing a precise correlation of the blocking potency with the degree of inactivation give strong support to this idea.

Our dose-response data are consistent with 1:1 binding of

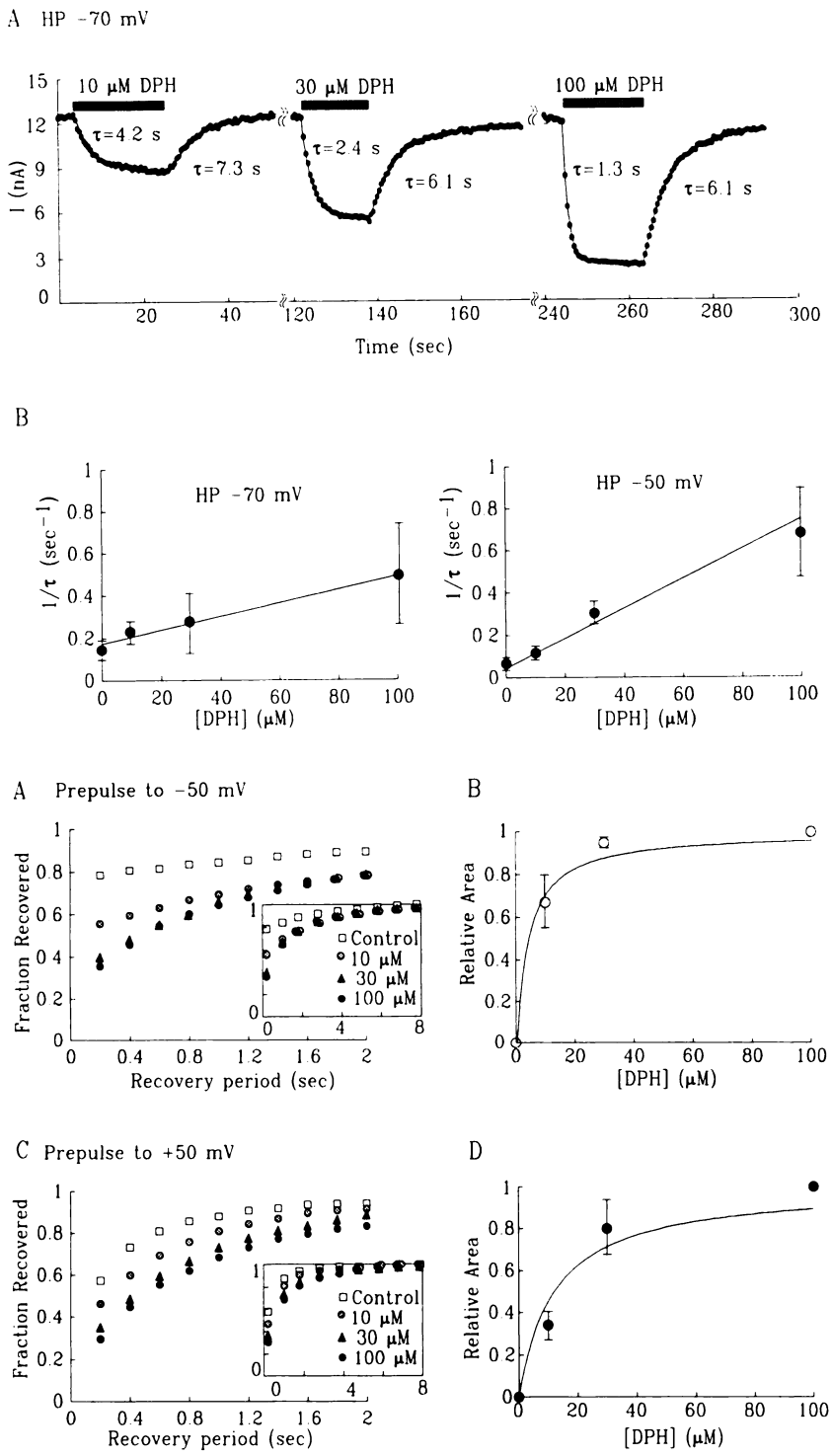


Fig. 6. Onset and recovery from phenytoin (*DPH*) block with rapid solution changes. A, The cell was held at −70 mV and pulsed to 0 mV for 5 msec every 0.5 sec. Peak test pulse current is plotted versus time. Applications of phenytoin were separated by about 1 min. Both onset and recovery were well fit by a monoexponential function in each case, with time constants indicated. B, The rates of phenytoin block versus phenytoin concentration for experiments like that in A are shown. The reciprocal of the blocking time constant is plotted against the phenytoin concentration (averages from three or four cells). The reciprocal of the average time constant for recovery (average over four cells of values for each cell averaged over multiple applications of phenytoin) is plotted for [DPH] = 0. The *solid lines* are best least-squares fits, with slopes of 3100 M^{-1} sec^{-1} at −70 and 7000 M^{-1} sec^{-1} at −50 mV. *HP*, holding potential.

Fig. 7. Concentration-dependent slowing of recovery by phenytoin at two different prepulse voltages. A, Recovery after a prepulse to −50 mV. The pulse protocol was as in Fig. 4C, with a 16-sec prepulse to −50 mV and variable gap length. *Inset*, same data on a longer time scale. B, Area between each time course and the time course in control solution (calculated from multiexponential fits for each course to reduce noise) normalized to the value for 100 μM phenytoin and plotted against the phenytoin concentration. The *line* is a rectangular hyperbolic fit to the *points*, 1/(1 + 4 μM/[DPH]). C, Same experiment (in the same cell) as in A but with prepulses to +50 mV. D, Plot as in B with data from C. The *line* was drawn according to the equation 1/(1 + 14 μM/[DPH]).

phenytoin to Na^+ channels. Based on the dose-response data at different holding potentials, we estimate that the K_d for binding to inactivated channels is at least as low as 7 μM and that the K_d for binding to resting Na^+ channels is at least as high as 600 μM. Comparing our results with those in other cell types, there are no obvious differences in the potency of phenytoin. When examined at holding potentials where channels are ~25% inactivated (somewhat different in each cell type), half-block was produced by ~30 μM phenytoin in mouse neuroblastoma cells (11) and by ~40 μM phenytoin in rat node of Ranvier (13). For hippocampal neurons, a K_d of ~30 μM can be estimated for such a holding potential by interpolating from the data in Fig. 2.

Strength of binding to slow and fast inactivated states. We can conclude that the requirement for long (>1-sec) depolarizations to enhance phenytoin block does not reflect exclusive binding to slow inactivated states, because the development of phenytoin block is faster than the development of slow inactivation. By comparing the dose dependence for prepulses to +50 mV with that for prepulses to −50 mV (where channels

are mostly in the fast inactivated state), it seems that binding to slow inactivated channels may actually be slightly weaker than that to fast inactivated channels (Fig. 7). Drug binding to slow inactivated channels might be even weaker than the K_d of 14 μM estimated from Fig. 7D, because there is a substantial fraction of fast inactivated channels with a prepulse to +50 mV.

We obtained similar estimates for the K_d of phenytoin binding to inactivated channels using different protocols, including determination of on and off kinetics of application at a holding potential of −50 mV (9 μM) (Fig. 6), equilibrium block at a holding potential of −50 mV (7 μM) (Fig. 1), shift of the midpoint of inactivation curves determined with 16-sec prepulses (7 μM) (Fig. 3), and dose-dependent slowing of recovery after a prepulse to −50 mV (4 μM) (Fig. 7). Even though these protocols used long depolarizations to detect high affinity binding of phenytoin, all of these measurements probably reflect primarily binding to fast inactivated states, because all involve voltages negative to −50 mV, where there is generally <20% slow inactivation (e.g., see Fig. 4D) (and, because phenytoin binding to slow inactivated states is weaker than that to fast inactivated states, phenytoin would not "pull" channels from fast to slow inactivated states).

Both the strength and slowness of phenytoin binding are likely to be important for its ability to produce anticonvulsant effects without disrupting normal brain function. The estimated K_d of ~7 μM for binding to inactivated channels is very close to therapeutic concentrations in cerebrospinal fluid (5, 6), so there would be significant block of inactivated channels under therapeutic conditions. The slow on rate of phenytoin binding to inactivated channels may be crucial for preservation of normal neuronal firing. At concentrations of 10 μM or lower, the on rate of ~10,000 M^{-1} sec^{-1} (at room temperature) means that neurons would have to be depolarized for >10 sec to have significant block by phenytoin. At body temperature binding would probably be faster, but depolarizations of several seconds might still be needed. Depolarizations long enough to produce significant block might be unusual in normal electrophysiological activity but more common during epileptic activity.

Inactivation stabilizer or activated channel blocker? There are two ways of thinking about the state-dependent binding of phenytoin to the Na^+ channel. One is the simplest form of the modulated receptor hypothesis (18, 19), that phenytoin binds more tightly to inactivated channels and thereby stabilizes channels in the inactivated state. Another, somewhat different, possibility (that retains the essential modulated receptor concept of altered drug receptors in different gating states of the channel) is that the affinity of phenytoin depends not on whether the channel is open or inactivated but rather on the degree of activation (i.e., forward movement of the activation gating charge) of the channel. In this case, phenytoin binding to the channel might be regarded as mimicking inactivation rather than stabilizing it. Both the development of and recovery from Na^+ channel inactivation can be quantitatively understood by a simple model where binding of the inactivating particle becomes progressively faster and tighter as the gating charges move forward to activate the channel (31). According to this model, recovery from inactivation is steeply voltage dependent over the range from −70 mV to −170 mV because the inactivation particle is "ejected" from the channel by backwards movement of the activation gating charges, and the recovery rate saturates at voltages negative to about −190 mV because unbinding of the particle, a non-voltage-dependent step, becomes rate-limiting at voltages where all of the activation charges move back quickly. Recovery from channel block by phenytoin is 200-fold slower than normal recovery from inactivation but shows very similar voltage dependence (31), suggesting that the activation-deactivation gating voltage sensors interact very similarly with the inactivating particle and the phenytoin molecule.

According to this picture, the correlation between inactivation and the strength of phenytoin binding arises because both depend on movement of the activation-deactivation gating charges, not because of an interaction of phenytoin with the inactivation "gate." In this model, the states in scheme 1 would represent the extremes of a continuum between very weak binding to resting states (with all gating charge in the "off" position) and tightest binding to fully activated states (with all gating charges in the "on" position), which would include both open and inactivated channels. Under normal circumstances, occupancy of open states would be too brief to allow significant phenytoin binding, so including only the inactivated state in scheme 1 would be a reasonable approximation. A prediction of this model is that phenytoin binding to activated channels would be equally strong if normal inactivation processes were removed. This is consistent with the experiments by Schauf (12) and Quandt (26), who found that phenytoin produced potent block when fast inactivation was removed by enzymes. (Slow inactivation was still present in their experiments, however, so it was not possible to establish whether phenytoin binds to open channels in the absence of all inactivation.)

Phenytoin and the normal inactivating particle might actually compete for the same receptor, or distinct receptors might be formed by the same movement of activation gating charges. If there are different receptors, there could still be reciprocal interactions between binding of phenytoin and binding of the inactivating particle. Such interactions could be tested by comparing the strength and kinetics of phenytoin block with and without inactivation. Interestingly, Quandt (26) found that, with fast inactivation enzymatically removed in neuroblastoma cells, 100 μM phenytoin blocked with a time constant of ~60 msec during a depolarization to −20 mV, which is >10 times faster than the time constant of ~1 sec that we found (Fig. 5A) with inactivation intact. The difference would be consistent with the normal inactivation process interfering somewhat with the access of phenytoin, but clearly the comparison needs to be made in the same preparation before this conclusion is accepted.

Slow kinetics of phenytoin binding and unbinding. Although the basic features of phenytoin interaction with the Na^+ channel may be similar to those of the normal inactivating particle, a major difference is that phenytoin binding and unbinding are several orders of magnitude slower that the development and recovery from fast inactivation. We estimate the rate constant for phenytoin binding to inactivated channels as ~10^4 M^{-1} sec^{-1} (Figs. 5B and 6B), which is 10^3 to 10^4 times slower than if drug binding were diffusion limited. An interesting possibility is that binding of phenytoin is competitive with that of the normal inactivating particle, which would occupy its receptor >99% of the time (because steady state noninactivating current for fast inactivation is <1%). This possibility seems unlikely, however. If it were true, removing fast inacti-

vation would speed the rate of phenytoin block by 100-fold, but the rate of block found in neuroblastoma cells with inactivation removed (26) is only about 10 times faster that we measured with inactivation intact. Also, if block of noninactivated channels occurred with a rate constant of 10^7 M^{-1} sec^{-1}, then 100 μM phenytoin would block the open channels at a rate of 1 $msec^{-1}$ and would therefore produce a faster rate of decay of Na^+ currents, which are normally "blocked" at a rate of ~1 $msec^{-1}$ by normal inactivation (near 0 mV). This is not the case, as seen in Fig. 1, where the time courses of macroscopic Na^+ currents are very similar with or without phenytoin.

More likely, the slow rate of phenytoin binding is an intrinsic property of the interaction between phenytoin and its receptor, perhaps implying strict stereospecific requirements so that only one of every 1000 collisions between phenytoin and the channel results in binding. Once bound, the phenytoin-channel complex is very stable, so that the unbinding rate is quite slow, being ~0.5 sec^{-1} near −100 mV and reaching a saturating value of ~20 sec^{-1} at voltages negative to −150 mV, where binding is maximally destabilized by full backwards movement of the gating charges (31).

Slow inactivation of Na^+ channels in hippocampal neurons. The slow inactivation of Na^+ channels in hippocampal neurons seems similar to that described in squid axons (23), *Myxicola* axons (12, 25), and neuroblastoma cells (26, 33). In all cases, the development of and recovery from slow inactivation occur with time courses of hundreds of milliseconds to seconds, i.e., 2–3 orders of magnitude slower than fast inactivation. As for fast inactivation (and also block by phenytoin), the rate of recovery from slow inactivation is voltage dependent. With recovery voltages negative to −130 mV channels recovered completely within about 0.5 sec. With more positive recovery potentials it took longer for the channels to recover. There was always a very fast component of recovery at the beginning, as if 20–40% of the channels remained in the fast inactivated state even with long strong depolarizations. There are apparently voltage-dependent steps in the conversion of channels between fast and slow inactivated states, because the slow inactivation curve (Fig. 4D) has a shallow voltage dependence over voltages positive to −50 mV, where fast inactivation is saturated. The steepness of the slow inactivation curve would be consistent with movement of about 1.5 equivalent charges across the electric field in conversion of fast inactivated channels to slow inactivated channels. An interesting possibility is that this charge movement arises from the same charges that underlie the steeply voltage-dependent activation-deactivation steps, moving a little further beyond the position for open and fast inactivated states of the channel. With this model, movement of the same gating charges from off to on positions would result in the formation of high affinity receptors for both the normal inactivating particle and phenytoin (and other drugs, such as local anesthetics, that bind tightly to activated channels). Further movement of the same gating charges would induce a conformational change into a slow inactivated state, where the phenytoin binding site would be slightly altered so that its affinity would be somewhat reduced. This model for slow inactivation would fit well with the observation of Rudy (25) that slow inactivation develops faster after enzymatic removal of fast inactivation. Because the receptor for the fast inactivating particle is created by the gating charges being in the on position, binding of the inactivating particle might tend to stabilize the channel in this position and retard further gating charge movements to the slow inactivated state.

There are intriguing parallels between the phenomenology of slow inactivation and Na^+ channel block by phenytoin. As with phenytoin block, slow inactivation would have almost no effect during normal action potential firing from well polarized neurons. However, prolonged depolarizations beyond −50 mV or so would put channels into slow inactivated states. This process would minimize Na^+ entry that would otherwise come, at sustained depolarizations, from the steady state (0.1–1%) currents resulting from not quite complete fast inactivation. Slow inactivation could thus be viewed as having the properties of an "endogenous anticonvulsant" that can limit Na^+ channel activation under conditions of pathological depolarization.

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