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Barbiturate-Induced Alterations in the Kinetic Parameters of Slow Outward Current in *Aplysia* Giant Neurons[1]

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ABSTRACT
Regulation of spike frequency adaptation has been postulated to be a neuronal mechanism of action of the barbiturates (Zbicz and Wilson, 1981). A barbiturate sensitive slow outward current is present in *Aplysia* giant neurons, and this current is very effective at regulating adaptation. In the current study a quantitative method of determining the effect of barbiturates was developed using a single-electrode voltage clamp. This method, tail current analysis, involved measuring the exponential decay of the slow outward current at the end of a depolarizing voltage-clamp command. The tail currents were made up of two decay phases with average half-times of 7 and 70 sec, and the tail current amplitude accurately reflected the magnitude of slow outward current. Barbiturates were found to cause a concentration-related, saturable and stereospecific increase in one component of the tail currents, the amplitude of the slow phase. With moderate concentrations of barbiturates there were no changes in the other parameters describing the exponential decay current. The rank order of potency of barbiturates for the enhancement of slow outward current was not well correlated with the lipid solubility of these drugs, indicating that slow outward current enhancement is not due to a nonspecific mechanism, but rather may be receptor mediated.

A slowly activating outward current has been demonstrated in *Aplysia* giant neurons (Cote *et al.*, 1978). The slow outward current is activated by near-threshold depolarizations in the voltage range of −40 to −30 mV. This range includes the average interspike potential that is attained during a prolonged train of action potentials in these molluscan neurons, indicating that slow outward current has functional significance in the control of firing rate.

Barbiturates, which have clinical use as anticonvulsants, sedatives and anesthetics, potentiate the voltage-sensitive development of slow outward current (Cote *et al.*, 1978; Zbicz and Wilson, 1981) with a resultant enhancement of the process of spike frequency adaptation. This action of barbiturates may be important in the mechanism of some of the clinical actions of this group of drugs. It has thus been hypothesized that these effects are related to the anticonvulsant actions of barbiturates (Zbicz and Wilson, 1981). This hypothesis is based on the observation that certain aspects of seizure activity in animal models of epilepsy as well as in human epileptic cortex are associated with prolonged periods of high frequency firing of individual central neurons (Ward, 1961; Kandel and Spencer, 1961; Sawa *et al.*, 1963). Pharmacological intervention which affects the ability of neurons to fire repetitively would then be expected to have anticonvulsant actions. However, it should be noted that if activation of slow outward current is an anticonvulsant mechanism, it is a mechanism which is unique to barbiturates, and is not shared by other anticonvulsant compounds such as hydantoins and benzodiazepines (Zbicz, 1979; Hoyer and Oosterode, 1981).

In order to understand this barbiturate action further and to elucidate the structural properties that were important in determining activity in enhancing slow outward current, it was desirable to develop a means of quantitating the effects of barbiturates on slow outward current. This report presents the results of a voltage-clamp study of the kinetics of slow outward current in *Aplysia* giant neurons, and the effects of barbiturates on the kinetics. Kinetic analysis provides a sensitive means by which to determine the concentration-dependent effects of the barbiturates on slow outward current. This preparation of *Aplysia* giant neurons was chosen for several reasons, including: 1) the presence of slow outward current in these neurons, 2) the ready identification of these neurons within the neural ganglia which allows the study of a uniform population of cells and 3) the size and accessibility of the soma of these giant neurons which allows simultaneous measurement of membrane currents, membrane potentials and ionic activities (both intracellular and extracellular).

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ABBREVIATIONS: A_1, amplitude of the fast component of tail current decay; A_2, amplitude of the slow component of tail current decay; GABA, γ-aminobutyric acid.

The kinetic analysis utilized in this study involved characterizing the tail currents which were produced after activation of slow outward current. The kinetic parameters describing the tail currents in the control situation were established, and the changes in these parameters induced by different barbiturates were used as a measure of the barbiturate effect. Barbiturates were found to cause concentration-dependent and stereospecific increases in one component of the tail current amplitude, with little effect on the kinetics. The activity of barbiturates in enhancing slow outward current, as determined by this method, was not well correlated with the sedative/anesthetic activities. These data, taken together with the fact that an anticonvulsant barbiturate with minimal sedative properties (diphenylbarbituric acid; Raines *et al.*, 1975, 1979) produces marked slow outward current enhancement (Huguenard and Wilson, 1985), suggests that enhancement of slow outward current may be associated with other, nonsedative, effects of the barbiturates, perhaps the anticonvulsant effects.

Methods

The preparation. Giant neurons R2 and LP1 (Frazier *et al.*, 1967; Hughes and Tauc, 1961) from the marine mollusc *Aplysia california* (Marine Specimens Unlimited, Pacific Palisades, CA) were used in this study. The ganglion of study (the abdominal ganglion for R2, or the left pleural ganglion for LP1) were dissected from 100- to 300-g animals and pinned to the bottom of the Sylgard (Dow Chemical, Midland, MI)-lined recording chamber. In some cases the connective tissue sheath was dissected with fine forceps and microdissecting vannas scissors (George Tiemann & Co., Plainview, NY) to expose the underlying neurons and expedite electrode impalement.

In the recording chamber, the ganglia were perfused constantly with constant temperature (20°C) artificial sea water of the following composition (millimolar): NaCl, 492; $NaHCO_3$, 2; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 70; and $MgSO_4$, 30. The artificial sea water contained twice the normal MG^{++} concentration in order to suppress synaptic activity. Although 2× Mg^{++} solutions were found to cause a minor suppression (approximately 10%) of the outward currents under study, the increased stability obtained by suppressing slow synaptic activity made this maneuver highly desirable. The pH of the control artificial sea water and each drug solution was adjusted to 7.8 immediately before each experiment. A volume of at least 100 ml (50 times the volume of the recording chamber) was always allowed to flow before the chamber superfusate was assumed to have equilibrated after any change in drug concentration. (Dye dilution experiments indicated that wash was essentially complete with a wash volume of approximately 20 chamber volumes.)

The drugs in this study were dissolved in artificial sea water at the desired concentrations. Solutions of the sodium salts of pentobarbital, secobarbital (Sigma Chemical Co., St. Louis, MO) and phenobarbital (compliments of Sterling-Winthrop, Rensselaer, NY) were obtained by dissolving them in artificial sea water. The free acid forms of the stereoisomers of secobarbital and pentobarbital (National Institute on Drug Abuse, Research Triangle Park, NC) were dissolved in 1 ml of 0.1 N NaOH and then diluted to the desired final concentration in artificial sea water. The diluent was also added to control solutions. Before any change in drug solutions, the preparation was allowed to come to equilibrium (as judged by the parameters obtained from tail analysis).

Electrophysiological procedures. A modified single electrode voltage clamp (Wilson and Goldner, 1975) was used for either controlling current or voltage of the giant neuron under study. The clamp circuitry was modified to provide an extracellular voltage reference which eliminated signal drift due to current passing through the bath ground circuit. The reference electrode was a broken tip microelectrode (0.1–0.5 megohm) which was placed in the chamber near the giant cell.

Microelectrodes were pulled on a Kopf vertical electrode puller from 1.5 mm outside diameter fiber-filled capillary glass and filled with 1.5 M KCl. Optimum microelectrode resistance for voltage clamping *Aplysia* giant neurons with the single electrode voltage clamp was found to be in the range of 0.5 to 1 megohm. Microelectrode polarization due to current passage was tested and the acceptable limit was set at less than 1 mV/100 nA of current. Generally, the magnitude of currents passed in these experiments was 50 nA or less, and therefore artifacts due to electrode polarization were minimized.

The tail currents that were recorded in this work were relatively small, usually less than 20 nA. To eliminate the possibility that a time-dependent polarization of some component of the recording circuit could produce similar tail currents, the following control was performed: at the end of an experiment, the microelectrode was withdrawn from the giant neuron of study but left submerged in the bath. A electrical cell model was placed within the ground return circuit of the bath, and the cell model was voltage-clamped. Voltage steps were imposed on the cell model such that current magnitudes greater than those seen with the largest physiological currents were obtained. Under these conditions, the time-dependent currents recorded over periods of up to 100 sec were less than 0.1 nA.

The experiments in this study were performed by voltage-clamping *via* a single microelectrode placed in the soma of the neuron of study. Because the giant neurons have extensive processes in the neuropil of the ganglia there is some concern regarding the degree of spatial control of membrane potential during voltage clamping in the soma. This was not a source of major error in this study for the following reasons: 1) the slow outward current exists *in* the soma (Huguenard *et al.*, 1985), 2) two-electrode voltage clamping of these neurons, which would be expected to result in somewhat better spatial and temporal control of the membrane potential, gave similar results (Zbicz and Wilson, 1981) and 3) in a few cases it was possible to study the barbiturate enhancement of slow outward current and tail currents in an intact neuron and then isolate the soma by axotomy and then repeat the study; in these cases the results obtained with axotomized neurons were quite similar to those obtained before axotomy.

Method of quantitating slow outward current. The very slow time course of slow outward current presented problems for the analysis of barbiturate effects. For subthreshold voltage clamp depolarizations in the range of −30 mV, the slow outward current activates very slowly, over a period of many minutes (see fig. 7). The final current appears to approach a maximum but does not stabilize, even after a period of 30 min. Therefore, the final current level cannot easily be used as a measure of barbiturate effects. The slope of outward current development has been used to measure slow outward current during a 60-sec period (Zbicz and Wilson, 1981) but there are at least two disadvantages to this method, especially during prolonged activation of slow outward current. First, the rise in current during depolarization is not linear; the slope gradually decreases with time as the current approaches a maximum over a period of 20 to 30 min. Second, the membrane currents during a prolonged depolarization appear to consist of at least two types of currents. Colmers *et al.* (1982) have demonstrated that, in cesium loaded *Aplysia* neurons, there exists a partial inactivation of a slow *inward* current during a prolonged depolarization. Therefore, changes in membrane current not only reflect slow outward current, but changes in other currents as well.

One method does provide a means of measuring slow outward current *in isolation*. After slow inward current activation in cesium-loaded cells there is *no* tail current (Colmers *et al.*, 1982). Therefore, by analyzing the tail currents obtained after a prolonged voltage clamp depolarization, one has a measure of the slow outward current which was activated during the depolarization (Evans *et al.*, 1981). Analysis of tail currents has been used in the present study to elucidate the action of barbiturates on slow outward current.

Plotting the tail currents on a semi-logarithmic graph revealed that the decay phase of slow outward currents could be represented by the sum of at least two exponential decay processes. An automated "peeling" routine was developed in order to accurately determine the kinetics and amplitudes (zero time intercepts) of tail currents (Zierler, 1981).

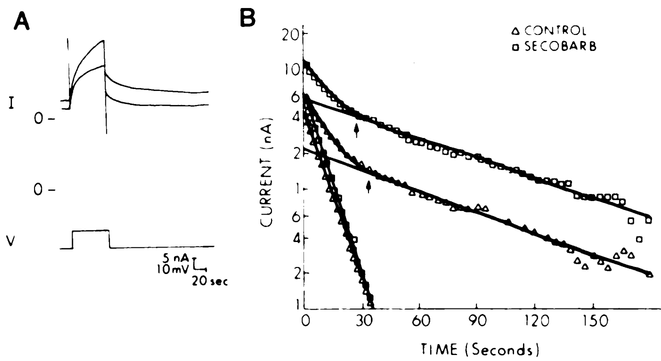

Fig. 1. Example of the change in tail current produced by barbiturate. A, tracings of slow outward current obtained in control and with 100 μM secobarbital. Upper traces are current (I) (up = outward), lower trace is voltage (V). Of the two current traces the lower, shallower trace is control and the upper, steeper trace is in the presence of secobarbital. B, semilog plots of tail currents from A. The arrows mark the division between the slow and fast component of tail current as determined by the analysis. In order to obtain the fast component of tail current, the residuals were subtracted from the best fit line of the late time points. The set of straight lines, as well as the curve fit, were obtained from the tail analysis routine. The results of the analysis for these two curves are shown in table 1. The stimulus was a 60-sec depolarization to −34 mV from a holding potential of −50 mV.

TABLE 1
Results of the analysis of tail currents shown in figure 1

Components	Amplitudes		Half-Times		Dev*
	1	2	1	2	
	nAmp		*sec*		
Control	6.21	2.31	6.74	51.7	0.092
Secobarbital, 100 μM	5.94	5.51	6.00	53.9	0.123

* Dev, deviation is an estimate of the goodness of fit of the results of the tail current analysis and is analogous to the S.D. It is derived by the following formula: $\text{Deviation} = \frac{SS}{n - (NC \times 2)}$ where SS is the sum of the squares of the residuals, n is the number of points in the curve and NC is the number of components (decay processes) in the curve.

Figure 1 shows an example of slow outward current and tail current obtained in control solution and with added barbiturate, in this case 100 μM secobarbital. The raw data are shown in figure 1A whereas figure 1B shows semi-logarithmic plots of the tail currents and the best fit curves obtained from the analysis program (solid lines).

The method of the tail analysis was similar to the peeling used in compartmental analysis: 1) the slowest component of decay was determined by performing a log-linear regression, 2) the contribution of the slow component was removed from fast components by subtraction and 3) the next slower component of decay was determined. The process was repeated until there were no more points. The arrows in figure 1b mark the boundary between "comparments," *i.e.*, the points to the left of the arrows were found to be significantly above the line obtained by performing a log-linear regression on the points to the right of the arrows. Table 1 contains the results of the tail current analysis for the two curves in figure 1. The results from this experiment were exceptional in that the only change produced by the barbiturates was an increase in the amplitude of the slow component; in most cases there were slight changes in the kinetics as well (*e.g.*, see table 2). The results obtained with the peeling routine were verified by the use of alternative curve fitting techniques including nonlinear least-square fitting methods. All methods yielded best fits with equivalent numbers of compartments (two), and the estimates of the decay parameters obtained with the different methods did not differ by more than 5 to 10%.

The results of tail analysis most often yielded two components with half-times of approximately 7 and 70 sec. However, in some cases, three components were described. In these cases it was found that the tails could also be reasonably well fit with a two-component tail. In all cases in which an equally good fit could be obtained with models of differing complexity, the least complex model (that with fewest components) was assumed.

In all experiments the interval between stimuli was carefully controlled to reduce any effect of potentiation and/or desensitization. In most cases the intersimulus interval was set to be 12 min, and was kept constant for the duration of an experiment. Voltage clamp currents were conditioned with a low-pass filter (50 Hz, −18 dB/octave) and recorded on a double wide (100 mm) channel on a chart recorder (Gould 2400). Tail currents were then digitized using a graphics pad (Apple Computer) with a resolution of 200 points/cm and the data were stored on floppy diskettes for analysis. Some experiments also utilized direct on-line collection of tail currents *via* an analog-to-digital converter (Mountain Computer, Scotts Valley, CA) installed in the computer.

Concentration-response curves were fit with a nonlinear least-squares fitting program (SAS NLIN) which determined the K_D and maximal effect. Unless otherwise noted, all observations in this study are representative of at least three experiments.

Results

These results represent data obtained from *Aplysia* giant neurons during a continuous study over a period of 18 months. No seasonal differences were observed. Upon the initial electrode impalement, only those neurons which met the following criteria were used in the study of slow outward current: 1) resting membrane potential more negative than −50 mV, 2) cell input resistance greater than 1 megohm, 3) the ability to fire trains of action potentials for prolonged periods of time (greater than 10 sec) and 4) a stable slow outward current (as measured by tail current analysis) over a 2-hr control period. Of the giant neurons studied, 83 met all of the above criteria and were chosen for further study of slow outward current and the effects of barbiturates.

Tail currents accurately reflect slow outward current amplitude. The first step in this study involved determining the accuracy with which tail current analysis estimates the magnitude of slow outward current. It was hypothesized that there was no instantaneous decay of slow outward current upon repolarization; *i.e.*, that the conductance underlying the slow outward current at the end of the depolarizing pulse is the same as the conductance underlying the tail current at the beginning of the repolarization. To test this hypothesis a neuron was voltage-clamped to a holding potential of −50 mV and step depolarized to −32 mV for intervals of 10 to 180 sec. The amplitudes of tail currents measured upon repolarization were compared to the amplitudes of the developing slow outward current in four cells. Figure 2 shows the results of a typical experiment. In figure 2A is shown the amplitude of slow outward current and tail currents *vs.* time of depolarization. There is a correlative rise in both curves with increased duration of depolarization.

Based on the assumption that both of these currents are due to flux of potassium ions through the membrane, one can convert the curves to conductances as shown in figure 2B. For this figure, first the leak current was calculated from the current-voltage relationship in the hyperpolarized region. Then the conductance was calculated (after subtraction of the leak current) based on the following relationship: $g_K = I_K(E_K - E_m)$, where g_K is the potassium conductance underlying slow outward current and tail current, I_K is the potassium current, E_K is the potassium equilibrium potential (−76 mV, Russell and Brown, 1972) and E_m is the membrane potential. After the first 10 sec

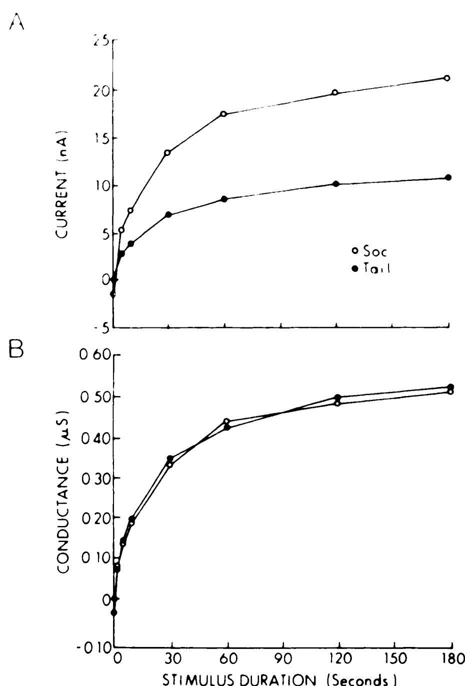

Fig. 2. Onset of slow outward current compared to the amplitude of tail current. A, ○ are the slow outward current (SOC) with increasing duration of depolarization. ●, total amplitude of tail current (A_1 plus A_2) which is obtained upon repolarization after increasing durations of depolarization. In order to directly compare the magnitude of slow outward current and tail current, both were converted to conductance in B. The average half-times for the two components in this experiment were 4.9 ± 0.3 and 84.3 ± 4.6 sec. The depolarized potential was −32 mV from a holding potential of −50 mV.

of depolarization the conductance calculated from the tail current is the same as the conductance calculated from the slow outward current. Similar results were obtained in experiments when 100 μM pentobarbital was added to the artificial sea water, *i.e.*, the time course of slow outward current development paralleled the time course of tail current development.

Illustration of the voltage dependence of slow outward current. To determine the optimum parameters for measuring the effects of barbiturates on slow outward current, the voltage-dependent onset and decay of this process were characterized. The voltage dependency of onset of slow outward current was determined by depolarizing giant cells to potentials within the activation range of slow outward current (−40 to −30 mV), and then measuring the tail currents to determine the amount of slow outward current which has developed at each potential. The results of tail analysis from a representative experiment are summarized in figure 3.

The amplitudes of the two components of tail current (A_1 and A_2 are the fast and slow components, respectively) are plotted *vs.* the depolarized potential in part A (holding potential, −50 mV). In control solutions, the slow component of tail has a higher threshold for activation (−32 mV) than the fast component (−36 mV). The addition 100 μM pentobarbital appears to shift the voltage sensitivity of both components, but the effect on the slow component is marked compared to the shift in the fast component. This is best seen in figure 3B in which the tail component amplitudes have been normalized to the percentage of increase above control level at each potential. Here the selective increase of the slow component (A_2), and the steep voltage dependence is clearly evident. There were negligible effects on tail kinetics, *i.e.*, the rate of decay of each component.

In order to determine the optimum *holding* potential for studying tail currents, the holding potential was varied in small increments around the resting potential, and tail current responses were obtained at each potential. An example of such an experiment is shown in figure 4. Here the giant neuron R2 was depolarized to −34 mV from holding potentials in the range of −60 to −36 mV. Both A_1 and A_2 increase in amplitude in the range of −60 to −44 mV. However, further depolarized holding potentials of −42 to −36 mV resulted in decreased amplitude of A_1, whereas holding potentials of −40 to −36 mV resulted in decreased amplitude of A_2. Inasmuch as these are potassium currents (Huguenard *et al.*, 1985), then the tail current magnitude can be maximized by depolarizing the cell to increase the driving force. This increase in magnitude can be seen clearly in the voltage range of −60 to −45 mV. The decreases in magnitude seen with further depolarizations are due to the activation of some slow outward current at the holding potential (*cf.* fig. 3). The tail currents will thus reflect the difference between the slow outward current activated by the depolarization and the steady-state slow outward current activated at the holding potential. In figure 4B are shown the half-times for the two components of tail decay obtained with different holding potentials. As was generally the case in this study, the alterations of the tail current were produced by changes in the amplitude but not the kinetics of exponential decay which make up the tail current.

Based on the above findings, the holding potential for barbiturate experiments was set at −50 mV and the slow outward current eliciting voltage was between −32 to −30 mV, or the most depolarized voltage that could be obtained without loss of voltage clamp control.

Effects of barbiturates on tail currents. Several barbiturates were examined in this study, including pentobarbital, phenobarbital, secobarbital and amobarbital. The general effects of barbiturates will be presented first, and then the specific results obtained with each compound will be discussed.

The results of tail analysis were used as a means of quantitating slow outward current and enhancement of slow outward current by barbiturates. In nearly every case, the major effect of pentobarbital and other barbiturates was an enhancement of the amplitude of the slower component of tail delay, A_2. Figure 5 demonstrates the time course of effects produced by barbiturate in a typical experiment (in this case phenobarbital). Notice the marked increase in A_2, compared to A_1, that is obtained with increasing concentrations of phenobarbital in the upper panel of figure 5. These raw current values have been normalized to percentage of control in the lower panel. Here it can be seen clearly that phenobarbital produces a concentration-dependent, selective increase in the slow component of tail current decay. Notice the slow reversal of barbiturate effects that is obtained over a period of 2 hr of wash with control artificial sea water. This is much slower than the bath turnover time of these experiments (less than 5 min).

The half-times of both the fast and slow decay components were relatively unaffected by changes in barbiturate concentration. However, at high concentrations (greater than 200 μM) there was a marked slowing in the slower component. Figure 6 depicts several tail currents on a semi-logarithmic plot. All of the tail currents were produced by the same stimulus, a 60-sec voltage clamp depolarization to −34 mV from a holding poten-

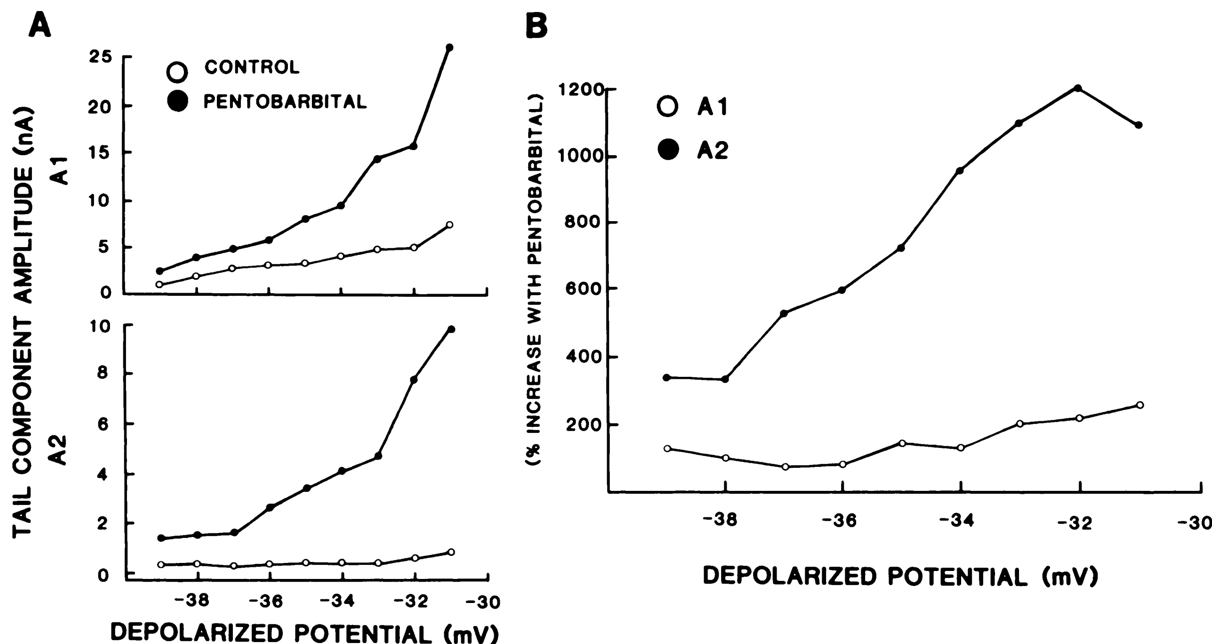

Fig. 3. Voltage sensitivity of the onset of two components of tail current; alterations by 100 μM pentobarbital. A, the amplitude of the fast component (A_1) and the slow component (A_2) of tail current are plotted against the depolarized potential. B, the changes produced by pentobarbital are normalized by plotting them as the percentage of increase above control levels. Pentobarbital is shown to alter the voltage dependence of onset of both components, but the effect on A_2 is more pronounced. Average half-times of the two components in this experiment were 8.2 ± 0.4 and 69.8 ± 3.2 sec. Holding potential, 50 mV.

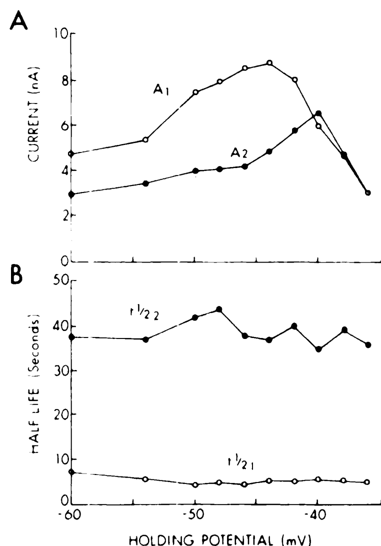

Fig. 4. Dependence of tail current on holding potential. The membrane holding potential was set at various levels between −60 and −36 mV and tail currents were obtained after eliciting slow outward current with a depolarization to −32 mV. A, amplitudes of the two components of tail current decay *vs.* holding potential. B, half-times of the two components of tail current decay *vs.* holding potential. The open symbols represent the parameters (amplitude and half-time) of the fast component of decay, whereas the closed symbols represent the parameters of the slow component of decay. The depolarized potential in this experiment was −32 mV.

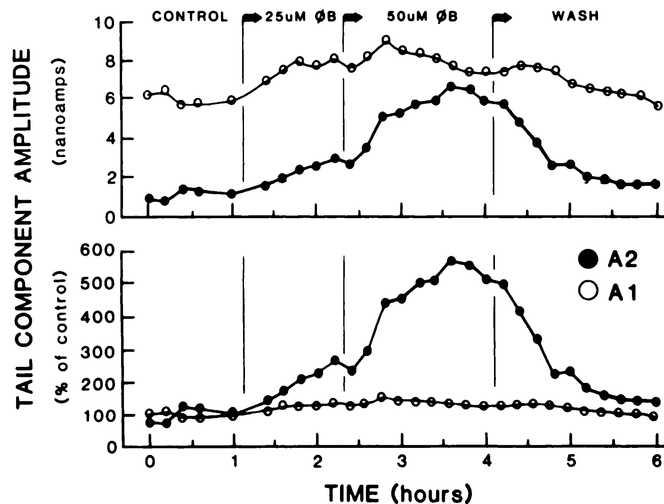

Fig. 5. Time course of phenobarbital effect during wash-on and wash-off. Control responses were obtained, followed by sequential application of 25 and 50 μM phenobarbital (φB), and then wash. In the upper panel the amplitudes of the two components of tail current (A_1 and A_2) during the course of the experiment have been plotted. In the lower panel these values have been normalized to percentage of control. The average half-times of the two components in this experiment were 8.6 ± 0.4 and 80.9 ± 6.5 sec. The stimulus was a 60-sec depolarization to −33 mV from a holding potential of −50 mV every 12 min.

tial of −50 mV. Increasing the concentration of pentobarbital up to 200 μM results in a selective increase in A_2, with little effect on kinetics or amplitude of A_1. However, when the barbiturate concentration was increased to 400 μM, there is a marked prolongation of the slow component. Also, with concentrations of barbiturates that slowed the tail current kinetics, there was generally an outward shift in the holding current. Table 2 is a summary of the results of the tail analysis from the single experiment shown in figure 6.

Again, it can be seen that the major changes induced by pentobarbital are concentration dependent increases in A_2, and a slight slowing of the kinetics with higher concentrations of pentobarbital.

Most experiments involving barbiturate enhanced tail cur-

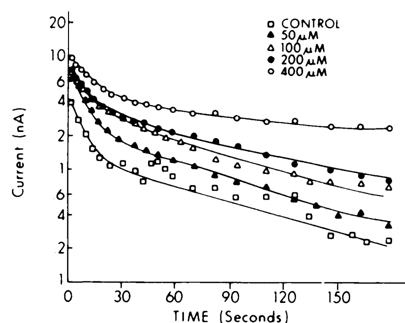

Fig. 6. Semilog plots of tail currents obtained in control and with several concentrations of pentobarbital. The stimulus was a 60-sec depolarization to −30 from a holding potential of −50 mV. The results of tail analysis for the curves in this figure are given in table 2.

rent had very similar results to those shown in table 2. For example, in 18 representative experiments conducted over a 35-day period, the amplitude A_2 in control was ± 1.10 nA (average ± S.D.), and the maximum enhancement by barbiturates was within the range of 400 to 500% of the control value. However, in few experiments (n = 3), the enhancement of A_2 was much greater than average, and sometimes the effect was as striking as a 2000% increase over control. The amplitude of A_1 in control was 3.76 ± 1.61 nA, and the maximum effect of barbiturates on this parameter was a 50 to 60% increase. In two cases the major effect of barbiturates was an enhancement of amplitude of the *fast component* of tail current, A_1, rather than the slow component, A_2.

In experiments in which spike frequency adaptation responses were obtained, there was a good correlation between enhanced slow adaptation (after the first 5 sec) and the effects on slow outward current tails. There were minimal effects on fast adaptation (between 0 and 10 sec). However, with high doses of barbiturates, there was an increase in the leak conductance of the cell, as evidenced by a steeper slope conductance in the hyperpolarized region of −60 to −90 mV. Under these circumstances there was a decrease in the *initial* firing rate, as well as late firing rate, in response to a prolonged constant current stimulus.

As described above, barbiturates had very little effect on the decay kinetics of slow outward current at moderate concentrations. Thus, we examined the effects of barbiturates on the *onset* kinetics of slow outward current. Because it was difficult to directly measure the onset kinetics of slow outward current, we devised experiments to study these kinetics indirectly using tail current analysis. Slow outward current was elicited with depolarizations of increasing duration, and the results of analysis for each tail current was used as a measure of the slow outward current which had developed during the corresponding time interval. These responses were obtained in control, and then the alterations produced by barbiturate were studied.

In figure 7 are shown the results of such an experiment. This figure shows the time-dependent changes in tail component amplitude obtained with prolonged depolarizations (up to 740 sec). The major effect of pentobarbital is that of a sharp increase in the rate at which A_2 rises with time of depolarization. With long depolarizations there is a barbiturate induced enhancement of the rate of development of A_1 as well as A_2. The major effect, however, is the enhancement of A_2. At 400 sec of depolarization, for example, there is approximately a 50% increase in A_1 but a *1000%* increase in A_2.

Specific Effects of Individual Barbiturates

Pentobarbital. The results from eight experiments in which concentration response data were obtained for pentobarbital are graphically depicted in figure 8. The amplitude of A_1 shows a concentration-dependent increase, although this effect was not saturable within the concentration range tested.

The relationship between concentration and A_2 is somewhat clearer. This curve shows the typical sigmoidal shape that is obtained with a log-linear plot of a saturable receptor activated process. However, the curve is rather steep, and rather than going from minimum to maximum effect within two orders of magnitude, the full range of effects occurs within *one* order of magnitude. The best fit curve for the concentration response curve of the amplitude of A_2 *vs.* pentobarbital concentration was obtained with a K_D of 98 μM and a maximum effect of 3.2 nA (not shown). As mentioned previously, there was little effect on either rate constant by pentobarbital until very high concentrations (>200 μM) were used. Also, it should be noted that the increase in A_1 produced by pentobarbital was somewhat atypical compared to other barbiturates. With most compounds, there was little effect on A_1 across the entire concentration range tested.

Phenobarbital. The effects of phenobarbital were tested in nine experiments. In the concentration response curves shown in figure 9 it can be seen that there is a saturable effect on the amplitude of A_2, with very little effect on A_1. For the effects on A_2, the best fit curve was obtained with an apparent K_D of 115 μM and the maximum increase was 3.02 nA (not shown).

Stereoisomers of barbiturates. We were able to obtain limited amounts of the stereoisomers of pentobarbital and secobarbital for this study. In every experiment (n = 5), and at every concentration where the R (+)- and S (−)-isomers of these two compounds were compared for activity in enhancing

TABLE 2
Results of tail current analysis from the experiment shown in figure 6

Components	Amplitudes[a]		Half-Times[a]		n
	1	2	1	2	
	nAmp		*sec*		
Control	4.76 (0.57)	1.01 (0.16)	3.72 (0.38)	57.1 (3.7)	6
Pentobarbital					
50 μM	6.87 (0.44)	2.18 (0.17)**	4.94 (0.47)	69.2 (17.9)	4
100 μM	6.74 (0.37)	3.30 (0.32)**	6.32 (0.60)**	67.9 (20.6)	6
200 μM	5.72 (0.21)	3.47 (0.20)**	6.93 (0.74)**	99.9 (14.8)	5
400 μM	6.33 (0.22)	4.88 (0.13)**	6.85 (0.40)**	147 (12)**	6

[a] Average and (S.E.M.).
** P < .001 compared to control, two-sided *t* test.

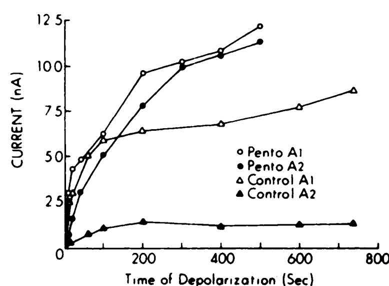

Fig. 7. Onset kinetics during depolarization; effects of pentobarbital (Pento) compared to control. The stimulus was depolarization to −30 mV from a holding potential of −50 mV. [Pento] = 100 μM. The average half-times of the two components in this experiment were 7.9 ± 0.3 and 66.3 ± 3.6 sec.

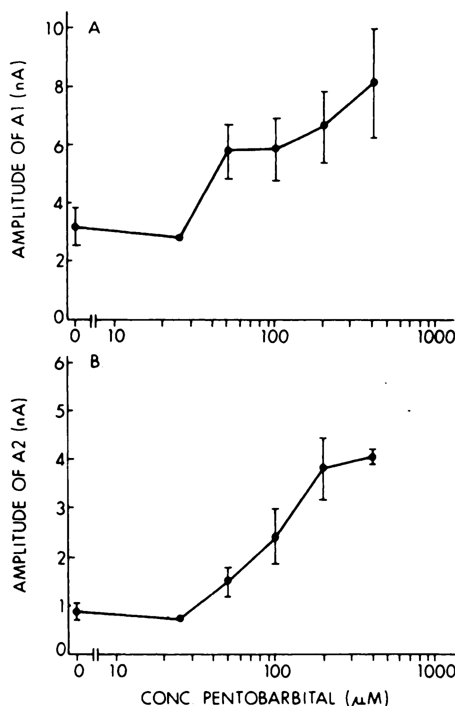

Fig. 8. Concentration (CONC)-response relationships for alteration of the amplitude of the two components of tail current by pentobarbital. The error bars represent ± S.E.M.; n = 8 neurons. Linear regression of the CONC-response curves yielded slopes of 2.77 (A_1) and 3.23 (A_2) nA/10-fold change in pentobarbital concentration. The correlation coefficients were 0.57 (P < .002) and 0.31 (P < .002), respectively.

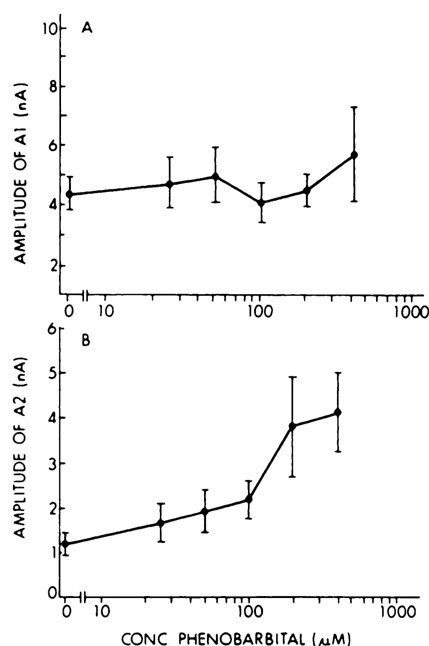

Fig. 9. Concentration (CONC)-response relationships for phenobarbital alteration of the amplitude of the two components of tail current. The error bars represent ± S.E.M., n = 9 neurons. Linear regression of the CONC-response curves yielded slopes of 0.11 (A_1) and 2.49 (A_2) nA/10-fold change in phenobarbital CONC. The correlation coefficients were 0.0009 (not significant) and 0.40 (P < .001), respectively.

tail current, the S (−)-isomer was more effective than the R (+)-isomer. Figure 10 shows typical results from an experiment in which secobarbital was tested. Figure 10A shows the slow outward current and tail current responses obtained with the two stereoisomers of secobarbital. With the R (+)-isomer, there is little difference between control, 10 and 30 μM, but 100 μM changes the responses markedly. The S (−)-stereoisomer appears to be more potent; graded increases in both slow outward current and tail current are seen with each increase in concentration. Figure 10B shows the adaptation responses obtained for the same experiment. Here the effects of either stereoisomer can easily be seen with each increase in concentration. At each concentration tested, the S (−)-isomer was more effective than the R (+)-isomer in the inhibition of sustained firing. Figure 11 shows the concentration-response relationship for the amplitude of tail components with the stereoisomers of secobarbital from the experiment shown in figure 10. Here it can easily be seen that the S (−)-isomer was more effective at every concentration than the R (+)-isomer at enhancing the amplitude of the slow component (A_2). In a few experiments, such as the one depicted in figure 11, there appeared to be affects on adaptation at concentrations which had no apparent effect on tail current parameters. This lack of sensitivity of tail current analysis in these cases was most likely due to the inability to depolarize sufficiently to simulate the average interspike potential achieved during the elicited spike train. Stronger depolarizations resulted in loss of membrane potential control due to incomplete space clamp, and therefore in these cells the slow outward current activation produced in voltage clamp did not perfectly model the membrane currents activated during a prolonged spike train. With the very strong voltage dependence of the barbiturate effect (see fig. 3), it is not surprising that insufficient depolarization (by even 1 or 2 mV) would result in difficulty in observing the effect on tail current. It should be emphasized that sufficient voltage clamp depolarizations were obtained in the majority of the neurons studied, and that the minimum effective concentrations for tail current augmentation generally coincided with the minimum effective concentration for effects on adaptation.

Other barbiturates. The effects of amobarbital and secobarbital on tail currents were tested in only a limited number of experiments and concentration-response relationships were not determined. In general, both of these compounds produced results more similar to pentobarbital than phenobarbital. That is, in addition to the increase in A_2, there was some enhancement of A_1. In three of five experiments with secobarbital, and in one of two with amobarbital, there was an increase in the amplitude of the fast component of greater than 50% compared to control. This compares to increases in three of five experiments with pentobarbital, zero of nine experiments with di-

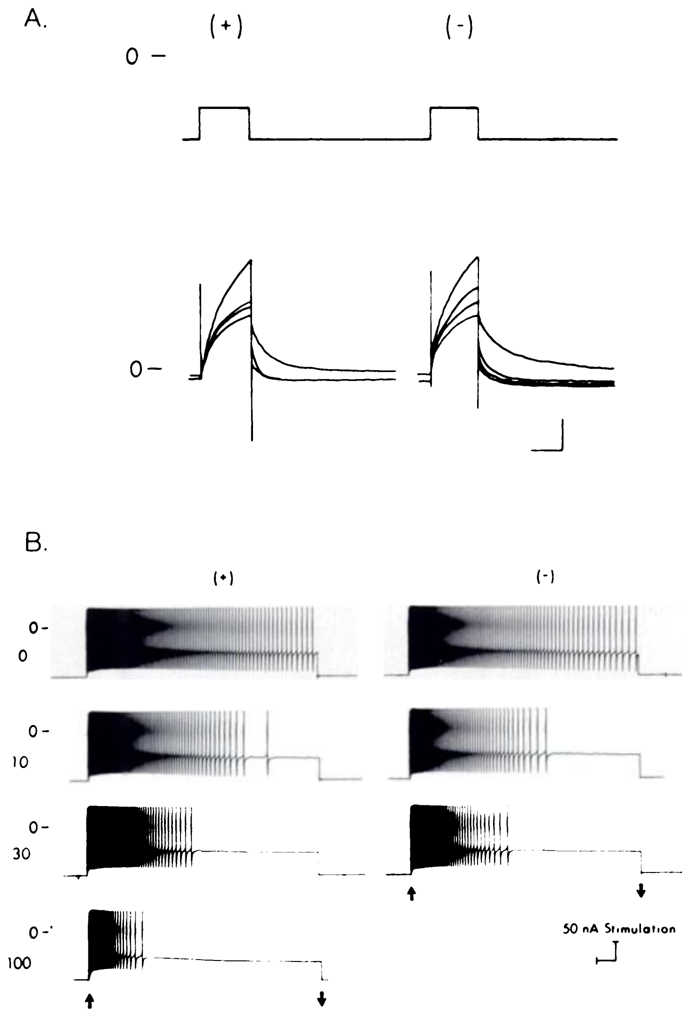

Fig. 10. Effects of stereoisomers of secobarbital on slow outward current and adaptation. A, slow outward current responses in control and with 10, 30 and 100 μM of the *R* (+)- and *S* (−)-isomers of secobarbital. Upper trace is voltage, lower trace is current. The stimulus in this case was a 60-sec depolarization to −30 from a holding potential of −50 mV. Calibration: vertical = 20 mV, 10 nA; horizontal = 30 sec. B, alterations in adaptation responses produced by stereoisomers of secobarbital. The change in adaptation can be seen most readily as the decreased duration of firing in response to the constant current pulse. The entire series of responses was performed for (+)-secobarbital, then a wash was obtained (0 μM (−)-secobarbital), and the series was repeated with the different concentrations of (−)-secobarbital. Concentrations (micromolar) shown to the left of the traces. The neuron died before it was possible to obtain an adaptation response in 100 μM (−)-secobarbital. The adaptation stimulus was 50 nA of depolarizing current in current clamp after being held at a −50 mV in voltage clamp. Calibration: horizontal = 5 sec; vertical = 20 mV.

phenylbarbituric acid (Huguenard and Wilson, 1985) and zero of nine experiments with phenobarbital.

Discussion

Slow outward currents have been demonstrated recently in a number of vertebrate systems, including guinea-pig and rat hippocampal pyramidal cells (Brown and Griffith, 1983; Bragdon and Wilson, 1982), rat hippocampal cells in culture (Segal and Barker, 1984), cat spinal motoneurons (Barrett *et al.*, 1980) and mouse neuroblastoma cells (Moolenaar and Spector, 1979). Although the slow outward currents in these vertebrate cells are much faster (usually less than 1 sec to peak) than the slow outward currents presented in this study (increasing over at least 10s of sec, see fig. 7), the firing behavior of vertebrate cells is clearly affected by these relatively slow currents. It is possible that a similar barbiturate sensitive outward current exists in the vertebrate central nervous system which would serve to regulate repetitive firing. At this point, however, we know of no reports of such a current. Alternative hypotheses that have been put forward for selective anticonvulsant barbiturate effects (especially phenobarbital) include the augmentation of inhibitory GABA responses and selective reduction in glutamate-mediated excitation at doses which exert no GABAmimetic actions (Macdonald and Barker, 1978, 1979; Schulz and Macdonald, 1981). Nevertheless, it remains unclear which properties of barbiturates are most important for anticonvulsant action.

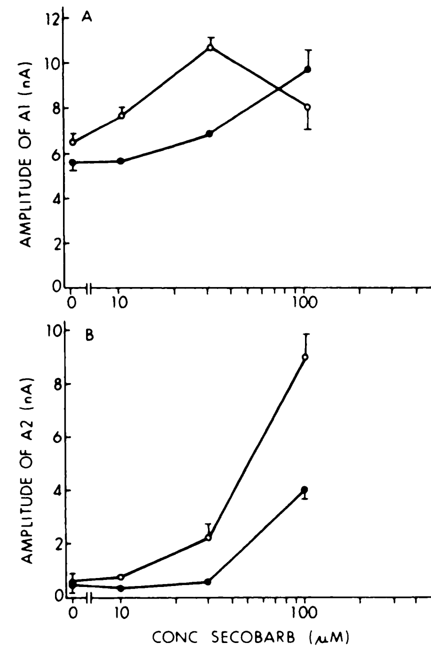

Fig. 11. Concentration (CONC)-response relationship for tail component amplitudes obtained with stereoisomers of secobarbital. Data from same experiment in figure 10. (O, *S* (−)-secobarbital; ●, *R* (+)-secobarbital.) The error bars represent ± S.E.M. of each value from within a single experiment in which five tail currents were obtained with each CONC.

In this study, we have validated the use of tail current analysis as a means of quantitating slow outward current. This was undertaken because slow outward current affects spike frequency adaptation in neurons, and this process is so profoundly enhanced by barbiturates (Zbicz and Wilson, 1981). Using this means, it has been shown that barbiturates result in a relatively specific alteration in one parameter describing the tail current, *i.e.*, an increase in the amplitude of the slow component. This increase is stereospecific with the *S* (−)-stereoisomers of pentobarbital and secobarbital being more potent than the *R* (+)-compounds. This stereospecificity matches that of the enhanced adaptation (fig. 10) and physiologically measured responses in other systems, such as enhancement of GABA responses in cultured spinal neurons and anesthesia in mice (Huang and Barker, 1980; Waddell and Baggett, 1973).

The enhancement of slow outward current by barbiturates, as well as being stereospecific, is concentration-dependent and approaches a maximum. Thus, it appears that slow outward

current enhancement by barbiturates is a receptor-mediated phenomenon rather than a nonspecific effect. However, if this is a receptor-mediated phenomenon, it is not a simple one. The steepness of the concentration-response curves requires a Hill coefficient of approximately 2 for the best fit to the data, and therefore there may be some type of cooperativity involved in the response. A further test of receptor mediation of this process is the determination of the correlation (or lack of it) between activity at enhancing the slow outward current and the octanol-water partition coefficient, the latter being closely associated with anesthetic activity (Hansch and Anderson, 1967). The potency series for slow outward current enhancement is: diphenylbarbituric acid > pentobarbital > phenobarbital, (K_D values of 3.1, 98 and 110 μM, respectively; diphenylbarbituric acid data from Huguenard and Wilson, 1985). The rank of the log of partition coefficients is the same: diphenylbarbituric acid > pentobarbital > phenobarbital (log partition coefficients = 2.19, 1.95, and 1.42, respectively). The partition coefficients were determined by the method of Hansch and Anderson (1967) and compiled by Henry *et al.* (1976). Although the rank order of potency (of slow outward current enhancement) is the same as the rank order of log partition coefficient, there was not a strong correlation between these two properties within this admittedly small sample of barbiturates ($r^2 = 0.68$).

The enhancement of the slow outward current is produced clearly by all barbiturates that have been studied, including two that are less sedative, phenobarbital and diphenylbarbituric acid. We conclude, therefore, that enhanced slow outward current is less likely to be associated with a general depressant effect leading to sedative/anesthetic actions, but rather is associated with other neuronal actions of the barbiturates, possibly some of the anticonvulsant effects. The reasons for this conclusion are 2-fold, 1) slow outward current enhancing activity is not well correlated a property of barbiturates (oil-water partition coefficient) which are related to general depressant action and 2) diphenylbarbituric acid which shows little or no sedative action in mice and rats, but is an effective anticonvulsant (Raines *et al.*, 1975, 1979), is very potent in the enhancement of slow outward current (Huguenard and Wilson, 1985).

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