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Voltage Clamp Analysis of the Inhibitory Actions of Diphenylhydantoin and Carbamazepine on Voltage-Sensitive Sodium Channels in Neuroblastoma Cells

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SUMMARY

The actions of diphenylhydantoin (DPH) and carbamazepine (CBZ) on sodium channels in mouse neuroblastoma cells (clone N18) were analyzed using the patch voltage clamp procedure in the whole cell configuration. DPH and CBZ reduced sodium currents without effect on the voltage dependence of sodium channel activation. Half-maximal inhibition was observed with approximately 30 μM of each drug. Depolarization increased and hyperpolarization reversed channel block by these two drugs in the voltage range from −90 to −45 mV. Repetitive stimulation at 2 Hz or greater enhanced inhibition of sodium channels. The half-time for recovery from voltage-dependent inhibition was greater for DPH (1.36 sec) than for CBZ (0.38 sec). A combination of prolonged depolarizing pulses of 15 mV with superimposed brief maximal depolarizations designed to mimic the electrical activity in an epileptic focus gave additive effects of voltage-dependent and frequency-dependent inhibition. The results support the previous proposal that DPH and CBZ are sodium channel-selective anticonvulsants and provide a potential basis for specific inhibition of neurons in epileptic foci. The mechanism of DPH and CBZ action is considered in terms of an allosteric or modulated receptor model of drug binding and action.

INTRODUCTION

The anticonvulsant agents DPH[1] and CBZ are both extensively employed in the management of grand mal and partial seizures. These agents inhibit sodium channels in nonmammalian nerve and skeletal muscle fibers (1–8). Neurochemical studies have shown that they block the binding of batrachotoxin and other sodium channel activators to their receptor site on sodium channels in rat brain synaptosomes and mouse neuroblastoma cells and prevent the persistent activation of sodium channels by these agents (9–11). The concentrations required to block sodium channels in rat brain are closely correlated with the brain levels of these drugs achieved during effective prevention of experimental seizures in rats, leading to the hypothesis that DPH and CBZ are sodium channel-selective anticonvulsants that modify sodium channel function as their principal mechanism of action (10, 11). Electrophysiological examination of the actions of these drugs in cultured mammalian spinal cord neurons and neuroblastoma cells has provided additional support for this hypothesis (12–14).

At therapeutic doses, the anticonvulsants have a specific action to prevent seizures without causing sedation or diminishing normal electrical activity in the brain. General inhibition of sodium channels in central neurons would be expected to cause nonspecific sedation. Therefore, the hypothesis that a subclass of anticonvulsant drugs may act primarily by inhibition of sodium channels requires consideration of possible mechanisms whereby their action might be selective for neurons responsible for the generation of seizure discharges. Neurons in experimental epileptic foci are subject to prolonged depolarizations which trigger trains of action potentials (15–17). These events are called paroxysmal depolarization shifts. These unusual membrane potential transients provide a possible basis for a specific action of anticonvulsant drugs on neurons involved in epileptogenesis.

In peripheral nerve, the action of local anesthetics and related compounds is dependent on membrane potential and frequency and duration of sodium channel activation (18–22). The affinity of the drug receptor is modulated by membrane potential and sodium channel state such that the drugs bind most rapidly to the open state of the channel and have highest affinity for the inactivated

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[1] The abbreviations used are: DPH, diphenylhydantoin; CBZ, carbamazepine; GABA, γ-aminobutyric acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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state. These properties result in increased inhibition of sodium channels upon depolarization and/or repetitive stimulation of the nerve fibers. In the present study, we have employed the patch clamp recording technique in the whole cell configuration (23) to examine the time- and voltage-dependent actions of DPH and CBZ on sodium currents in cultured mouse neuroblastoma cells. Particular attention has been given to comparison of the concentration dependence of these drug actions with therapeutic levels of drug *in vivo* and to determination of the voltage- and frequency-dependent actions of these agents which may confer selective block of sodium channels in the repetitively firing, depolarized neurons that are considered to play an important role in epileptogenesis.

EXPERIMENTAL PROCEDURES

Cell culture. N18 neuroblastoma cells were cultured in 100-mm-diameter Petri dishes for 6 to 8 days until confluence was reached. Details of the culture techniques have been described previously (24). Culture medium consisted of 5% newborn calf serum, 95% Dulbecco's modified Eagle's minimum essential medium. Three to 8 hr prior to recordings, cells were harvested by mechanical trituration in Ca^{2+}-Mg^{2+}-free phosphate-buffered saline and reseeded in 35-mm-diameter plastic dishes containing 1 ml of the culture medium at a cell density of 1×10^5 cells/ml. Reseeding enabled us to record from isolated spherical cells as required for voltage clamp measurements. Typically, cells 20–30 μm in diameter were used for recording.

Voltage clamp recording. For recordings, the culture medium was replaced with 2–3 ml of recording solution (150 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 5 mM Na^+-Hepes, pH 7.4), and the plastic dish was mounted on a stage of an inverted phase contrast microscope. All recordings were performed at room temperature (20 ± 1°).

The voltage clamp was based on the one-pipet, gigaseal whole-cell recording technique of Hamill *et al.* (23). Glass hematocrit microtubes were pulled in two steps, and tips of the pipets were fire-polished under a light stream of air. The measured resistances of pipet tips following fire-polishing were 0.2–1.0 megaohms in recording solution. Gentle suction applied after the pipet was in contact with the cell surface sufficed to form a seal and rupture the patch of membrane in the orifice. After 10–15 min, the total input resistance of pipet and cell rose to 10^8–10^{10} ohms. The internal pipet medium contained 160 mM CsF and 10 mM NaF. This solution blocked potassium currents and allowed stable sodium current recordings for up to 3 hr. The voltage clamp circuit used a single bath electrode and the patch pipet and included a circuit for compensation of up to 1-megohm series resistance in the current-passing pathway. Series resistance was compensated and leak currents were subtracted electronically in all experiments. Other details of recording arrangements were described previously (25). In most experiments, cells were maintained at a holding potential of −75 mV. For measurement of voltage dependence of sodium current activation, the membrane was hyperpolarized in a prepulse to −105 mV for 90 msec to remove inactivation of sodium channels and then depolarized to test potentials from −60 to +90 mV for 10 msec. For measurement of the voltage dependence of sodium channel inactivation, the prepulse potential was varied from −120 to −15 mV for 90 msec followed by a test pulse to +7.5 or +15 mV for 10 msec. For experiments examining the voltage or frequency dependence of drug action on peak sodium currents, the stimulus conditions are described in the figure legends.

Drug treatment. All drugs were added to the Petri dish by perfusion. At least 15 min were allowed for equilibration prior to recording. All control measurements were performed in the same cell prior to the addition of drugs. DPH and phenobarbital were purchased from Sigma, diazepam was kindly supplied from Hoffmann-LaRoche (Nutley, NJ), and CBZ was a gift from Dr. A. Camerman (University of Washington, Seattle). Stock solutions of DPH, CBZ, and diazepam were prepared by dissolving the compounds in ethanol followed dilution into aqueous medium with gentle warming. Aliquots of these solutions were added to the mammalian Ringer's solution. Control experiments showed that the final concentration of ethanol (0.1–1.0%) did not affect sodium currents. The sodium salt of phenobarbital was added directly to the recording solution.

RESULTS

Effect of anticonvulsants on the inward sodium current. Stimulation of neuroblastoma cells with successive depolarizing pulses (−60 to +90 mV) evokes a family of inward currents which are illustrated as a series of superimposed oscilloscope traces in Fig. 1. Typically, maximum inward current (3–5 namp) is evoked in response to test pulses between 0 and +15 mV. These inward currents are reversibly inhibited by 1 μM tetrodotoxin. Their rapid time course and sensitivity to the toxin indicate that these currents are carried by sodium ions moving through voltage-sensitive sodium channels.

DPH (40 μM) reduces peak inward current to 42% of its control value without altering the time course of the remaining current (Fig. 1). CBZ has similar effects (data not shown). The peak sodium currents elicited by depolarizing test pulses to various potentials in experiments like the one illustrated in Fig. 1 are plotted as a function of pulse voltage in Figs. 2A and 3A for DPH and CBZ, respectively. With both drugs, the sodium current is proportionately reduced at each membrane potential tested and the reversal potential of the sodium current is unaffected. The same data are presented in normalized plots of peak sodium conductance versus pulse voltage in Figs. 2B and 3B. These data show that the voltage dependence of sodium channel activation is unaffected by DPH and CBZ under conditions where a substantial fraction of the sodium current is blocked.

The concentration dependence of the reduction of peak sodium current by DPH and CBZ is illustrated in Fig. 4. Half-maximal inhibition is observed at approximately 30 μM of each drug and a clear 20% inhibition is observed at 3 μM. These values are in excellent agreement with those derived from neurotoxin binding and ion flux measurements on synaptosomes and neuroblastoma cells (9–11).

Previous studies have shown that diazepam and phenobarbital are capable of inhibiting voltage-sensitive sodium channels in both neuroblastoma cells and rat brain synaptosomes (10, 11). However, the concentrations required to block channels are substantially in excess of those found in the brains of laboratory animals protected against experimentally induced seizures (10). At concentrations between 1 and 2 μM, diazepam does not affect sodium currents in cells maintained at a holding potential of −75 mV (data not shown). At 200 μM, a concentration well above the therapeutic range, phenobarbital produces inhibition of sodium currents in cells maintained at a holding potential of −75 mV (data not shown). Valproic acid and ethosuximide have no effect on sodium channels at concentrations as high as 1 mM (10, 11) and were not examined in this study. Taken together with earlier work, these results confirm our

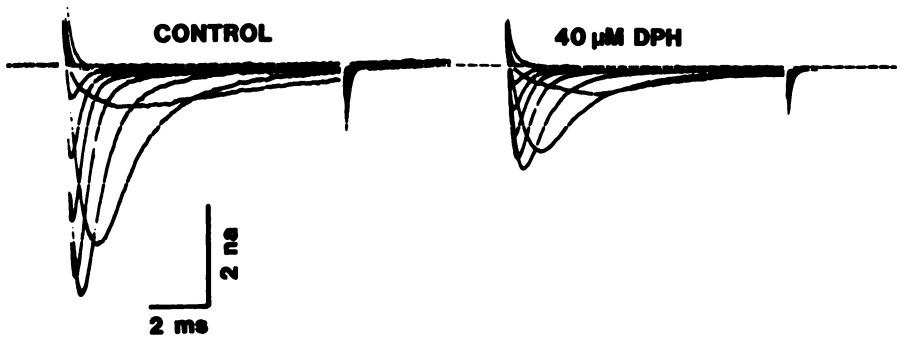


FIG. 1. *Effect of DPH on sodium currents of N18 neuroblastoma cells under voltage clamp*

Cell cultures were prepared and whole cell patch recordings were made as described under Experimental Procedures. Cells were maintained at a holding potential of −75 mV, hyperpolarized to −105 mV for 90 msec, and depolarized to potentials from −60 to +90 mV in 15-mV intervals to evoke the sodium currents at a frequency of 1 Hz. Data from one cell are presented and are representative of six cells studied under identical conditions. Left, family of currents in the absence of added drugs. Calibration: 2 msec, 2 namp. Right, family of currents in the presence of 40 μM DPH in the same cell. Calibration as in the control.

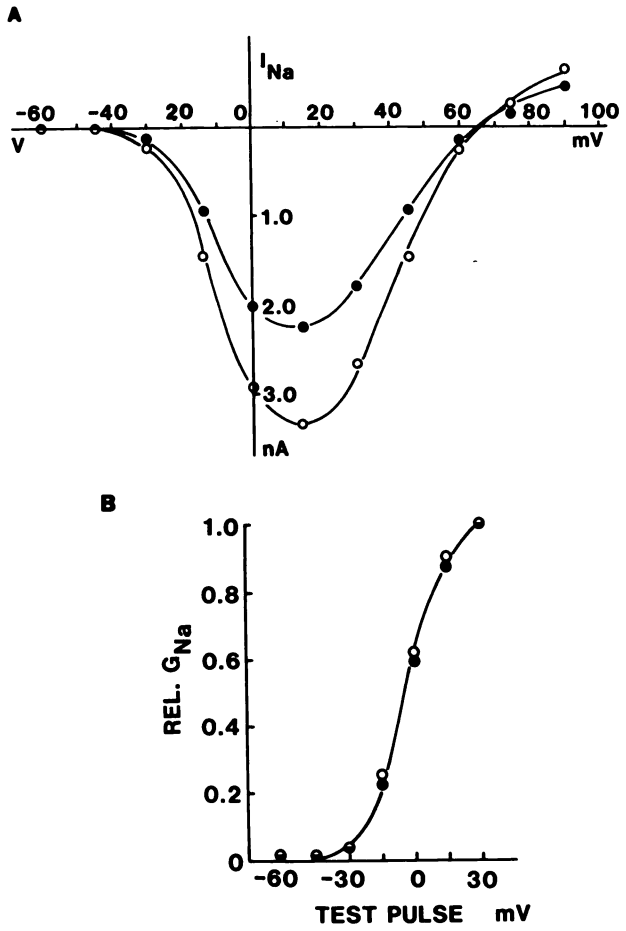


FIG. 2. *Effect of DPH on the voltage-dependent activation of sodium currents in N18 cells*

Data from one cell are presented and are representative of six cells studied under identical conditions. Standard errors of the means of normalized currents were less than 10%. A, current-voltage relationship of peak sodium current activation in the absence (O) and presence (●) of DPH (10 μM) was determined as in Fig. 1. B, normalized conductance-voltage relationship in the absence (O) and presence (●) of DPH (10 μM) in the same cell used in A.

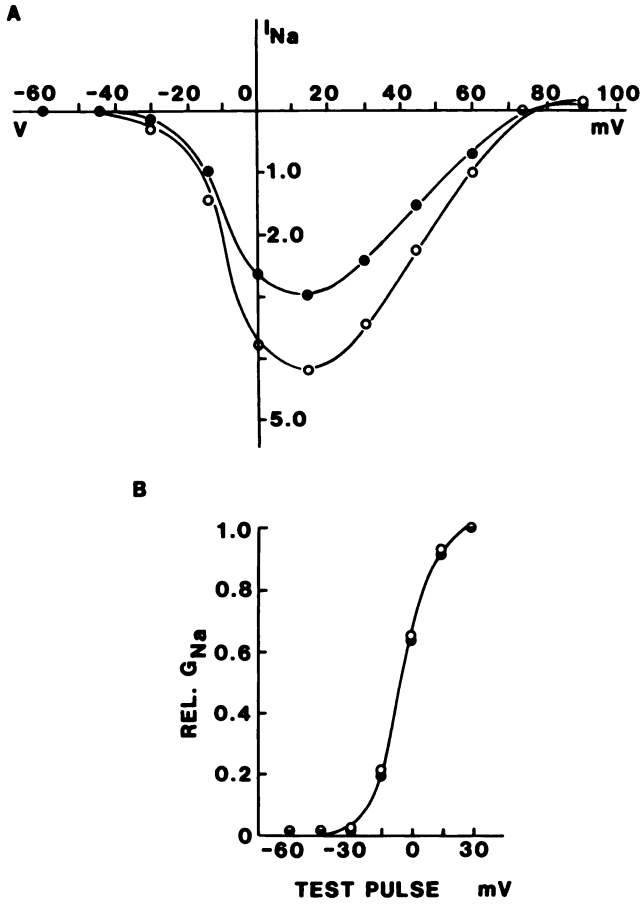


FIG. 3. *Effect of CBZ on the voltage-dependent activation of sodium currents in N18 cells*

Data from one cell are presented and are representative of six cells studied under identical conditions. Standard errors of the means of normalized currents were less than 10%. A, current-voltage relationship of sodium current activation in the absence (O) and presence (●) of CBZ (20 μM) was determined as in Fig. 1. B, normalized conductance-voltage relationship in the absence (O) and presence (●) of CBZ (20 μM) in the same cell used in A.

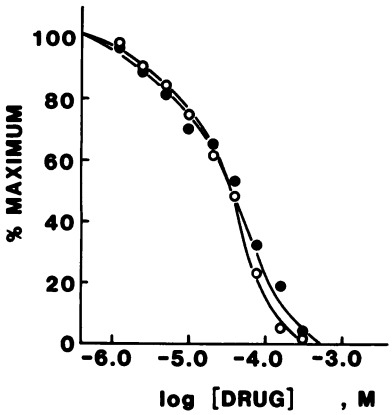


FIG. 4. *Inhibition of maximum inward sodium currents by DPH and CBZ*

The effect of DPH (○) and CBZ (●) on maximum currents in response to +15-mV test pulses was studied according to the protocol outlined in Fig. 1 in the presence of the indicated concentrations of DPH or CBZ. Each point represents the mean of normalized data from four to six separate cells. Standard errors of the means of the normalized data were less than 10%.

conclusion that only DPH and CBZ of the several major classes of anticonvulsants have significant inhibitory actions on sodium channels at therapeutic concentrations.

Effects of DPH and CBZ on steady state inactivation. A significant fraction of sodium channels is inactivated at the resting membrane potential of most neurons. Therefore, modulation of the level of steady state inactivation can have important effects on cellular excitability. Steady state sodium channel inactivation was measured by varying the potential of a 90-msec prepulse from −120 to −15 mV prior to a 10-msec test pulse to +7.5 mV to activate sodium channels maximally. Initial observations showed that, in this cell type, there is primarily a single rapid time constant for inactivation (25). An additional slower component of inactivation was prominent in studies of N1E-115 neuroblastoma cells treated with dimethyl sulfoxide (14). Steady state inactivation was described by the midpoint E_h and slope K_h of the inactivation curve obtained from the following equation (26):

$$h_\infty = \frac{1}{\left[1 - \exp\frac{(E - E_h)}{K_h}\right]}$$

Both DPH and CBZ produced hyperpolarizing shifts in the steady state inactivation curve (Fig. 5, A and B, respectively). In the absence of anticonvulsants, the membrane potential at which half of the sodium channels is inactivated (E_h) is −67.0 ± 3.2 mV. DPH (40 μM) and CBZ (20 μM) shifted E_h to −85 and −88 mV, respectively, without a marked change in the shape of the inactivation curve (Fig. 5). The concentration dependence of these effects is illustrated in Fig. 6 for two different cells. The threshold concentration for DPH-induced shifts in the inactivation curve was approximately 5–10 μM. At these concentrations, maximum inward sodium currents were reduced by approximately 20–25% (Fig. 4). Similarly,

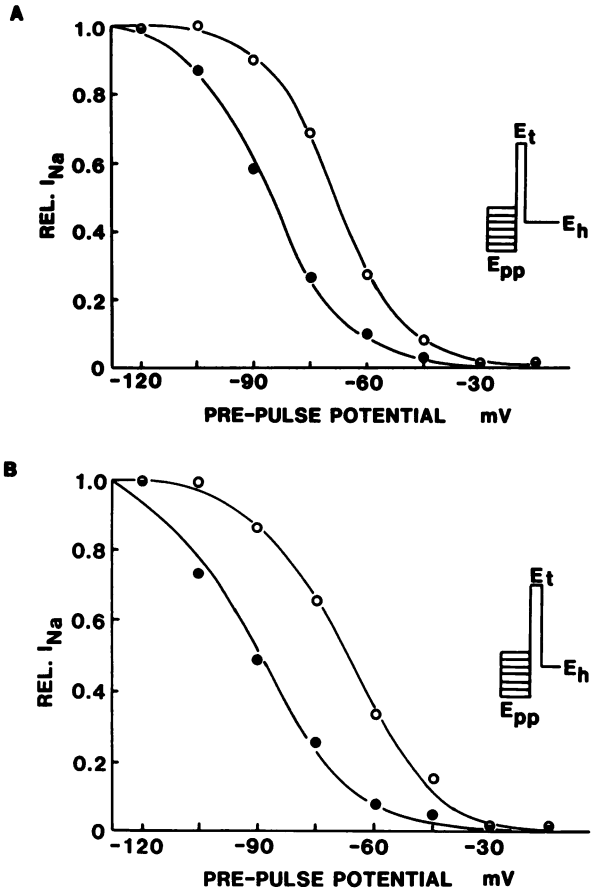


FIG. 5. *Effect of DPH and CBZ on steady state inactivation of inward sodium currents in N18 cells*

Availability of channels was modulated by varying the prepulse (E_{pp}, 90 msec) between −120 and −15 mV prior to a 10-msec test pulse (E_t) to +7.5 mV. Cells were maintained at a membrane potential of −75 mV (E_h) between stimuli. Data from a single cell are presented which are representative of six cells studied under identical conditions with each drug. A, steady state inactivation relationship before (○) and after (●) administration of 40 μM DPH. B, steady state inactivation relationship before (○) and after (●) administration of 20 μM CBZ.

CBZ shifted the inactivation curve by 5–8 mV at concentrations (5–10 μM) which produced approximately 20% inhibition of maximum inward sodium currents (compare Figs. 4 and 6). Half-maximal shifts in E_h were observed at 18.5 and 14.5 μM, respectively, for DPH and CBZ. At equimolar concentrations, CBZ produced somewhat larger shifts of E_h than did DPH (15 vs. 18 mV; see Fig. 6). Thus, the reduction of peak inward sodium current by these anticonvulsant drugs is accompanied by a substantial shift in steady state inactivation of sodium channels to more negative membrane potentials.

Voltage-dependent inhibition of inward sodium currents by DPH and CBZ. Neurons in experimental epileptic foci generate prolonged depolarizations and rapid trains of action potentials (15–17). These characteristics distinguish them from normal neurons and may provide a mechanism of selective action of antiepileptic drugs on sodium channels in epileptogenic neurons. In order to test whether the electrical activity characteristic of epileptic foci might alter the action of DPH and CBZ, we have examined the effects of holding potential and fre-

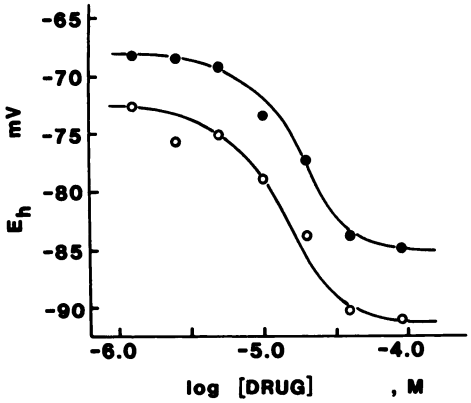


FIG. 6. *Dose-response relationships for DPH and CBZ on producing hyperpolarizing shifts in the steady state inactivation relationship in N18 cells*

The voltage dependence of steady state inactivation was measured as described in Fig. 5. The effect of the indicated concentrations of DPH (●) and CBZ (○) on the voltage required for half-maximal inactivation of maximum inward current (E_h) are presented. Control value for E_h was −67.0 ± 3.2 mV. See text for further details. The results are from a single cell and are representative of six cells studied with each drug.

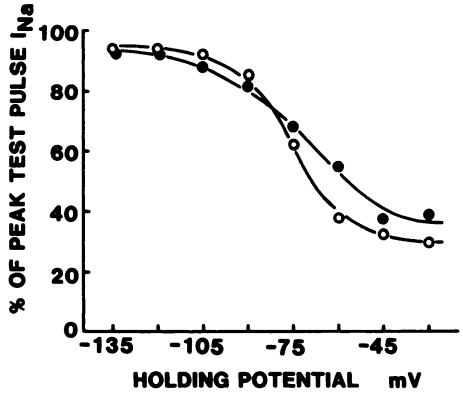


FIG. 7. *Voltage-dependent inhibition of inward sodium currents by DPH and CBZ*

Families of inward sodium currents were generated in response to test pulses (10 msec) from a range of holding potentials (−135 to −30 mV) as described in Fig. 1. A 90-msec prepulse at −105 mV preceded each test pulse. A period of 1 min was allowed between changes in holding potential. The effect of DPH (●) and CBZ (○), both at 20 μM, on maximum inward currents (measured at +15 mV) at different holding potentials is shown. Each curve presents the mean of normalized values from six cells. Standard errors of the means of normalized data were less than 10%.

quency of action potential generation on the action of these drugs. When N18 cells are maintained at a holding potential of −75 mV, peak sodium currents are inhibited by about 35–40% in the presence of 20 μM DPH or CBZ (Fig. 4). In fact, the extent of block of sodium currents is highly voltage-dependent. In Fig. 7, amplitudes of peak sodium currents in the presence of DPH (20 μM) or CBZ (20 μM) are shown as a percentage of sodium currents in the absence of these agents at holding potentials over a range of −135 to −30 mV. As the holding potential is made more negative, inhibition by both drugs is progressively reversed. The sodium current is restored to 95% of the control value in the absence of drug by hyperpolarization to −120 mV and is reduced to 35–40% of the control value by depolarization to −45 mV. The action of CBZ is more sharply voltage-dependent than that of DPH (Fig. 7). Thus, the inhibition of sodium currents by DPH and CBZ is strikingly modulated by membrane potential. This mechanism may contribute to selectivity of drug action.

Frequency-dependent block of inward sodium current by DPH and CBZ. Previous studies have indicated that DPH produces a slight frequency- or use-dependent inhibition of sodium currents in axons and in N1E-115 neuroblastoma cells (7, 14). In the absence of added drug, a repetitive volley of depolarizing test pulses (0 mV, 40 msec) produces only a very small alteration (2–3%) of current in N18 cells after 20 pulses delivered at 2 Hz (Fig. 8). CBZ (80 μM) produces a modest frequency-dependent inhibition (8–10% decrease in current at the end of 20 test pulses; Fig. 8) at 2 Hz. The frequency-dependent actions of DPH (40 μM) are more pronounced, with 18–20% reduction in current following 20 test pulses (Fig. 8). These frequency-dependent actions of DPH and CBZ are dependent on a number of variables including the duration of the test pulse, the frequency of stimulation, and the holding potential. For both drugs, frequency

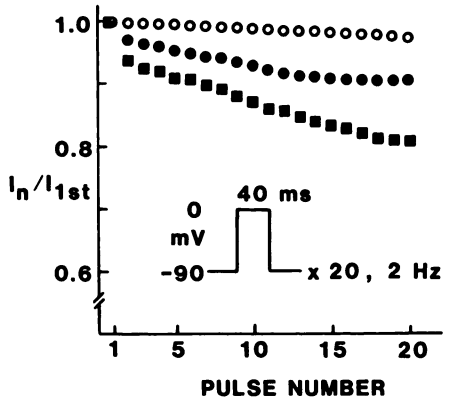


FIG. 8. *Frequency-dependent inhibition of inward sodium currents by CBZ and DPH*

Voltage clamp test pulses from −90 to 0 mV of 40-msec duration were applied repetitively at 2 Hz in trains of 20 pulses. Peak sodium current at each pulse number is normalized to the current evoked by the first pulse. The decline in current is measured before (○) and after (●) 80 μM CBZ or 40 μM DPH (■). The results from an individual cell are presented and are representative of four cells studied at 80 μM CBZ and five cells studied at 40 μM DPH.

dependence can be abolished by shortening the test pulse, decreasing the rate of stimulation below 1 Hz, or changing the holding potential to values more negative than −90 mV.

An alternative method for measuring frequency dependence is to stimulate the cell with paired hyperpolarizing and depolarizing pulses. For local anesthetic and antiarrhythmic drugs, this procedure leads to a reversal of drug-induced block of sodium channels by reversal of inactivation of the drug-modified channels during the strong hyperpolarization, followed by activation of the modified channels during depolarization, allowing rapid drug dissociation (19, 21). Switching off the hyperpolarizing prepulse while maintaining the depolarizing pulse

leads to increased block of sodium current that is dependent upon the voltage and frequency of depolarization. Return to the hyperpolarizing prepulse reverses the frequency-dependent block. Fig. 9 illustrates examination of DPH and CBZ inhibition of sodium channels in N18 cells using this procedure. In the control condition (○), removal of the hyperpolarizing prepulse results in an immediate reduction in current to about 70% of maximal current which reaches steady state with the first pulse (Fig. 9). This is due to increased sodium channel inactivation without the prepulse. When the prepulse is switched on again, the current returns to maximal levels with the first test pulse. No cumulative effects are observed during repetitive stimulation. In the presence of DPH (40 μM), about 10 test pulses are required to reduce sodium current to a new steady state level when the prepulse is removed (Fig. 9, ●). Similarly, about seven test pulses are required for full recovery of maximum current in the presence of the drug when the prepulse is reinstated (Fig. 9, ●). A similar pattern is observed for CBZ (80 μM), although fewer pulses are required for steady state reduction and recovery of the sodium current upon respective removal and introduction of the prepulse (Fig. 9, □). In each case, DPH and CBZ cause a greater increase in block of sodium currents at the steady state due to the sum of voltage- and frequency-dependent modes of inhibition. These results support the conclusion drawn from the data of Fig. 8 that inhibition of sodium channels by DPH and CBZ is indeed frequency-dependent.

The block of sodium channels by CBZ reaches steady state in a smaller number of pulses than that of DPH (Fig. 9). This difference in rate of drug action may be due to more rapid dissociation of the bound drug from drug-modified, inactivated sodium channels. This possibility can be examined more directly in experiments in which the membrane potential is held at −45 mV for 3 sec to inactivate sodium channels and produce maximum drug inhibition, and then stepped to −105 mV. Subsequent test pulses (0 mV, 5 msec) at 50 msec and each second thereafter reveal that recovery from block in the presence of 40 μM DPH requires three or four pulses while the recovery in the presence of CBZ (80 μM) is complete on the second pulse. An estimate of drug dissociation rates can be made by normalizing these results. This is done by assigning the extent of current block prior to the first pulse as 100% and that when maximum currents reappear as 0%. A simple monoexponential relationship is observed for DPH (Fig. 10). From this function, estimates for the half-time of drug dissociation from inactivated channels are 1.36 and 0.38 sec for DPH and CBZ, respectively. Evidently, the more rapid dissociation of CBZ from the drug-modified inactivated state of the sodium channel accounts for its more rapid approach to steady state inhibition during repetitive stimulation.

Effect of DPH and CBZ on sodium currents evoked by stimuli in the form of paroxysmal depolarization shifts. The results presented so far show that the inhibition of sodium channels by DPH and CBZ is modulated by both the membrane potential and by the frequency of impulse generation. During a paroxysmal depolarization shift in neurons of an epileptic focus, high frequency action potentials are superimposed upon a prolonged depolarization. Therefore, we have examined the effects of combining these two forms of stimulation on DPH and CBZ action. When neuroblastoma cells are stimulated in the absence of drugs with a series of short-spaced (13 msec) test pulses (0 mV, 2 msec) superimposed upon a 15-mV

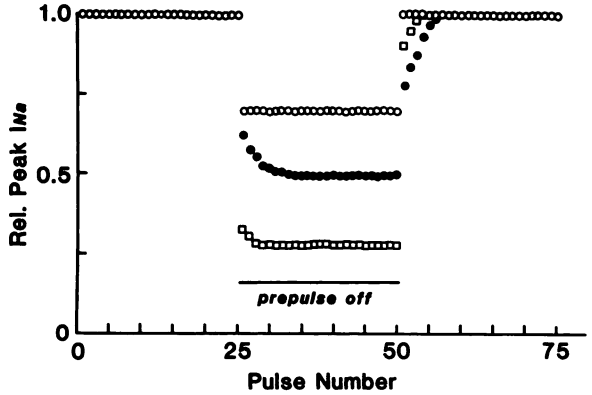


FIG. 9. *Onset and recovery of frequency-dependent inhibition of sodium channels by DPH and CBZ*

Neuroblastoma cells were incubated for 15 min in control medium (○), or in the presence of 40 μM DPH (●) or 80 μM CBZ (□). A train of 25 stimuli at 2 Hz consisting of a prepulse to −105 mV for 90 msec followed by a test pulse to 0 mV for 5 msec was applied and the resulting peak sodium currents were recorded and assigned a value of 1.0. Then 25 stimuli without the prepulse were applied followed by an additional 25 stimuli with the prepulse. Normalized sodium currents under each condition are presented. The results from an individual cell are presented and are representative of four cells studied at 80 μM CBZ and five cells studied at 40 μM DPH.

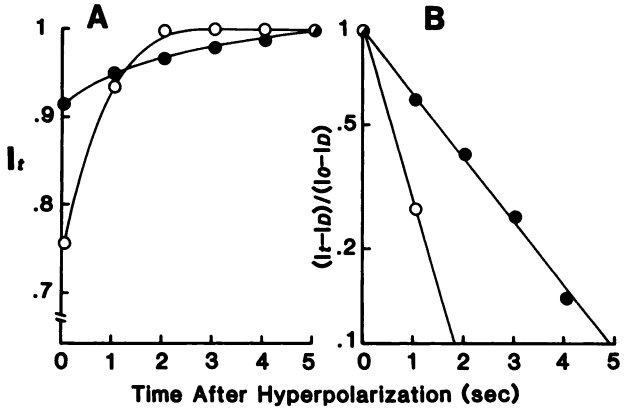


FIG. 10. *Rate of reversal of the inhibition of DPH and CBZ after repolarization*

Neuroblastoma cells were incubated for 15 min in control medium or in medium containing 40 μM DPH (●) or 80 μM CBZ (○). Individual cells were then depolarized to −45 mV for 3 sec to give maximum sodium channel block and repolarized to −105 mV. Test pulses to 0 mV for 5 msec were then given at 50 msec and each second thereafter and peak sodium current was recorded. In the absence of drugs, recovery of the sodium current is complete before 1 sec. The results from two individual cells are presented and are representative of five cells studied under each condition. A, time course of recovery of sodium current. B, semilogarithmic plot of recovery. I_0, sodium current before depolarization; I_D, sodium current following depolarization; I_t, sodium current at the indicated time following repolarization.

depolarizing step from a holding potential of −90 mV, the level of current is rapidly attenuated and forms a steady state response after five pulses (Fig. 11). This new steady state level is about 50% of maximum current. In the presence of DPH (40 μM) or CBZ (80 μM), the current in response to the second pulse diminishes to about 37 and 22%, respectively, compared to drug block following the first pulse (Fig. 11). Over the course of 10 test pulses, the current continues to decline in the presence of these drugs (Fig. 11). Similar drug responses are observed when the interval between test pulses is varied between 5 and 18 msec. These results indicate that the effects of membrane potential and repetitive stimulation to enhance sodium channel inhibition by DPH and CBZ are additive. The effect of membrane depolarization is predominant while repetitive stimulation causes an additional increase in the fraction of channels blocked. Evidently, the pattern of electrical activity observed in neurons of experimental epileptic foci is appropriate to enhance block of their sodium channels by anticonvulsant drugs.

DISCUSSION

DPH and CBZ are sodium channel-selective anticonvulsants. Several lines of evidence now point to inhibition of voltage-sensitive sodium channels as the primary mechanism of action of these two drugs. They are effective blockers of voltage-activated sodium currents in several nonmammalian axon preparations (1–8). They bind to and block sodium channels in rat brain at concentrations achieved during effective prevention of maximal electroconvulsive shock-induced seizures as assessed from neurotoxin binding and $^{22}Na^{+}$ influx experiments (9–11). Moreover, DPH blocks action potentials and repetitive firing of intact mammalian dorsal root ganglion or spinal cord neurons maintained in cell culture at therapeutically relevant concentrations (12, 13). Further evidence supporting designation of the sodium channel as the primary receptor for these drugs has now

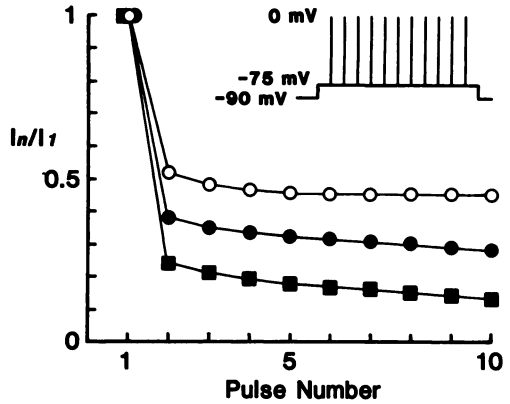


FIG. 11. *Effect of DPH and CBZ on current responses to paroxysmal depolarization shift stimulation*

Cells are depolarized to −75 mV from a holding potential of −90 mV. Following depolarization, brief depolarizing test pulses (0 mV, 2 msec) are delivered every 13 msec (i.e., 66.6 Hz) and current responses are expressed as a ratio of current at a given pulse to the current evoked from the first pulse. ○, control; ●, 40 μM DPH; ■, 80 μM CBZ. The results from two individual cells are presented and are representative of five cells studied under each condition.

been derived from patch voltage clamp analysis of DPH action on sodium currents in clone N1E-115 neuroblastoma cells (14) and of DPH and CBZ action on sodium currents in clone N18 neuroblastoma cells in this study. Dose-response curves for block of sodium currents under voltage clamp give apparent K_D values of 30 μM for both drugs (Fig. 4), well within the range of drug levels reached in rat brain during prevention of maximal electroshock seizures. Taken together, these results leave little doubt that sodium channels in rat brain are significantly inhibited by effective doses of DPH and CBZ.

A more quantitative estimate of the fractional inhibition of sodium channels that is required for effective prevention of seizures can be obtained by comparison of our dose-response curves for DPH and CBZ action with the pharmacokinetic studies of Masuda *et al.* (27). These investigators found that the minimum plasma concentrations of DPH and CBZ that were effective in preventing maximum electroshock seizures in rats were 22 and 16 μM, respectively. These correspond to brain levels of 21 and 25 μmol/kg of brain tissue. Values for mice were similar. A substantial, but unknown, fraction of this drug is likely to be associated with the membrane fraction of neurons and glia rather than free in fluid spaces. However, the sodium channel is an intrinsic membrane protein that is exposed to the hydrocarbon phase of the neuronal surface membrane and at least some local anesthetics appear to gain access to their receptor site on the sodium channel via the lipid phase (21). Thus, in the absence of information to the contrary, it seems appropriate to compare brain levels in micromoles/kg of tissue to the drug concentrations added in *in vitro* experiments. Concentrations of 30 μM DPH or CBZ give half-maximal block of sodium currents in neuroblastoma cells at a holding potential of −75 mV (Fig. 4). At 21 to 25 μM as achieved *in vivo*, approximately 40 to 45% of sodium channels would be blocked. Neurons with more negative membrane potentials and slower firing rates would have fewer sodium channels inhibited; neurons with less negative membrane potentials and more rapid firing rates would have more complete inhibition.

In contrast to this marked inhibition of sodium channels at therapeutic drug concentrations under physiological conditions, other neuronal processes that have been suggested to have a role in anticonvulsant action are not markedly affected at therapeutic concentrations. DPH at 100 μM did not alter calcium-dependent action potentials or enhance GABA-mediated inhibition in cultured rat dorsal root ganglion neurons, although pentobarbital did under identical conditions (12). No effect of DPH (140 or 200 μM) was observed on inhibitory transmission in the rat hippocampus or in frog motoneurons (28, 29). Effects of DPH on GABA-mediated inhibitory responses of cultured rat spinal cord neurons occur at 3- to 5-fold higher concentration than effects on repetitive firing rates that are thought to be due to actions on sodium channels (13). Effects of DPH on calcium influx into neuronal membrane preparations and on block of release of neurotransmitters are observed at higher concentrations that correlate with appearance of the toxic effects of the drug (30–33). Thus, the effects of DPH and CBZ

on repetitive firing and voltage-sensitive sodium channels occur at the lowest concentrations of any of the described actions of the drugs. These results support designation of DPH and CBZ as sodium channel-selective anticonvulsants.

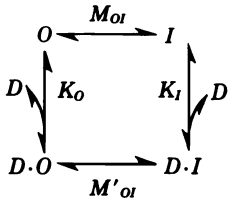
Unlike DPH and CBZ, other classes of anticonvulsants do not have marked effects on sodium channels. Phenobarbital and other barbiturates, which are thought to act by enhancing inhibitory responses to GABA, affect sodium channels only in concentrations well above 100 μM (10, 11). These actions correlate with the anesthetic rather than the anticonvulsant effects of the barbiturates. Similarly, diazepam and other benzodiazepines also block sodium currents but only at 100-fold higher than therapeutic concentrations (8, 10, 11). Valproic acid, ethosuximide, and trimethadione have no effect on sodium channels at accessible concentrations (10, 11). Evidently, DPH and CBZ have specific effects on sodium channels that are not shared by other anticonvulsants at therapeutic concentrations. This action may be essential for their effects on partial and focal motor seizures which are not shared by other anticonvulsants.

Depolarization and high frequency stimulation enhance DPH and CBZ action. As originally described for local anesthetics and antiarrhythmic drugs, several lines of evidence now indicate that inhibition of sodium channels by anticonvulsant drugs is both voltage- and frequency-dependent. In frog myelinated nerve and skeletal muscle fibers, the inhibition of sodium currents by DPH was found to be enhanced by long depolarizing pulses and reversed by hyperpolarizing pulses (3, 5, 8). Inhibition was enhanced by repetitive stimulation (7). More recently, similar effects have been described for CBZ, and the voltage dependence of the action of DPH and CBZ has been ascribed to preferential binding of the drugs to the inactivated state of the sodium channel (8). Our results and those of Matsuki *et al.* (14) on DPH confirm and extend those findings to intact mammalian neural cells and allow a quantitative comparison with pharmacokinetic data on the same species. The voltage dependence of DPH and CBZ action at a therapeutic concentration (20 μM; Fig. 7) indicates that the abnormal changes in membrane potential during paroxysmal depolarization shifts would be expected to modulate the fractional block of sodium channels from 15 to 60%. Sodium channels in neurons experiencing such sustained depolarizations would be selectively inhibited as compared to neighboring normal cells.

In addition to prolonged depolarizations, neurons in epileptic foci also generate an abnormally high frequency of action potentials (17). Repetitive firing at rates greater than 1 Hz also enhances the inhibition of sodium channels by therapeutic concentrations of DPH and CBZ (Fig. 8). This effect is additive with the effect of prolonged depolarization to give more complete inhibition of sodium channels in depolarized, repetitively firing neurons. Taken together, the increase in the inhibition of sodium channels in neurons subject to prolonged depolarization and rapid firing rates may be sufficient to explain the specificity of DPH and CBZ in blocking seizures without markedly altering normal aspects of brain function.

Mechanism of action of DPH and CBZ on sodium channels. The mechanism of action of DPH and CBZ has now been analyzed by two distinct approaches: voltage clamp mesurement of effects on sodium currents in this and previous (1–5, 7, 8, 14) reports and neurochemical studies of interactions with neurotoxins that act at specific receptor sites on sodium channels (9–11). In this section, we consider a unified model of drug action which accommodates the qualitative features of the results of these two different approaches.

Previous ion flux and neurotoxin-binding experiments have characterized DPH and CBZ as allosteric inhibitors of the persistent activation of sodium channels by batrachotoxin, veratridine, and related neurotoxins that act at neurotoxin receptor site 2 (9–11). These neurotoxins activate sodium channels by binding selectively, with high affinity, to the open state of sodium channels and shifting sodium channels to that state according to the laws of mass action (34). The mechanism proposed for this allosteric competitive inhibition is preferential binding of DPH and CBZ to nonconducting states of sodium channels causing stabilization of the channels in nonconducting (closed or inactivated) states (9–11). Since these neurochemical measurements were all made at membrane potentials more positive than −60 mV, sodium channels were inactivated (see Fig. 5). Therefore, the action of batrachotoxin and related toxins in these experiments is to shift sodium channels from the inactivated to the open state. DPH and CBZ oppose this action by binding preferentially to the inactivated state of the sodium channel and stabilizing it (10). Such an allosteric mechanism can be illustrated by Scheme 1 which illus-

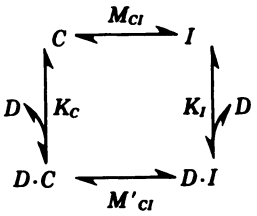


SCHEME 1

trates transitions between open (O) and inactivated (I) states of the sodium channel as influenced by drugs. Here, the equilibrium between O and I states (M_{OI}) is modified by the presence of a drug that binds with unequal affinity to the O and I states according to $M'_{OI}/M_{OI} = K_I/K_O$ (9–11, 34).

We consider next the dependence of the steady state inhibition of sodium channels by DPH and CBZ on membrane voltage observed in this and previous (3, 5, 8, 14) studies. This voltage dependence is expressed in two ways in our experiments: changes in the holding potential alter the fractional inhibition of sodium channels (Fig. 7) and DPH and CBZ appear to shift the voltage dependence of sodium channel inactivation due to the increased binding of the drugs at the more depolarized potentials in the inactivation curve. Similar characteristics of block of sodium channels by tertiary amine and neutral local anesthetics (18–22) were interpreted in

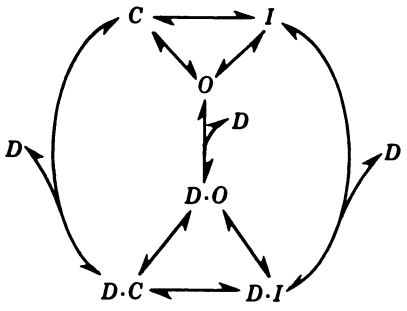
terms of a "modulated receptor" hypothesis which postulated preferential binding of the drugs to the inactivated state of sodium channels (21). This hypothesis, like the mechanism of neurotoxin action described above, represents an extension of the concepts of allosteric modulation of enzyme activity to voltage-sensitive ion channels. Voltage-dependent block of sodium channels by DPH and CBZ can be represented by a cyclic model like that of Scheme 1 but containing only the closed (C) and inactivated (I) states of the channel (Scheme 2). As



SCHEME 2

before, $M'_{CI}/M_{CI} = K_I/K_C$. Thus, drugs which bind selectively to the inactivated state ($K_I < K_C$) make the transition from closed to inactivated states more probable ($M'_{CI} < M_{CI}$). This transition then occurs at more negative membrane potentials, shifting the inactivation curve to the left (Fig. 5) and increasing drug block at more positive membrane potentials where inactivation is favored (Fig. 7). Evidently, the allosteric inhibition of neurotoxin activation of sodium channels by DPH and CBZ, the voltage-dependent channel block by these drugs, and the shift of the inactivation curve to more negative membrane potentials all result from preferential binding to the inactivated state of the sodium channel.

The final aspect of anticonvulsant action which we consider is frequency dependence. This property of DPH and CBZ is also shared with tertiary amine local anesthetics and has been considered previously in the context of the modulated receptor hypothesis (21). Frequency dependence arises when the interaction between drug and receptor is dependent upon the state of the sodium channel and is too slow to reach equilibrium within a single cycle of sodium channel activation and inactivation, that is, within a few milliseconds. Since both activation and inactivation processes are involved, a more complete model of sodium channel function containing closed, open, and inactivated states is required. Scheme 3 is adapted from Ref. 21. Frequency dependence may be



SCHEME 3

considered to arise from the preferential binding of DPH and CBZ to the inactivated state of the sodium channel that is generated during each depolarization and the relatively slow dissociation of the bound drug molecules from the inactivated state of their receptor (Fig. 10) which allows accumulation of drug-bound, inactivated sodium channels from stimulus to stimulus. Sodium channels in the closed state first activate and then inactivate. DPH or CBZ rapidly bind to the inactivated state, stabilizing those channels in an inactive form and preventing them from returning to the closed state to be activated by subsequent stimuli. Accumulation of sodium channels in the drug-bound, inactivated state continues until a steady state is reached in which the rate of entry of sodium channels into this state during a stimulus is equal to the rate of dissociation of drug from the drug-bound, inactivated sodium channels between stimuli, allowing them to return to the closed state. Thus, the extent of frequency-dependent block depends on pulse duration and frequency and on drug dissociation rate. The underlying basis of this property of DPH and CBZ action, like those considered previously, is preferential binding to the inactivated state of the sodium channel.

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