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Modification of Slow Inactivation of Single Sodium Channels by Phenytoin in Neuroblastoma Cells

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SUMMARY

Modifications of Na^+ channels by phenytoin (PT), an anticonvulsant drug, were examined. Previous work using voltage-clamp methods indicated that PT could interact with inactivated states of the channel to reduce excitability. Single-channel analysis was used to test the idea that the fast inactivation process was not required for modification of the channel. The hypothesis that PT could interact with open or slow inactivated states to produce a drug-bound, long duration, nonconducting state was also tested. Currents due to the opening of single Na^+ channels were measured in inside-out patches of membrane excised from N1E-115 mouse neuroblastoma cells grown in tissue culture. After the removal of the fast inactivation process enzymatically, the average Na^+ current in response to a step depolarization decayed due to the slow inactivation process. The time constant of decay decreased as a function of the concentration of PT. The average current appeared to be caused by extensive reopening of Na^+ channels. During maintained depolarization, the reopening of Na^+ channels occurred in bursts interrupted by long silent periods, due to the slow inactivated state. PT decreased the burst duration and increased the interval between bursts. The average open time of Na^+ channels was reduced in the presence of PT. All of the alterations were enhanced as the concentration of PT was increased. The amplitude of current through the open channel was not effected by PT. PT was able to modify the Na^+ channel in the absence of fast inactivation. The results suggest that PT can bind to the Na^+ channel and produce a nonconducting state from which the probability of a channel opening is small. These modifications could underly the selective block of action potentials during chronic depolarization of the membrane or during high frequency discharge.

PT belongs to a class of compounds used in the management of epilepsy (1). A considerable amount of evidence has now been obtained that indicate that the anticonvulsant action of PT is in large part due to block of repetitive firing of action potentials in neurons (2). A remarkable action of PT is to selectively block action potentials occurring late during a train, at high frequencies, or following chronic depolarization (3, 4).

Voltage-clamp work has indicated that PT causes a reduction in current generated by voltage-sensitive Na^+ channels. However, effects on Ca^{2+} channels have also been found (5). The peak Na^+ conductance has been found to be reduced although the time course of activation and fast inactivation were not effected (6–8).

More recent studies have examined the action of PT in greater detail on single neurons using the voltage-clamp technique (9, 10). These voltage-clamp experiments indicate that PT can modify the gating of the Na^+ channel by promoting a condition resembling inactivation. The antiarrythmic action of PT in heart may involve a similar mechanism (11).

PT induced block of Na^+ current is enhanced when the membrane is depolarized repetitively. Block of Na^+ current by PT exhibits a progressive increase during a series of depolarizations in voltage clamp experiments. If conditioning pulses are used to induce block, increases in the duration or frequency of conditioning pulses accentuate the reduction of Na^+ current. These observations are consistent with the idea that PT interacts with inactivated states, inasmuch as inactivation is enhanced at more depolarized potentials and with increases in duration. Recovery from conditioned block induced by PT is markedly slowed compared with recovery from normal inactivation. This last effect accounts for the increased block as the frequency of conditioning pulses is increased, because less time is allowed for recovery.

The hypothesis that PT binds primarily to inactivated states of the channel, producing a drug-bound state having properties resembling an inactivated state, would therefore account for the block of repetitive firing of neurons by PT. This hypothesis predicts actions on Na^+ channels that can be measured using single channel analysis. PT should promote the time-dependent reduction in probability of an open channel, as does inactivation. Further, in the steady state during exposure to PT, a channel should be in a nonconducting state from which the probability of opening is small a greater portion of time.

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ABBREVIATIONS: PT, phenytoin; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; DMSO, dimethylsulfoxide.

Willow *et al.* (10) concluded that PT interacts with various states of the Na^+ channel, including the state associated with fast inactivation. However, Na^+ channels typically have both a fast and a slow process for inactivation (12) and those in neuroblastoma cells are no exception (13, 14). In NIE-115 neuroblastoma cells, previous evidence (9) suggested that the slow inactivation process was modified by the action of PT. The membrane potential at which half of the Na^+ channels become inactivated due to the process of slow inactivation shifted to a more hyperpolarized value in the presence of the drug, although the curve describing fast inactivation did not. Second, block of Na^+ current was markedly enhanced as the duration of the conditioning pulse was increased from 40 to 80 msec (10°), even though the time constant of fast inactivation is less than 20 msec under these conditions (14). Moreover, the time course for recovery from block was greater than 0.5 sec (−100 mV), although the time constant for recovery from fast inactivation is 30 msec. In *Myxicola* axons, effects of PT on slow inactivation were observed even though fast inactivation was removed with pronase (15).

In order to test whether the fast inactivation process was required and to investigate the action of PT on single Na^+ channels, fast inactivation was removed. Fast inactivation can be specifically removed with enzyme treatment (16), leaving slow inactivation intact (17). Although removal of fast inactivation before exposure to PT provides a test of the idea that this state is not required for modification of the channel by PT, the lack of fast inactivation excludes the development of a complete description of the interaction of PT with all states of the channel. Removal of fast inactivation has a number of practical advantages. First, the duration of the open state is increased. Second, Na^+ channels continue to open during maintained depolarization, facilitating steady state measurements (18).

The use of single channel analysis has advantages for determination of the mechanism of PT action. First, actions on the conductance of the open channel can be separated from those on the probability of a channel being open. Second, changes in the number and duration of open and closed states can be determined. Third, long term depolarizations can be employed without alterations in the concentration gradients for ions during prolonged currents. Fourth, the technique is optimized to study events having a low probability of occurrence. Some of the results have been previously presented in an abstract (19).

Methods

Preparation. Neuroblastoma cells (NIE-115) grown in tissue culture were used in this study. The conditions for growth were identical to those previously used to study Na channels in this preparation (18). Cells were grown in media consisting of Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, and 20 mM HEPES at a temperature of 37° and a pH of 7.4. Humidified air having a CO_2 content of 10 percent was employed. Fungizone (2.5 mg/ml), penicillin (100 units/ml), and streptomycin (100 μg/ml) were also added to the growth media. Cells used in experiments were differentiated by growing them on glass cover slips in media with reduced fetal calf serum (1% and 2% DMSO (20) for 3 days to 2 weeks.

Recording conditions. The patch-clamp technique was used to record membrane currents due to the opening of single Na^+ channels (21). The "inside-out" configuration, in which the cytoplasmic surface of the membrane was oriented toward the bath, was employed in all experiments in order to apply enzymes and PT to the cytoplasmic surface. The details of the recording technique are identical to those given previously (18). Briefly, glass pipettes used for patch clamping were approximately 1 μm in diameter. The seal resistance between the pipette and membrane was typically 10 to 20 GΩ. Currents were recorded with an amplifier having frequency compensation to 1.5 KHz. Unless stated otherwise, all signals were filtered to 1 KHz (−3 dB) before digitization using an eighth-order Bessel filter (Frequency Devices 902 LPF).

The pipette (external) solution contained (mM) NaCl, 125; KCl, 5.5; $CaCl_2$, 3; $MgCl_2$, 0.8; HEPES, 20; dextrose, 25. Sucrose was added to bring the osmolarity to 330 mOsm and the pH was adjusted to 7.3 using NaOH. The internal solution applied to the bath just before excising the bath consisted of 150 mM glutamic acid, 20 mM ethyleneglycol-bis(aminoethylether)tetraacetic acid, 20 mM HEPES, and 1 mM NaHEPES. The pH was adjusted to 7.2 with the addition of CsOH. The use of Cs^+ eliminated currents due to K^+ channels from the records. Unless noted in the legends, experiments were performed at room temperature (22°). As described in Results, papain (0.088 to 0.116 units/ml; Cooper Biochemicals, Malvern, PA) was added to the internal solution along with 3 mM cysteine (free base). Phenytoin (5,5-diphenylhydantoin) was obtained from Sigma Chemical Co. (St. Louis, MO) and stored as a 20 mM stock solution in DMSO. Where indicated, PT was added to the test solution just before an experiment. Control experiments indicated that 0.5% DMSO, the maximum concentration applied during tests of the activity of PT, had no effect on properties of Na^+ channels modified by papain.

Analysis. An analysis of intervals between opening and closing events in these records was performed by the computer, which determined when currents crossed an amplitude half the distance between the baseline current and that generated by the open channel (22). Channel openings from continuous records stored on video tape were reconstructed for automatic computer analysis using a technique similar to that given by Sigworth (23). These records were routinely refiltered using a 500 Hz cutoff frequency during playback in order to further enhance the signal to noise ratio for the automated analysis. This procedure brought the effective cutoff frequency to 447 Hz. Open or closed states having a duration less than 0.4 msec were therefore not included in the analysis of continuous records. The limitation does not affect the measurement of burst durations and intervals between bursts considered in this paper.

Plotted histograms are cumulative. The reported values are the probability that the event is greater than the time given on the abscissa. Fitting of the histograms to exponential distributions was done employing a program that used a least squares criterion (24). An estimate of the goodness-of-fit was determined by calculating χ^2 (23). The probability that the fitted and actual distributions were identical was at least 0.9. As noted in Results, in some cases a second exponential component was added in order to obtain a fit having a probability of 0.9 or greater.

Results

PT modifies the time course of average Na^+ current. In order to examine the effects of PT on single Na^+ channels in the absence of fast inactivation, this process was removed. Typically, the fast inactivation process can be eliminated by applying selected proteolytic enzymes or protein reagents to the internal surface of the membrane. For the experiments reported here, papain was used for this purpose (18, 25).

The procedure used to accomplish this in excised patches of membrane from neuroblastoma cells is shown in Fig. 1. The internal surface of an inside-out patch of membrane was exposed to papain while the Na^+ current was monitored in response to a step depolarization. In this case, the patch contained numerous Na^+ channels. Before the application of papain,

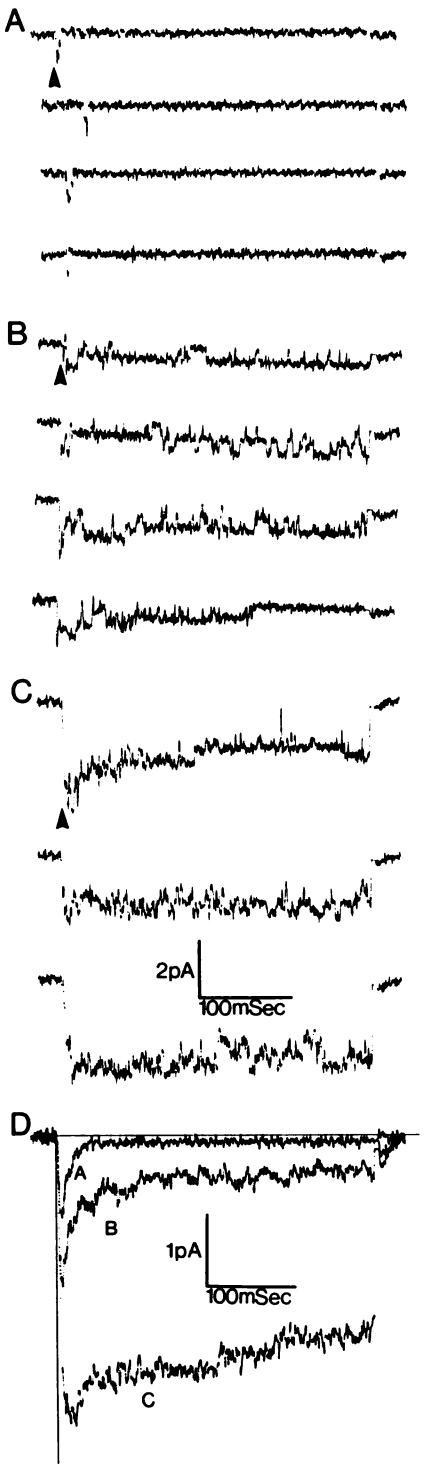


Fig. 1. Time course of removal of fast inactivation by papain. Membrane current was recorded from an inside-out patch of membrane from a neuroblastoma cell. A–C, Example records in response to a step depolarization to −30 mV from a holding potential of −120 mV, applied at 5-sec intervals, are shown. The *arrows* mark the onset of a 350-msec voltage pulse. A, A series of control records were measured before the addition of papain (0.09 units/ml) to the internal perfusate. B, Example records from a series recorded between 260 and 355 sec after papain was introduced. C, Records from a later series obtained 360–480 sec after addition of papain. D, Average membrane currents from series A, B, or C. Average currents were filtered using a 500 Hz gaussian digital filter. Temperature, 10°.

channels only opened during the beginning of the depolarization, as shown in Fig. 1A. The time course of changes in these currents due to papain is shown in Fig. 1, B and C. Two effects were noted that indicated that fast inactivation was no longer operating. First, Na^+ channels continued to open at later times throughout the depolarization. Second, the average duration that any individual channel was open increased. The peak current therefore increased due to the summation of channels opening with a higher probability and having an increased open time.

The average currents are shown in Fig. 1D. Under control conditions, the current declined due to the fast inactivation process. The time constant of decay of current was 11 msec. At an early time during the application of papain, two time constants of decay can be seen. The shorter time constant was 14 msec and represents the fast inactivation of unmodified channels. A longer time constant was apparent after fast inactivation was removed in a portion of the channels. The longer time constant was about 1.1 sec and was caused by the slow inactivation process (18). With prolonged exposure to papain, most channels were modified, inasmuch as a smaller fraction of current declined with the short time constant. The longer time constant was again about 1 sec, indicating that papain did not produce graded changes in the time constant but caused a greater fraction of current to decay with a fixed time constant. This suggests that each channel was modified in an all-or-none manner. A similar explanation was given by Gonoi and Hille (25). The effect of papain was irreversible.

Rather than entering the fast inactivated state, channels can reopen throughout the depolarization under these conditions (see Ref. 18 for a complete description). The increase in open time is due to the elimination of the reaction pathway from open to inactivated states, which then decreases the average rate of closure (26).

In voltage-clamp experiments from the whole cell, block of Na^+ current in response to a test pulse during exposure to PT increased as the duration of a conditioning depolarization was prolonged. After removal of fast inactivation, it was possible to determine the time course of action of PT directly by observing the decline of Na^+ current during a prolonged depolarization. Fig. 2 shows examples of individual and average membrane currents recorded from an inside-out patch in response to a step depolarization.

Na^+ currents in response to a step depolarization were measured under control conditions and during the addition of 30 or 100 μM PT to the internal perfusate. Under control conditions, this current decayed due to slow inactivation, with a time constant of 357 msec. This time constant was shorter than that in Fig. 1 primarily because a higher temperature was used in this experiment. When PT was present in the internal solution, the time constant of the decline in current decreased. Increases in the concentration of PT produced a faster decay. The time constant decreased to 125 msec during exposure to 30 μM PT, and 58 msec in the presence of 100 μM PT. The effect was reversible when PT was removed. Individual records revealed that the probability of entry into a conducting state at a later time during the depolarization was reduced in the presence of PT. However, the lifetime of an individual open state also appeared to be reduced (see below). The amplitude of current through the open channel was not altered by PT. For the experiment illustrated in Fig. 2, the mean amplitude of current

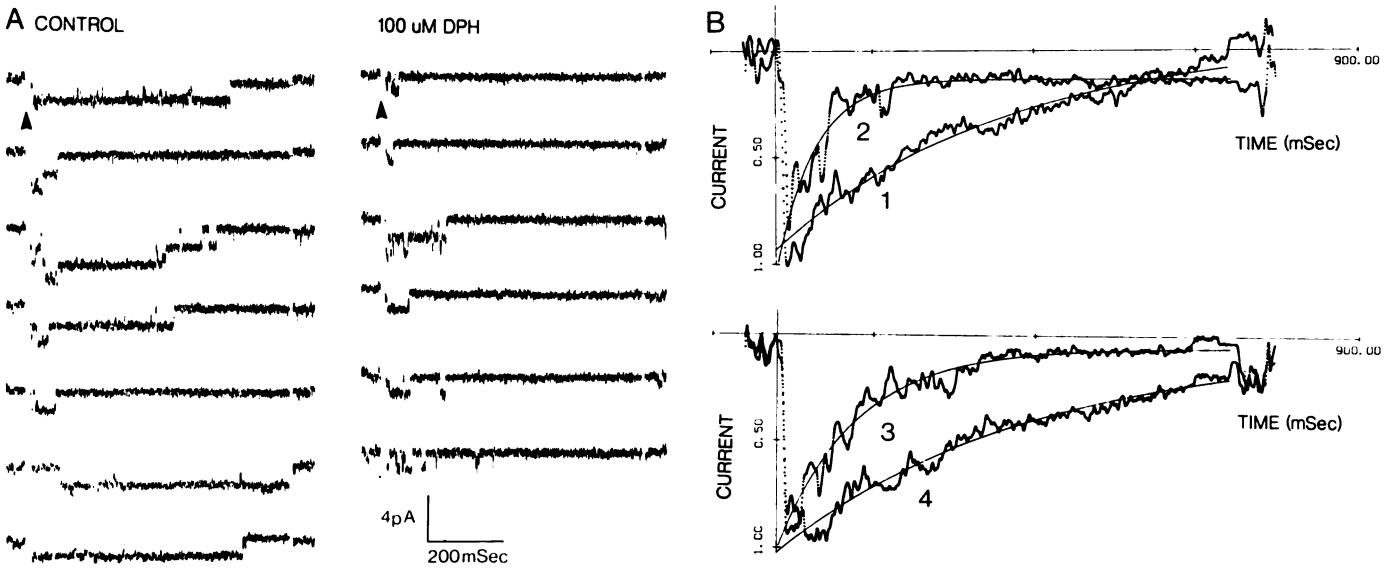


Fig. 2. PT accelerates the decay of Na^+ current following depolarization. A, Individual membrane current records in response to step depolarizations to −20 mV are shown before (*left*) and during (*right*) the application of 100 μM PT (*DPH*). *Arrows* mark the onset of a 700-msec voltage step. B, Average currents obtained from the experiment shown in A are plotted for control conditions (*curve 1*), during 100 μM (*curve 2*) or 30 μM PT (*curve 3*), and after PT was washed out (*curve 4*). Average time constants of decay were 357 msec in control, 125 msec in 30 μM PT, 58 msec in 100 μM PT, and 400 msec during recovery. *Fitted curves* are superimposed on the data. The results illustrated were typical for each of five patches that were tested using similar experiments.

was 1.44 ± 0.19 pA under control conditions and 1.48 ± 0.22 pA in the presence of 100 μM PT.

This experiment indicates that the fast inactivation process is not absolutely required for the effects of PT on the Na^+ current. The drug causes a time-dependent modification of the channel, to promote a nonconducting state from which the probability of a channel opening is small. The state responsible for the slow inactivation process is similar in nature (18).

Burst activity of Na^+ channels is altered by PT. The nature of the drug-bound nonconducting state was examined by determining the action of PT on spontaneous bursts of Na^+ channels. During maintained depolarization under control conditions, but following removal of fast inactivation, a series of openings of a Na^+ channel occurs, followed by a relatively long duration closed period (18). During these bursts, the Na^+ channel normally cycles rapidly between open and closed states until the slow inactivated state is entered. The burst duration is equal to the time constant of the decay of Na^+ current after a step depolarization from a negative holding potential. This last observation indicates that the burst is terminated by entry into the state associated with slow inactivation. The burst resumes when the channel enters the closed or open states from this inactivated state. The mean duration of the interval between bursts gives an indication of the rate of reaction out of the inactivated state.

The effect of PT on the burst activity of Na^+ channels is illustrated in Fig. 3. A continuous segment of a recording of membrane current under control conditions is shown in Fig. 3A. Also shown is a segment after the addition of 60 μM PT to the internal perfusate. Note that inward current generated during a burst was followed by a relatively long period in which no channel openings were observed. PT reduced the overall probability of a channel being open in the steady state. For example, in the experiment illustrated in Fig. 3, the probability of a channel being open was reduced from 0.036 under control conditions to 0.0025 during exposure to 60 μM PT. These values are the probability for each channel, corrected for the number of channels in the patch. The reduction in the probability of a channel being open was only slowly reversible after PT was washed out. In the experiment illustrated, the probability of an open channel increased to 0.0045 when measured during a 16-min period starting 13 min after PT was removed.

The reduced probability occurred due to three effects. First, the interval between bursts increased; second, the duration of bursts decreased; third, the lifetime of the open state was reduced. In order to investigate the effects of PT on the kinetics of bursts, it is necessary to separate events into different classes. The histogram of all durations of nonconducting events under control conditions is shown in Fig. 3B. Because these events can range from less than 1 msec to tens of seconds, the time axis is plotted on a log scale. The bin width was arranged so that the number of bins per decade was constant. Although most closed states were less than 60 msec in duration, the histogram revealed a distinct population of events having a long duration. This fraction of closed events represents the interval between bursts. Shorter duration events represent closed states within a burst. The probability of events having a duration greater than 60 msec was similar to that for states greater than 300 msec, indicating that closed states within bursts could be separated for analysis from intervals between bursts without ambiguity.

Because bursts of openings were separated by long duration intervals, it was possible to determine the duration of a burst as the sum of a series of open and closed times, bracketed by nonconducting events having a duration greater than 100 msec. This criterion for separating bursts and intervals was found to be valid for all of the patches studied. The ability to identify these events was not altered by PT, as shown in Fig. 3B. Each burst appeared to be due to the opening of a single Na^+ channel, inasmuch as the simultaneous opening of more than one chan-

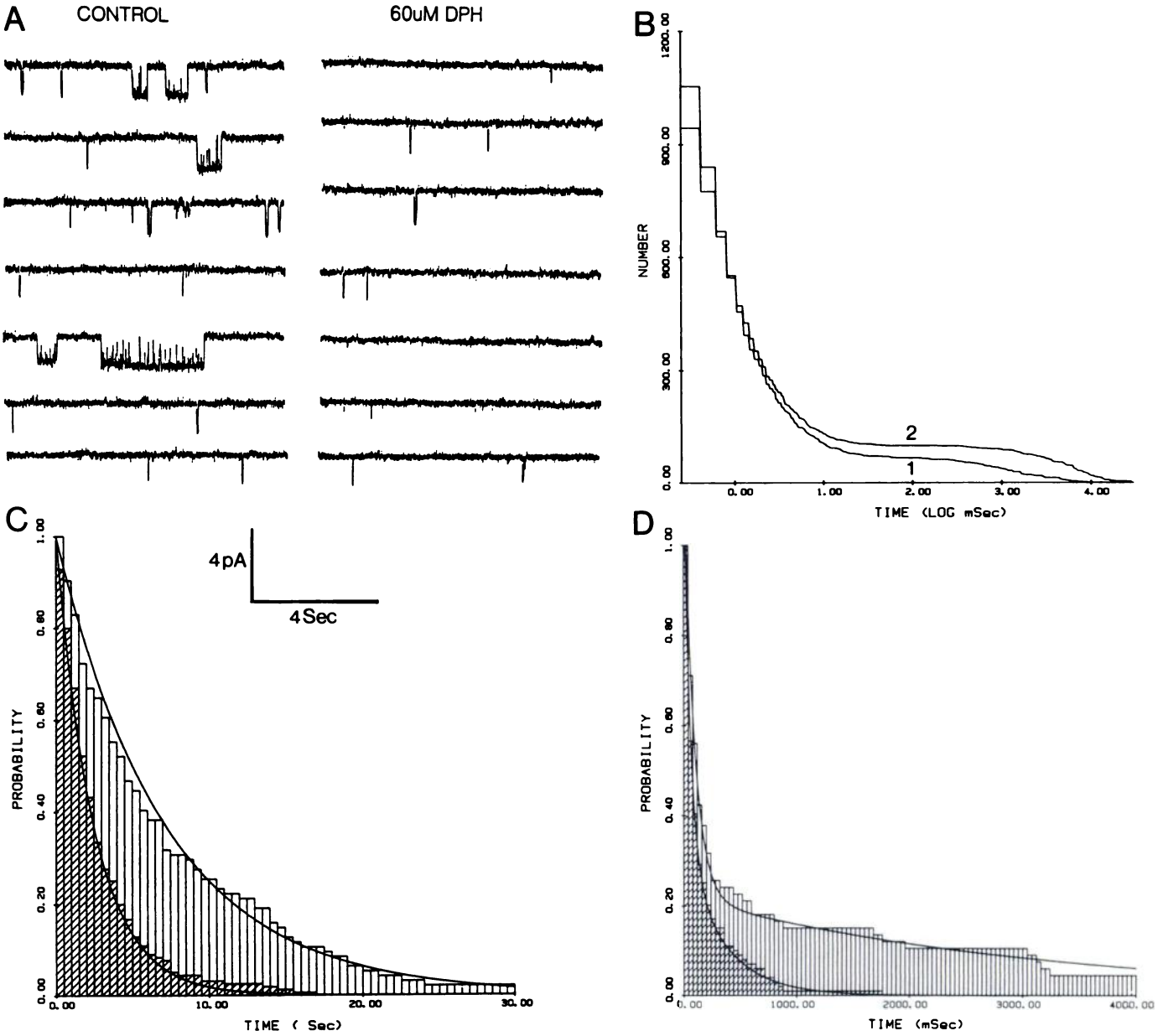


Fig. 3. PT reduces burst duration and increases the intervals between bursts. A, Segments of a continuous recording (*top* to *bottom*) of membrane current during a maintained depolarization (0 mV) are shown before (*left*), and during the application of 60 μM PT (*DPH*) (*right*). B, The histogram of all closed times during a steady state measurement under control conditions (*curve 1*, 1055 events) is compared with that measured in the presence of 60 μM PT (*curve 2*, 944 events). The number of events is plotted on a log time scale from 0.25 to 30,000 msec, using a resolution of 75 bins per decade. The data illustrated are consistent with observations from 20 patches in which the action of PT was examined in the steady state. C, Probability distribution histograms for interburst intervals (closed events greater than 100 msec) in the presence (□, 94 events) and the absence of PT (▨, 144 events). The *solid lines* give the best fit to an exponential distribution for each. The mean interburst interval was 2.77 sec under control conditions and 6.75 Sec in the presence of PT. D, Probability distributions for burst durations during steady state measurements at 0 mV are shown for control conditions (□, 66 events), or during the application of 60 μM PT (▨, 99 events). The best multi-exponential fit to the probability distributions are also given. Control, $P(t) = 0.8 \exp(-0.011\,t) + 0.2 \exp(-0.00032\,t)$; 60 μM PT, $P(t) = 0.67 \exp(-0.018\,t) + 0.33 \exp(-0.0029\,t)$.

nel was very rarely observed and the overall probability of opening was low.

The effects of PT on the duration of the intervals between bursts, as well as burst duration, are also shown in Fig. 3. In Fig. 3C, histograms of the intervals between bursts obtained in the presence and absence of PT are superimposed. Each probability distribution was fitted by a single exponential function in order to determine the mean duration. The mean duration of the intervals increased from 2.77 to 6.75 sec when exposed to PT.

Channels modified by PT therefore had a reduced rate for exit from a long duration, nonconducting state. This state therefore resembles that associated with slow inactivation. The state could prevent channels from reopening during relatively long duration voltage steps, such as those used for the experiment in Fig. 2. No clear evidence was obtained for two kinetically distinct long duration, nonconducting states in the presence of PT, inasmuch as 38 histograms having a sufficient number of bursts from 16 patches could all be adequately fit (see Methods) with only a single exponential.

The burst duration was dramatically reduced by PT. It should be noted that, under control conditions, two populations of bursts were typically observed at all potentials, as shown in Fig. 3D. The two populations could be distinguished by their different mean rates for termination of the burst. The histogram of burst durations during exposure to 60 μM PT indicated that the durations of both populations were reduced.

The decrease in the duration of a burst during exposure to PT is consistent with the more rapid decay of average Na^+ current during a step depolarization (Fig. 2). After a step depolarization, channels are free to open and reopen from closed states of the channel. Reopening is terminated by entry into the state associated with slow inactivation, or due to modification by PT. The apparent increase in the rate of inactivation would also reduce the probability of a channel reopening during a burst measured under steady state conditions.

Concentration dependency of PT action. It is important to determine the range of concentration of PT over which the effects on burst duration and interval between bursts can be observed. Further, the duration of either one or any group of states is inversely related to the sum of the rates of reaction for exit from these states (27). Rates of drug binding reactions are dependent on the concentration of drug. It is therefore of interest to determine whether the durations of bursts or intervals between bursts are graded with the concentration of PT, because this would suggest that the associated reactions are influenced by the rate of association of PT with the channel. Fig. 4 plots burst durations and intervals that were obtained for a number of experiments using different patches. The interval between bursts of Na^+ channels, as well as the duration of bursts, were dependent on the concentration of PT. As the concentration of PT was increased, the average burst duration decreased and the average interval between bursts increased. Most patches contained many channels; however, the intervals between bursts that are reported are those expected for a single channel (see the legend to Fig. 4).

PT reduces the open time of Na^+ channels. Many other compounds that reduce Na^+ current do so by blocking current through an open channel. In this case, either the amplitude of current or lifetime of the open channel is reduced. The possibility that PT could alter the lifetime of open Na^+ channels was therefore tested. An experiment to test the effect of PT on the open time of Na^+ channels is shown in Fig. 5. The open time was measured using step depolarizations. The advantage to this approach is that the temperature could be reduced to 10°. This procedure was undertaken to slow gating reactions so that the influence of the high frequency response of the recording system on the measurement would be reduced. If the temperature was reduced in experiments in which bursts were measured during a maintained depolarization, the interval between bursts increased, limiting the amount of data that could be collected during the time the patch was stable.

Fig. 5A shows individual records obtained under control conditions, as well as during exposure to 20 μM PT. The histograms of open times for the two conditions are superimposed in Fig. 5B to enable a direct comparison. In order to exclude the possibility that the simultaneous opening of more than one channel contributed to the histograms, the holding potential was reduced so that the probability of opening during the depolarization was low. Histograms had multiple components under both conditions. Inasmuch as a burst is due to the activity of a single channel and each burst can contain both classes of open states, each channel must have multiple open states under these conditions (18). The primary effect of PT was to reduce the proportion of open states having a longer mean duration. Overall, PT reduced the mean open time from 19.2 to 8.5 msec. Although inaccurate in quantitative detail, a similar effect on the open time of Na^+ channels measured during spontaneous bursts at depolarized holding potentials. The data therefore indicate that PT can reduce the probability that channels are open for long times.

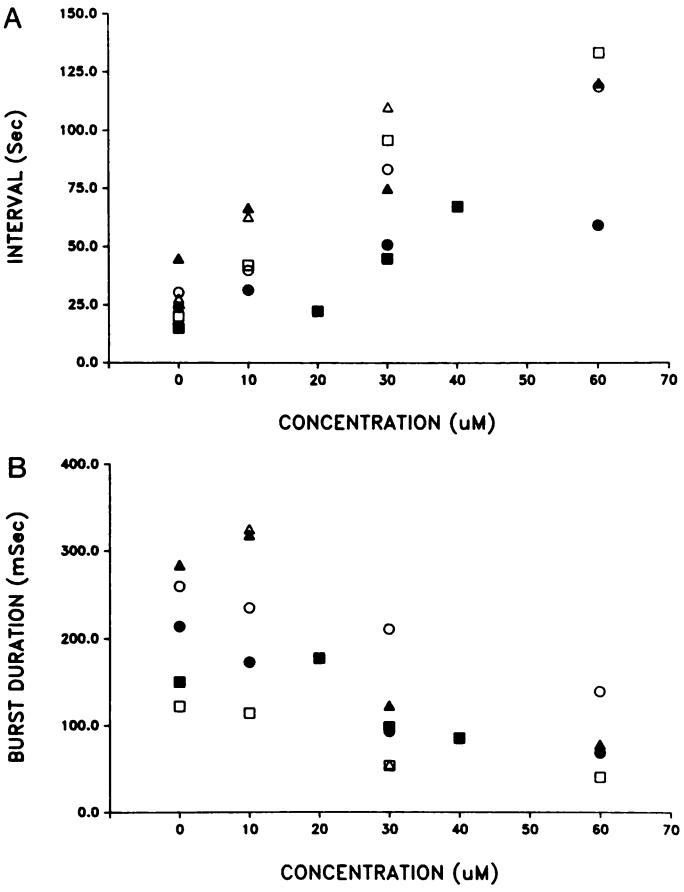


Fig. 4. Concentration dependency of the modification of Na^+ channels by PT. A, The mean interval between bursts was measured in the steady state as a function of the concentration of PT. The intervals have been corrected for a single channel by multiplying the measured interval by the number of channels in the patch. In order to determine the number of channels in a patch, the maximum number of channels open simultaneously during evoked responses to −10 or 0 mV from a holding potential of −120 mV were measured after fast inactivation was removed with papain. Under these conditions, the initial probability of opening of Na^+ channels was high and the lifetime of each open channel was long, so that overlapping currents due to the opening of all the channels were readily observed. Data from experiments on separate patches of membrane are plotted using different symbols. The membrane potential for all experiments was −30 mV. B, The weighted average durations of bursts are plotted. *Symbols* identify the corresponding experiments in A. The burst duration for control conditions in the experiment identified with the *unfilled triangles* is not plotted but was 1069 msec.

Discussion

Models for interactions of PT with the Na^+ channel. The results described in this paper can be used to construct possible models for the mechanism of action of PT-induced

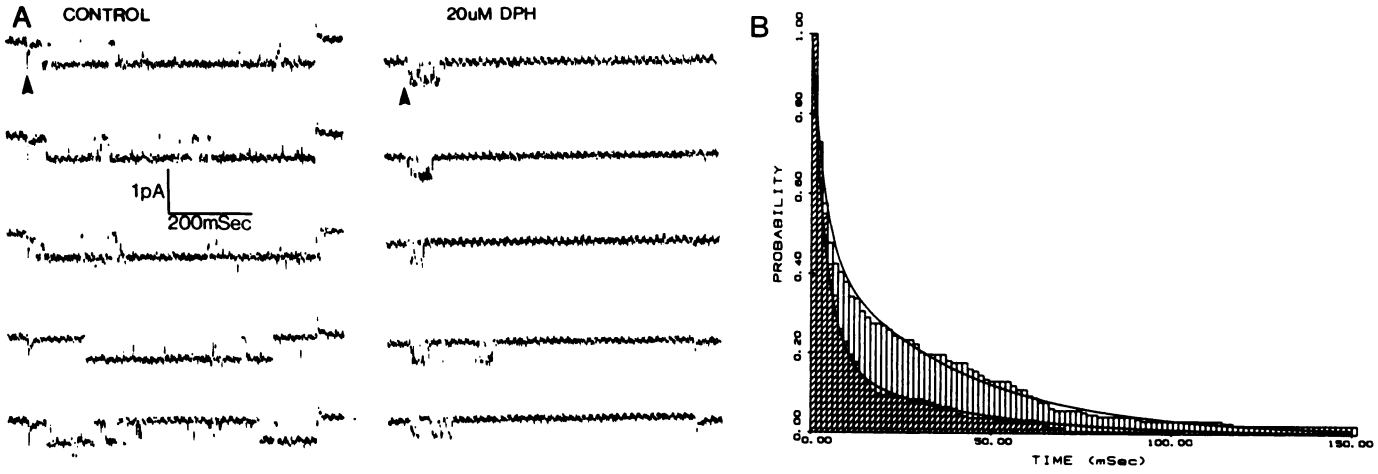
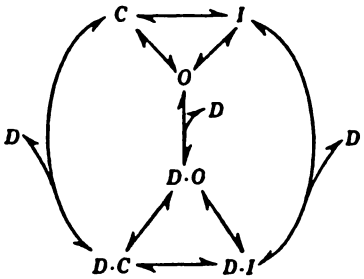


Fig. 5. PT reduces the duration of the open state of Na^+ channels. The open time of Na^+ channels was measured during step depolarizations to −20 mV. A, Examples of individual current records are given under control conditions (*left*) and during the application of 20 μM PT (*DPH*) (*right*). *Arrows* mark the onset of a 700 msec depolarization. B, Superimposed probability distributions of open times measured with (▩, 214 events) or without (□, 193 events) PT. For the control histogram, *solid lines* give the function $P(t) = 0.53 \exp(-0.28\,t) + 0.47 \exp(-0.027\,t)$; for the data obtained with PT, $P(t) = 0.81 \exp(-0.25\,t) + 0.19 \exp(-0.03\,t)$. Temperature, 11.5°. PT has a similar effect on open time in two other patches that were examined using this protocol.

block of the Na^+ channel. One model that would explain the action of PT has been given by scheme 3 of Willow *et al.* (10; see also Ref. 28), which is reproduced here. *C*, *O*, and *I* represent the closed, open, and inactivated states of the channel, respectively. *D* represents the drug. In this general case, PT can bind to any state of the channel. One modification of this scheme necessary to discuss the data presented here is that the inactivated state is associated with slow, rather than fast, inactivation. Of course, because fast inactivation was removed in the present study, additional interactions with the fast activated state are not excluded.



Evidence in the present study supports a number of the proposed interactions of states of the Na^+ channel with PT. First, PT probably interacts with the slow inactivated state to produce a second, drug-bound, "inactivated" state. The best evidence for the existence for this pathway is that the interburst interval increases with the concentration of PT. If PT could only modify a channel by binding to open or closed states, the interval would be set by the rate of dissociation of the drug and would be independent of concentration. The finding that only one component was observed for the intervals between bursts also supports the observation that a pathway exists between the normal and drug-bound inactivated states.

Second, PT likely binds to open or closed states. The graded reduction in burst duration with concentration is consistent with this idea. Because a burst is due to reopening of the channel, the burst could be shortened by binding of PT to either closed (along the pathway for activation of the channel) or open states. Either interaction would increase the probability that the channel enters the drug-bound inactivated state.

The reduction of mean open time by PT also implies an interaction with open or closed states. A decrease in the open time could be caused by block of the open channel. A reduction in the lifetime of the open state can occur when a channel blocker binds to this state, resulting in a channel that is gated open but nonconducting (27, 29). PT may therefore block a channel during the time it is gated open. For many open channel blockers, this drug-bound state is not able to undergo closure, leading to a burst with increased duration (27). If the action of PT is to block the open channel, it does not increase the duration of bursts, indicating that this drug-bound state can undergo slow inactivation. However, each channel has multiple open states (18) and the probability of a long duration open state was reduced rather than the actual duration. Therefore, an alternative idea more consistent with the data is that PT binds to a closed state and reduces the probability of entry into a subsequent open state of long duration.

Although the single channel data fit the scheme proposed by Willow *et al.* (10), the data could also be explained by a second type of model. PT could modify any channel in the steady state so that all gating reactions are altered. There is some precedent for this second type of action, because batrachotoxin causes a persistent activation of Na^+ channels by a relatively irreversible alteration in the rates of activation and inactivation (30). In this case, the drug-bound open state would be conducting, and the dissociation reactions would be very slow. The graded reduction in the burst duration and increase in the interval between bursts as a function of concentration would then be due to an increased probability of observing channels with altered characteristics. Because Na^+ channels exhibited two populations of bursts in the absence of PT, it is difficult to determine whether two populations of normal and modified channels coexisted in one patch. Distinct populations of modified and unmodified channels would likely exhibit different intervals between bursts. However, this prediction was incon-

sistent with the data inasmuch as only a single population of intervals between bursts existed during exposure to PT.

The single channel data help to explain the previous voltage-clamp data (9, 10). The existence of a second slow inactivated state in the presence of PT readily explains the shift of the membrane potential for half steady state slow inactivation to a more negative value. Single channels appeared to be inactivated a greater portion of time in the presence of PT at any voltage, explaining the shift. The increase in the interburst interval correlates with the finding that recovery from inactivation is slowed by PT. Block of Na^+ current by PT is frequency dependent and is enhanced as the duration and frequency of conditioning depolarizations is increased. The faster onset in the apparent rate of inactivation, as well as the slower recovery, would contribute to this frequency-dependent block.

Possible complications for Na^+ channels having fast inactivation. The present report deals exclusively with the interactions of PT with the Na^+ channel that has been modified to eliminate the process of fast inactivation. The rational for this approach was given in the Introduction. However, the question remains whether the interactions described here could be altered in intact Na^+ channels.

The binding site for PT is not likely altered by treatment with papain. Three points argue against this possibility. First, identical actions of PT were observed after removing fast inactivation using other compounds, including trypsin or *N*-bromoacetamide. A study of the modulation of the block of analogs of local anesthetics by the fast inactivation gate has been justified in a similar manner (31). Second, the use of these different compounds does not alter the efficacy of PT. The concentration of PT that produces the effects reported in this paper is within the range found to produce effects on the Na^+ channel having fast inactivation (9, 10). Third, previous evidence indicates that PT acts in its neutral form (32) and appears sensitive to the electric field of the membrane (9). The drug may thus partition in the membrane to exert its action. Evidence indicates that the enzyme used here to eliminate fast inactivation does so through an action at the internal surface of the membrane (see the discussion in Ref. 33). The observation that PT can exert its action by interaction with the slow inactivation process within the membrane and the observation that slow inactivation is not removed by application of enzymes acting at the external surface of the membrane are well correlated.

Interaction of the drug with the open channel is probably not extensive in channels having a reduced open time due to fast inactivation. Removal of fast inactivation causes the open time to increase by a factor of 2 to 10, depending on the membrane potential (34). Only the component of open times with a longer duration was selectively reduced by PT. The observation that PT does not cause a reduction in the component of brief open times is also consistent with the observation that PT did not appear to have a component of open-channel block in voltage-clamp experiments in which fast inactivation was not removed (9).

Therapeutic action of PT may relate to modified slow inactivation. The use of PT in the management of seizure activity relates to its unique ability to produce its block only under conditions of prolonged depolarization, or during a high frequency discharge of action potentials. For example, McClean and MacDonald (3) found that the rate of rise of the action potential was reduced by PT in proportion to the amount the resting potential was depolarized. Further, a reduction in the frequency of action potentials occurred over a time course of tens of milliseconds during a constant depolarization (37°) under control conditions. The onset in spike frequency adaptation was accelerated by PT. This action of PT was enhanced as the membrane was further depolarized. PT also slowed the recovery of the rate of rise of the action potential following a conditioning train from control values of tens of milliseconds to hundreds of milliseconds. The study by Adler *et al.* (4) confirmed that block of driven action potentials by PT occurred earlier after the start of a train, as the frequency was increased.

The effects of PT described in this study help to clarify the basis for this specificity. As noted above, PT reduces the duration of the open state only when the lifetime is long, or extensive reopening of the channel occurs. Evidence suggested that the drug-bound channel could close. Further, PT was found not to reduce the conductance of the open state. Thus, little effect should be observed on activation of the Na^+ channel. The rate of rise of an action potential should therefore not be reduced in the absence of resting inactivation.

As the membrane potential is reduced, single Na^+ channels are more likely in a drug-bound inactivated state. The second slow inactivated state was produced with a concentration of PT equivalent to the therapeutic levels required for the production of its its anticonvulsant activity. This effect would be predicted to reduce the rate of rise of action potentials as the resting potential is depolarized, because less are available to open during an action potential.

During a prolonged depolarization, the apparent rate of onset of inactivation of any Na^+ channel is increased in the presence of PT. During maintained depolarization under current-clamp conditions, Na^+ channels would accumulate in inactivated states sooner, causing an exaggerated adaptation of the spike frequency. The action of PT resembles the intrinsic slow inactivation process. This similarity suggests that the slow inactivation process of Na^+ channels could normally promote spike frequency adaptation.

Because single Na^+ channels recover more slowly from inactivation in the presence of PT, recovery of the rate of rise of the action potential following a conditioning train would be predicted to be slowed. This observation is also consistent with the slowing of recovery from inactivation with repolarization during voltage-clamp experiments (9).

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