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Cellular Bases of Barbiturate and Phenytoin Anticonvulsant Drug Action

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Although anticonvulsant drugs are the mainstay of therapy for epilepsy, their mechanisms of action remain uncertain for several reasons. First, most anticonvulsant compounds have been shown to have multiple physiological, biochemical, and pharmacological actions. It has been difficult, therefore, to determine which specific drug actions produce clinical effects. Second, investigation has often been performed on isolated peripheral or invertebrate neuronal or muscle preparations or on neurons in the intact vertebrate central nervous system. In the former case, the number of drug actions that can be studied is limited, since the pharmacology of these cells is quite unlike that found in the vertebrate central nervous system. In the latter case, the preparation is technically difficult to study and, therefore, limited experimental control is possible. Third, investigators have identified single actions of anticonvulsants and suggested a role in anticonvulsant action without determining if the drug effect is produced at concentrations achieved in the central nervous system during clinical use. Finally, anticonvulsants are often studied at concentrations achieved in serum during clinical use. However, for anticonvulsants that are protein bound, it is important to use therapeutic free-serum concentrations, since cerebrospinal fluid drug levels reflect free, not total, serum drug concentrations.

We have employed an *in vitro* mammalian neuronal preparation to study anticonvulsant drug action. Mouse spinal cord neurons in primary dissociated cell culture develop electrical excitability, synaptic connectivity, and postsynaptic chemosensitivity to a variety of putative neurotransmitters (1,2). Stable intracellular recordings can be made from individual neurons for several hours, and drugs and neurotransmitters can be applied by iontophoresis, local pressure ejection, or superfusion of the entire culture. Furthermore, with this neuronal preparation, it is possible to apply known concentrations of anticonvulsants directly to neurons in serum-free medium, thus obviating many pharmacokinetic problems of whole animal studies (serum protein binding, blood–brain barrier, and drug metabolism). In this paper we will review our investigations of barbiturate mechanisms of action and present recent studies of phenytoin actions.

Barbiturate Actions

Long-acting barbiturates such as phenobarbital (PhB) and mephobarbital are useful as chronic anticonvulsants since they have minimal sedative action at therapeutic concentrations. Intermediate- and short-

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acting barbiturates such as pentobarbital (PB), thiopental, or amobarbital are also effective anticonvulsants, but cannot be used clinically due to their prominent sedative effects. What is the basis for this difference in action among these barbiturates?

To assess barbiturate actions on physiological neuronal activity, we determined the effect of PhB and PB on normal spontaneous activity (3) (Fig. 1). Spontaneous activity of spinal cord neurons in cell culture consists of a random admixture of excitatory and inhibitory postsynaptic potentials and spontaneous action potentials. Both PhB and PB produced dose-dependent reduction in spontaneous activity, resulting in an increasing percentage of quiescent neurons at increasing barbiturate concentrations. PB was substantially more potent than PhB, increasing the percentage of quiescent neurons from 0 to 100% over concentrations of 100–500 μM (ED_{50}, 250 μM). PhB produced a similar effect at higher concentrations of 500–2,500 μM (ED_{50}, 900 μM). PB produces anesthesia at brain levels of about 100–200 μM, and thus production of 20–30% quiescent neurons should correlate with anesthesia. Anticonvulsant action would have to occur at much lower concentrations to avoid sedative toxicity.

As a model of anticonvulsant action, we investigated the effect of barbiturates on bicuculline (BICUC)-induced paroxysmal activity (3). BICUC is a convulsant that antagonizes GABA responses (4–6) by competing with GABA to bind to GABA receptors (7,8). In addition, at somewhat

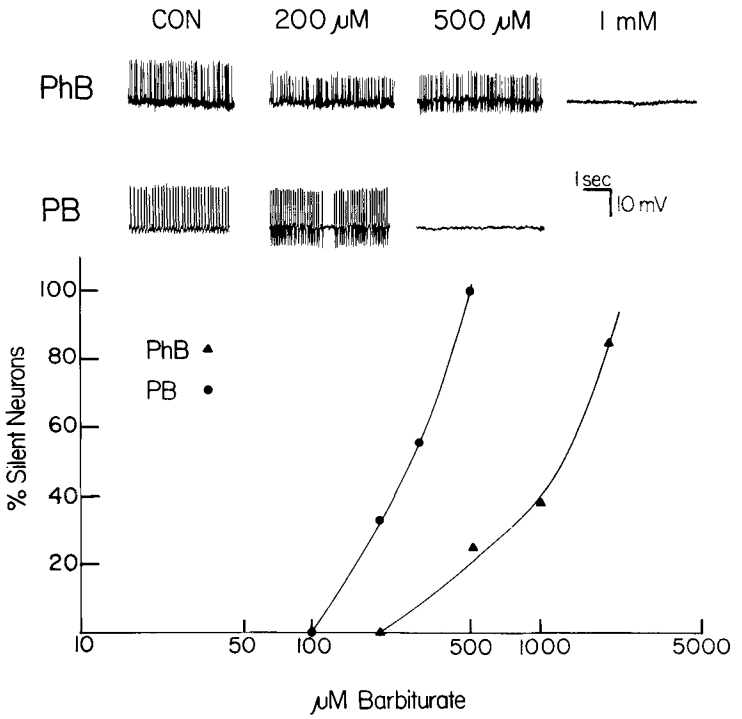

FIG. 1. Phenobarbital (PhB, triangles) and pentobarbital (PB, circles) reduced spontaneous activity. Spontaneous activity was recorded from spinal cord neurons in control medium (phosphate buffered saline, PBS) and in PBS containing barbiturates. Specimen records are intracellular recordings made with glass micropipettes filled with 4 *M* potassium acetate displayed on a rectilinear polygraph. Each point in the graph represents the mean % silent neurons obtained from 4 to 7 neurons (total of 22 for PB and 18 for PhB). CON, control. (From Schulz and Macdonald (3), with permission.)

higher concentrations, BICUC reduces membrane potassium conductance (9,10). When added to medium bathing spinal cord neurons in cell culture, BICUC produces large, randomly occurring depolarizations that evoke volleys of action potentials (5,9). These paroxysmal depolarizing events (PDE) appear to be similar to convulsant-induced paroxysmal bursting in spinal motor neurons (11) and to convulsant-induced paroxysmal depolarization shifts recorded in cortical neurons (12,13). When BICUC was added at 40 μM, all neurons developed PDE (9). Both PhB and PB (25–500 μM) abolished PDE in a dose-dependent fashion (Fig. 2). PB (ED_{50}, 75 μM) was slightly more potent than PhB (ED_{50}, 90 μM), but both drugs restored normal spontaneous activity. Therapeutic anticonvulsant serum levels of PhB vary from about 40 to 160 μM (10–40 μg/ml), but since PhB is about 50% protein bound, free PhB levels would be in the range of about 20 to 80 μM. Thus, PhB (and PB) reversed convulsant-induced paroxysmal activity and restored normal spontaneous activity at clinically relevant concentrations.

What are the bases for barbiturate anticonvulsant, sedative-hypnotic, and anesthetic actions? Barbiturates have multiple actions on both synaptic and nonsynaptic membranes (Table 1). Barbiturates act (a) *postsynaptically* to enhance GABAergic inhibition (3,14–19), to reduce glutaminergic (15,17–19) and cholinergic (20, 21) excitation, and to directly activate membrane GABA receptor–coupled chloride ion channels (3,15,19); (b) *presynaptically* to reduce release of neurotransmitters (22–25) including GABA (26,27), glutamate (27–29), aspartate (28–30), acetylcholine (28,31–34), and norepinephrine (35); and

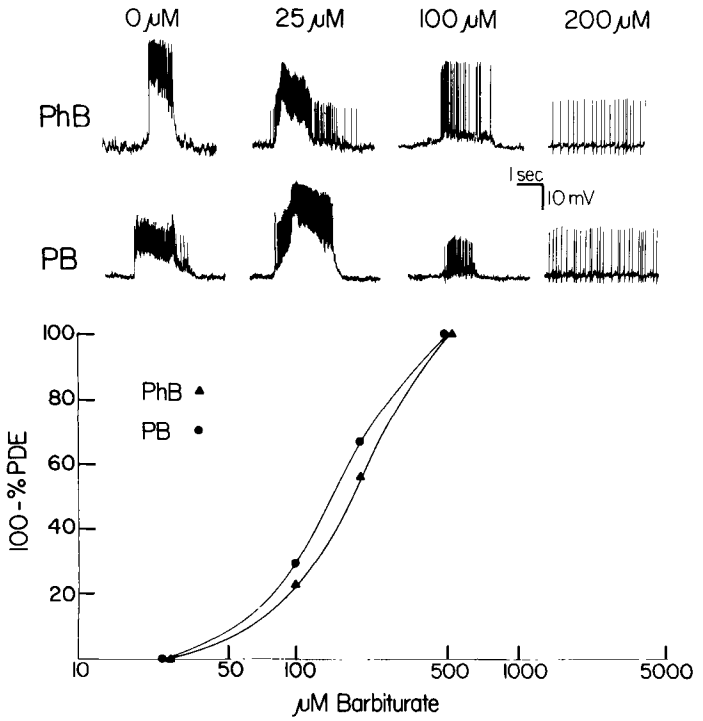

FIG. 2. PhB and PB abolished bicuculline (BICUC)-induced paroxysmal depolarizing events (PDE). When 40 μM BICUC was added to PBS, 100% of spinal cord neurons developed PDE. See legend to Fig. 1 for recording details, symbols, and abbreviations. (From Schulz and Macdonald (3), with permission.)

TABLE 1. *Barbiturate actions*

Alteration of *postsynaptic* neurotransmitter action
Enhancement of GABA-mediated inhibition
Antagonism of excitatory synaptic transmission
Direct enhancement of membrane chloride ion conductance
Reduction of *presynaptic* calcium entry and neurotransmitter release
Reduction of *nonsynaptic* membrane sodium and potassium ion conductances

(c) *nonsynaptically* to reduce sodium and potassium conductances (36–38).

To investigate postsynaptic barbiturate actions on neurotransmitter responses, we determined the effect of barbiturates on GABA, glycine, β-alanine, and glutamic acid responses evoked on spinal cord neurons (3,18,19). Both PhB (20–500 μ*M*) and PB (10–400 μ*M*) augmented GABA responses at low concentrations without altering glycine or β-alanine responses, with PB having a slightly lower ED_{50} (50 μ*M*) than PhB (75 μ*M*) (Fig. 3). Both PhB and PB antagonized glutamic acid responses at barbiturate concentrations effective in augmenting GABA responses. Thus, both

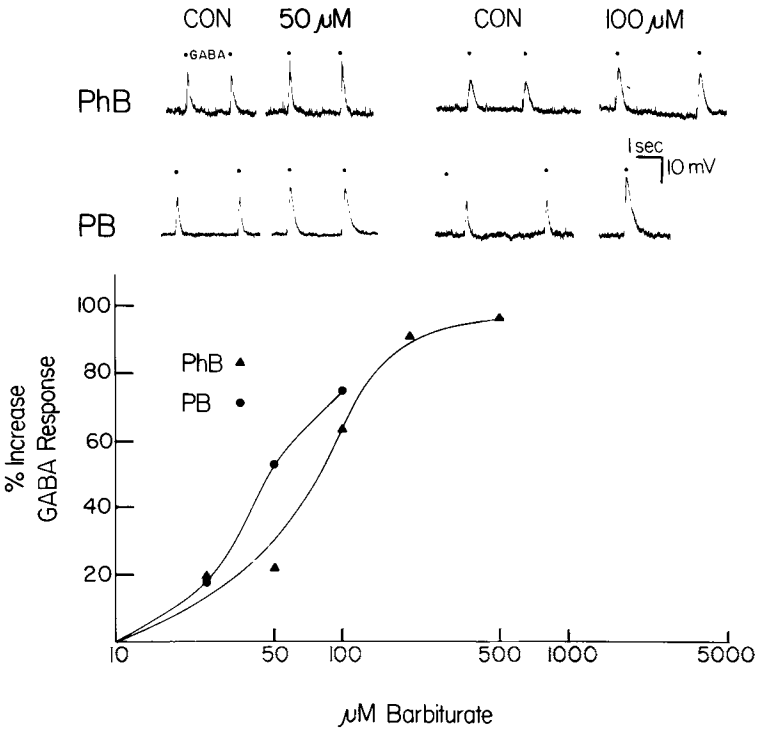

FIG. 3. PhB and PB augmented postsynaptic GABA responses. Intracellular recordings were made with glass micropipettes filled with 3 *M* KCl from spinal cord neurons bathed in PBS containing 10 m*M* Mg^{2+} to suppress spontaneous activity. GABA was applied by iontophoresis for 100 ms and barbiturates were applied by local pressure injection from large-tipped glass pipettes. GABA responses were depolarizing due to intracellular chloride leak from the recording micropipettes. Each point on the plot represents the average augmentation of GABA responses on three to eight spinal cord neurons with two to four trials per neuron (33 trials for PhB and 15 trials for PB). See legend to Fig. 1 for symbols and abbreviations. (From Schulz and Macdonald (3), with permission.)

PhB and PB modulated postsynaptic amino acid responses at concentrations which reversed BICUC-induced PDE and which are achieved in the central nervous system when serum barbiturate concentrations are therapeutic. It is therefore likely that barbiturates have anticonvulsant action due to augmentation of postsynaptic GABAergic inhibition, as well as to antagonism of postsynaptic glutaminergic excitation.

In addition, both PhB and PB were shown to reduce calcium-dependent action potential duration (39) and to increase membrane chloride ion conductance (3,19) (Fig. 4). Release of neurotransmitter is calcium dependent (40), requiring entry of calcium through voltage-dependent presynaptic calcium channels (40–42). Since barbiturates reduce calcium-dependent action potential duration (39) and calcium uptake by synaptosomes (43–45), it is probable that they block neurotransmitter release by reducing presynaptic voltage-dependent calcium ion entry. The direct increase in

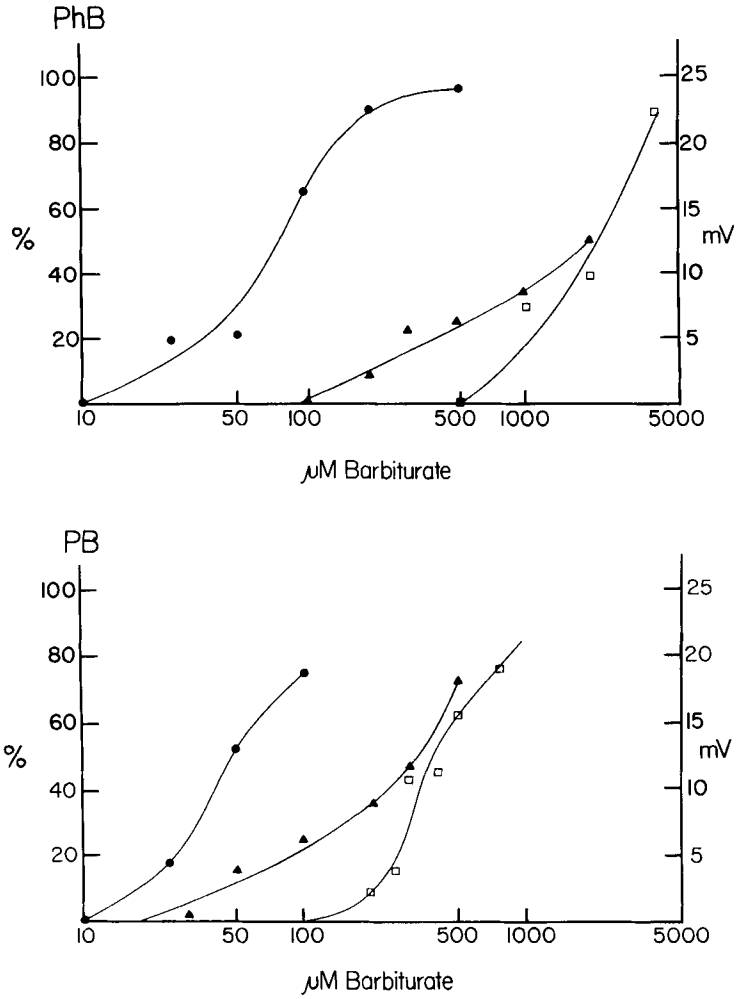

FIG. 4. Dose-dependency of phenobarbital (PhB, **top**) and pentobarbital (PB, **bottom**) enhancement of GABA responses (circles), reduction of calcium-dependent action potentials (triangles), and membrane depolarization (mV) (squares). (From Heyer and Macdonald (39), with permission.)

chloride ion conductance produced by barbiturates was blocked by the GABA antagonists penicillin and picrotoxin (19), suggesting that the barbiturates bound to GABA receptors. Increasing postsynaptic membrane chloride ion conductance would produce membrane hyperpolarization and a decrease in membrane excitability. PhB produced these actions only at high concentrations, reducing calcium-dependent action potential duration from 100 to 5,000 μM (ED_{50}, 900 μM) (39) and increasing chloride ion conductance from 500 to 4,000 μM (3) (Fig. 4, top). PB was much more potent in both actions, reducing calcium-dependent action potential duration from 25 to 600 μM (ED_{50}, 170 μM) and increasing chloride ion conductance from 100 to 500 μM (Fig. 4, bottom). For both barbiturates, reduction of calcium-dependent action potential duration and increase in membrane chloride ion conductance correlated with reduction of spontaneous activity (Figs. 1 and 4). From these data, we concluded that sedative-hypnotic actions of barbiturates may be due to slight reduction of neurotransmitter release in addition to modulation of postsynaptic neurotransmitter responses. Anesthesia may be produced by a larger reduction of neurotransmitter release, in addition to modulation of postsynaptic neurotransmitter responses and direct membrane hyperpolarization. Finally, the only difference between anticonvulsant and anesthetic barbiturates is the dose-dependency of these actions. PhB has a relatively large therapeutic index between drug concentrations producing postsynaptic modulation of neurotransmitter responses and those producing reduction of presynaptic neurotransmitter release and postsynaptic membrane hyperpolarization (Fig. 4, top). For PB, there is a poor therapeutic index (Fig. 4, bottom).

Phenytoin Actions

Phenytoin (PT) is useful in the management of a wide variety of partial and generalized seizure types similar to those responsive to phenobarbital. However, PT has less sedative action than the barbiturates. The mechanism of PT anticonvulsant action is unclear. PT limits generalization of seizures without suppressing seizure foci, and thus is unlike barbiturates, which elevate seizure threshold (46). PT and PhB also have different effectiveness against seizures produced in experimental animals. PT is effective against maximal electroshock seizures (MES) but ineffective against pentylenetetrazol (PTZ) seizures; PhB is more effective against PTZ seizures than against MES (47,48). Thus, despite having similar clinical anticonvulsant efficacy, their mechanisms of action are undoubtedly dissimilar.

To investigate the clinically relevant actions of PT it is necessary to determine the concentration of PT in the central nervous system when serum levels are in the therapeutic range. PT is a lipid soluble compound that is highly protein bound (~90%). At therapeutic serum levels (10–20 μg/ml or 40–80 μM), free-serum PT levels are about 1–2 μg/ml (4–8 μM). Cerebrospinal fluid levels are equal to free-serum levels, but brain concentrations are variable and range from 75 to 120% of total serum concentration (49). Thus, does PT have its relevant actions at total serum, free-serum, or brain concentrations? As one approach to this issue, we determined the dose-dependency of PT actions on spontaneous activity and on convulsant-induced paroxysmal activity recorded from mouse spinal cord neurons in cell culture (50). When added to the protein-free bathing medium, PT did not alter normal spontaneous activity at concentrations up to 2 μg/ml (Fig. 5). However, above 2 μg/ml, there was a progressive reduction in spontaneous activity and an increase in the percentage of neurons with no spontaneous activity. At 5 μg/ml, about 50% of neurons were quiescent. Based on these results, it is unlikely that PT has its clin-

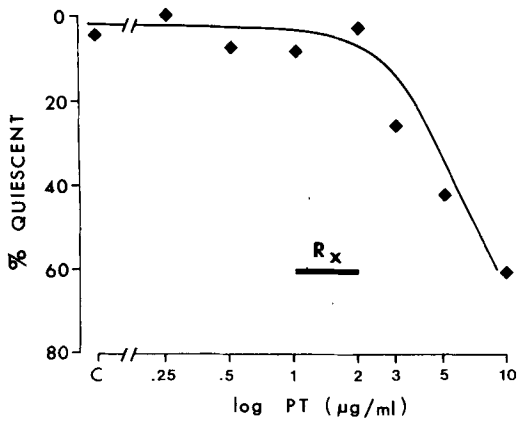

FIG. 5. Phenytoin reduced spontaneous activity. Phenytoin produced a dose-dependent increase in the number of quiescent neurons. However, at or below 2 μg/ml, PT did not alter spontaneous activity.

ical action at brain or total serum concentrations, since all spontaneous activity would be suppressed. Thus, it is likely that the free-serum (and cerebrospinal fluid) levels of 1–2 μg/ml are the relevant concentrations.

If free-serum concentrations of PT are relevant for anticonvulsant action, what effect does PT have on convulsant-induced paroxysmal activity at these concentrations? At 2 μg/ml, PT did not suppress PDE (Fig. 6, top). The paroxysmal activity was often increased in frequency by PT. When higher concentrations of PT were used (5–20 μg/ml), PDE was suppressed in a dose-dependent fashion, but the paroxysmal activity was replaced by quiescence (Fig. 6, bottom). Thus, unlike the barbiturates, PT does not act by suppressing convulsant-induced paroxysmal activity.

How then does PT produce anticonvulsant effects? Four basic actions of PT have been described and proposed as the anticonvulsant mechanism of action (Table 2). First, PT has been shown to reduce the rise in intracellular sodium produced by MES (51). Two alternative mechanisms for this have been proposed. PT and the inactive PT metabolite 5-hydroxyphenyl-5-phenylhydantoin (HPPH) enhance Na-K ATPase activity (52,53) (and therefore presumably increase active extrusion of sodium ions) by reducing the inhibitory effect of high sodium concentrations. However, this effect was not specific for PT, since HPPH was also active (53). Thus, it is unlikely that PT has anticonvulsant action by enhancing active sodium extrusion. Alternatively, PT has been shown to reduce the maximal rate of rise, overshoot, and

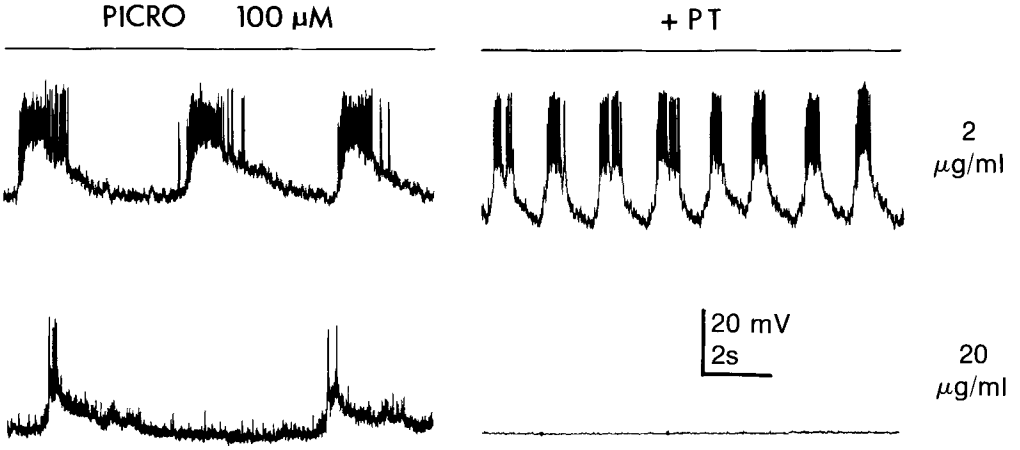

FIG. 6. Phenytoin (PT) did not suppress paroxysmal depolarizing events (PDE) at therapeutic free-serum concentrations. PDE were produced by addition of 100 μ*M* picrotoxin (PICRO) to phosphate buffered saline bathing medium. PT at 2 μg/ml did not suppress PICRO-induced PDE (**top**) but did suppress PDE at 20 μg/ml (**bottom**). Intracellular recordings were made with micropipettes containing 4 *M* potassium acetate.

TABLE 2. *Phenytoin actions*

Reduction of *nonsynaptic* sodium ion movements
Enhancement of active sodium ion transport
Reduction of sodium ion channel conductance
Reduction of *presynaptic* calcium ion entry and neurotransmitter release
Alteration of *postsynaptic* neurotransmitter action
Enhancement of GABA-mediated inhibition
Antagonism of excitatory synaptic transmission
Reduction of repetitive firing and posttetanic potentiation

amplitude of sodium-dependent action potentials (54–57) and to decrease inward sodium ion fluxes (58,59). Second, PT has been shown to reduce calcium-dependent potentials (60), to reduce inward calcium fluxes (61–63), and to reduce presynaptic release of neurotransmitters (64–67). Third, PT has been shown to modify postsynaptic neurotransmitter responses. PT enhanced postsynaptic GABA responses in the crayfish stretch receptor neuron (68,69) as well as cortical inhibition in the cat (70). However, PT did not alter GABA responses evoked on rat dorsal root ganglion neurons (71) nor on rat hippocampal pyramidal neurons (72). PT reduced excitatory postsynaptic responses to acetylcholine at the neuromuscular junction (67) and the excitatory actions of glutamate and acetylcholine in rat cortex (73). Finally, PT has been shown to reduce repetitive firing (74–76) of neurons, nerve terminals, and muscle cells, and to reduce posttetanic potentiation (76,77) evoked in cat spinal cord and sympathetic ganglia.

Using spinal cord neurons in cell culture, we studied several of these actions of PT to determine which were produced at low PT concentrations (1–2 μg/ml) (50). PT reduced the maximum rate of rise of sodium-dependent action potentials (an index of inward sodium current) and reduced the duration of calcium-dependent action potentials (an index of inward calcium current) only at PT concentrations above 2.5 μg/ml. The reduction of action potentials correlated with reduction of spontaneous activity. GABA and glutamate responses were not altered by PT at 2 μg/ml. Thus, it is unlikely that PT has anticonvulsant action by blocking sodium channels or calcium channels or by modulating postsynaptic GABA responses. However, repetitive firing produced by depolarizing currents was substantially reduced by PT at 1–2 μg/ml (Fig. 7). In control medium, a 2-s depolarization of sufficient amplitude evokes a continuous train of action potentials. PT at 0.2 μg/ml did not modify this firing pattern (Fig. 7A). However, PT at 1.0 and 2.0 μg/ml reduced the duration of repetitive firing dose-dependently (Fig. 7B and C). HPPH at 10 μg/ml did not modify repetitive firing (Fig. 7D). The effect of PT on repetitive firing was fully reversible following washout of the PT. Thus, PT may have anticonvulsant activity due to its ability to limit high frequency repetitive firing, a characteristic firing pattern in epileptogenic foci. The mechanism by which PT produces this limitation of repetitive firing is uncertain and is currently under investigation. Possibilities include (a) a shift of the voltage-dependency of slow inactivation of sodium channels, (b) reduction in the rate of removal of sodium channel inactivation, (c) development of use-dependent sodium channel block, or (d) enhancement or production of a slow outward calcium- or voltage-dependent potassium conductance.

Conclusions

The results obtained thus far suggest that although barbiturates and phenytoin have multiple actions on neuronal membrane properties, electrical excitability, and

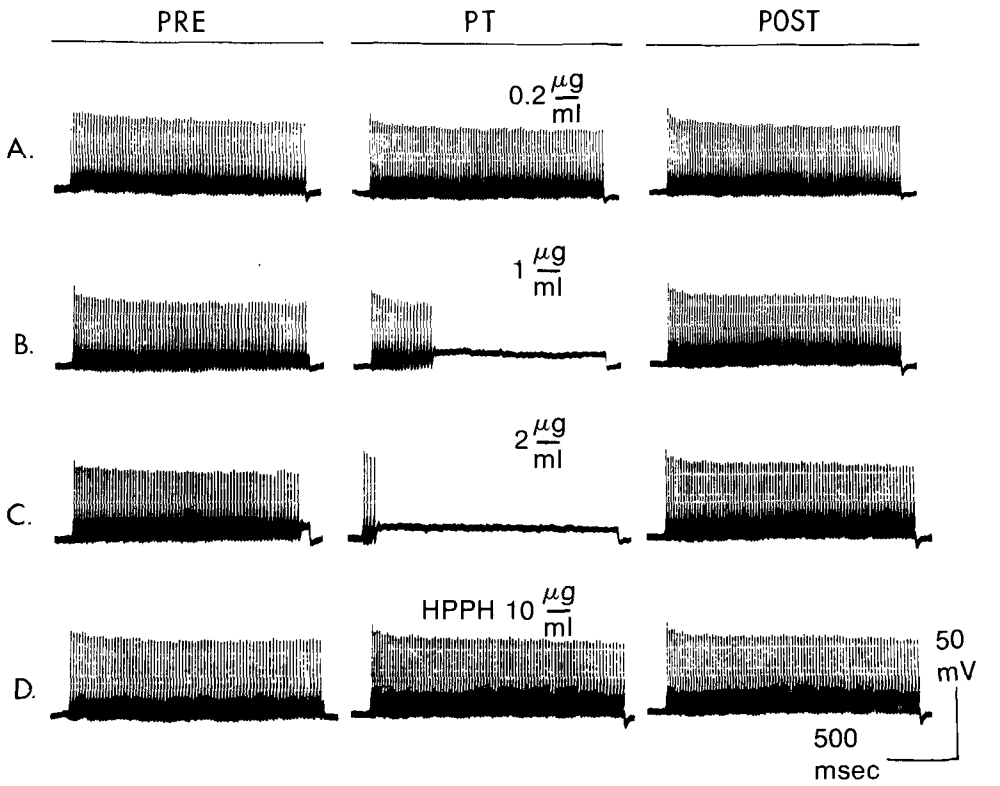

FIG. 7. Phenytoin suppressed repetitive firing. Intracellular recordings were made from spinal cord neurons, and long (2.0 s) depolarizing current pulses were applied using the single electrode bridge technique. Recordings shown are photographs of oscilloscope traces (A–D). Continuous recordings were made from individual neurons prior to (PRE), during (PT) and following (POST) superfusion of hydantoin-containing bathing medium. The recordings in A–D were made from different neurons. See text for details.

synaptic transmission, their specific anticonvulsant actions can be postulated to be postsynaptic modulation of neurotransmitter actions and suppression of repetitive firing, respectively. These conclusions should be considered as working hypotheses to be evaluated using more intact, vertebrate, central neuronal preparations such as hippocampal or neocortical slices *in vitro* or intact cortex *in vivo*. It is certainly possible that alternate mechanisms will be identified in the more intact preparations, but the investigation should be facilitated by comparisons to data obtained from mammalian neurons in cell culture.

A clear objective of this research in the future is the identification of the therapeutic and toxic actions of all major anticonvulsants using this *in vitro* preparation. Once these mechanisms are identified, it should be possible to study specific physiological actions of drug congeners to identify which portions of the drug molecules are relevant for each of the therapeutic and toxic drug actions. Furthermore, it should be possible to formulate specific drugs that would have improved therapeutic indices and therefore would be clinically more useful. Finally, it should be possible to study the interactions of anticonvulsants with different mechanisms of action to determine which drugs have synergistic anticonvulsant action without synergistic toxicity.

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