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Barbiturate Effects on Acetylcholine-Activated Channels in *Aplysia* Neurons

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SUMMARY

The effects of pentobarbital, phenobarbital, amobarbital, and diphenylbarbituric acid were studied by examining the average lifetime and conductance of acetylcholine-activated sodium channels in *Aplysia* neurons. Although none of the barbiturates tested modified the conductance of single-ion channels, pentobarbital and amobarbital had profound effects on channel lifetime. In the absence of barbiturate, relaxations in response to voltage jumps during steady-state current responses to acetylcholine have a single-exponential time course. In the presence of pentobarbital (75–500 μM), current relaxations consist of two exponential components that take the same direction as control relaxations. The faster component becomes faster and relatively larger at higher pentobarbital concentrations, while the slower component always has the same time constant as control. These results are not consistent with a sequential model of channel blockade described for local anesthetics, in which blocked channels must become unblocked before channel closure can occur. Kinetic data are better explained by a cyclic model in which blocked channels have the same probability of closing as nonblocked channels. Alternatively, the results can also be explained by a two-site model in which one binding site regulates the susceptibility of channels to the effects of the barbiturate, whereas occupation of the second site determines the extent of changes in channel lifetime. The effects of amobarbital were qualitatively similar to those of pentobarbital, while phenobarbital and diphenylbarbituric acid did not alter current relaxations at similar concentrations.

INTRODUCTION

Barbiturates may exert their anesthetic and anticonvulsant effects by attenuating excitatory synaptic transmission in the central nervous system. Barbiturates act postsynaptically to suppress excitatory responses to ACh[2] and glutamate in the mammalian central nervous system (1, 2), to glutamate in cultured mouse spinal neurons (3), to ACh and carbachol at the frog neuromuscular junction (4, 5), to ACh and glutamate in *Helix* (6), and to ACh and γ-aminobutyric acid in *Aplysia* (7, 8).

The finding that barbiturates attenuate excitatory responses to several different transmitters suggests that barbiturates may not be acting at the postsynaptic receptor, but may be altering the properties of ion channels opened by transmitter. These experiments were intended to explore the effects of pentobarbital, phenobarbital, amobarbital, and diphenylbarbituric acid on the average lifetime and conductance of ACh-activated sodium channels in *Aplysia* neurons.

Single-channel conductance has been calculated from measurements of the average currents flowing through individual ion channels, as determined from the size of current fluctuations produced by ACh. During a steady-state current response to ACh, currents flowing through a large number of channels summate to produce a mean response. As individual channels open and close at random, however, the total current will fluctuate slightly about its mean level. The amplitude of these current fluctuations, measured in terms of the variance about a mean level, is an indication of the magnitude of the currents flowing through single-ion channels (9).

The average lifetime of open channels has been determined from relaxation experiments based on the finding that channel lifetime is voltage-dependent (10). During steady-state current responses to low concentrations of ACh, voltage clamp command pulses produce a change in current that normally consists of two components. After subtraction of capacitive transients, the current first appears to change almost instantaneously due to a

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[2] The abbreviations used are: ACh, acetylcholine; PB, sodium pentobarbital; PhB, sodium phenobarbital; AmoB, sodium amobarbital; DPB, diphenylbarbituric acid.

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change in driving force on the permeant ions, then relaxes exponentially to a new steady-state level. This relaxation occurs because the channels remain open longer at hyperpolarized potentials than at depolarized ones, and the time constant of the relaxation reflects the average lifetime of an open channel at the new potential. If the channel opening reaction is written simply as

$$\text{closed} \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} \text{open}$$

where α and β are the observed rate constants for transition between the two states, and α decreases with membrane hyperpolarization, then the time constant of current relaxations is simply $\tau = 1/(\alpha + \beta) \cong 1/\alpha$ for $\beta \ll \alpha$ (see Methods) (11–13).

We have found that relaxations measured in the presence of barbiturates consist of two exponential components, and the time course of these relaxations has been compared with the predictions of several models of drug action.

In particular, the results have been considered in terms of a sequential channel blocking model suggested previously for barbiturates (14, 15):

$$\text{closed} \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} \text{open} \underset{b}{\overset{fd}{\rightleftharpoons}} \text{blocked}$$

where α, β, f, and b are rate constants, and d is the drug concentration. According to this sequential model, receptor-channel complexes can be blocked only after they have been activated by transmitter and are in an open, conducting state. Blocked channels must again pass through a conducting state before they can close. This model predicts that current relaxations in response to voltage jumps should have a double exponential time course defined by (16, 17):

$$\frac{1}{\tau_f}, \frac{1}{\tau_s} = \frac{1}{2}(\alpha + \beta + fd + b) \pm \frac{1}{2}[(\alpha + \beta + fd + b)^2 - 4(\alpha b + \beta fd + \beta b)]^{1/2}$$

where $\tau_f < \tau_s$ and corresponds to the plus sign before the square root term. These experiments were therefore designed specifically to determine whether barbiturates are also channel blockers in *Aplysia*, although other possible models of drug action were also considered.

METHODS

The methods used in these experiments have been described previously (18). Briefly stated, right pleural-pedal ganglia were dissected from *Aplysia californica*, and the connective sheath surrounding the right pleural ganglion was slit with a razor blade to expose individual cell bodies. ACh (Sigma Chemical Company, St. Louis, Mo.) was applied by iontophoresis to neurons in the lower portion of the pleural ganglion which responded with an increase in permeability to sodium ions (10). The iontophoretic electrode was positioned far from each cell to permit the gradual development of a steady-state response over a period of several seconds. Except at high barbiturate concentrations (0.5–1 mM), steady-state responses were always much less than the maximal response that could be elicited to ensure that $\beta \ll \alpha$.

Cells were clamped with a single-electrode voltage clamp (19) switching at a frequency of 5 kHz. Approximately 2 msec are required to establish the new voltage following a voltage clamp command pulse, and current relaxations were measured beginning 2 msec following the voltage jump. Extensive testing has demonstrated that the single-electrode voltage clamp provides an accurate reflection of the time course of current relaxations once the new voltage is established (18).

Relaxation experiments utilized 50-mV hyperpolarizing voltage clamp commands. The command pulse was slowed to prevent ringing, and currents were filtered through a 750-Hz six-pole low-pass Bessel filter (Frequency Devices). Voltage jumps were performed in the absence and presence of ACh, and the difference between the two traces, representing the ACh-induced current (11), was recorded in analogue form by an *X-Y* plotter (MFE Corporation, Salem, N. H.). ACh-induced current relaxations were smoothed by eye and digitized using a Hipad digitizer, and time constants were determined by computerized nonlinear regression analysis. Single-exponential relaxations seen in the absence of barbiturate were described by a time constant, τ_{con}, where the current I at time t is $I(t) = Ae^{-t/\tau_{con}}$, and A is the relaxation amplitude. Double-exponential relaxations measured in the presence of barbiturate were expressed as the sum of two components, so that $I(t) = A_f e^{-t/\tau_f} + A_s e^{-t/\tau_s}$, where $\tau_f < \tau_s$.

Elementary currents (i_{el}), or the average currents flowing through single-ion channels, were calculated from the ratio σ^2/I, where σ^2 is the variance of current fluctuations produced by ACh (9), determined using an RMS/DC converter (Analog Devices, Norwood, Mass.). Currents were filtered through a 1.5-Hz high-pass first-order filter and a 200-Hz low-pass Butterworth active filter (48db/oct, Frequency Devices), and measured values of σ^2 were corrected to compensate for attenuation of low-frequency components below 1.5 Hz. Elementary conductance (γ) was calculated from the slope of a plot of i_{el} as a function of membrane potential.

Barbiturate solutions were always prepared immediately prior to use. Unless specified, each barbiturate was applied at its pK_a: 8.0 for PB (Sigma Chemical Company), 7.3 for PhB (courtesy of Sterling Winthrop, New York, N. Y.), 7.9 for AmoB (courtesy of Eli Lilly, Indianapolis, Ind.), and 9.4 for DPB (courtesy of Dr. A. Raines).

RESULTS

Elementary conductance. None of the barbiturates tested modified γ. Figure 1 illustrates the results of a typical experiment in which γ was 9.4 pS. In the presence of PB, i_{el} was unchanged at several membrane potentials. If channel blockade does not occur, then channels are occluded in an all-or-none fashion and do not pass through a state of partial conductance.

PB relaxations. In the absence of barbiturate, relaxa-

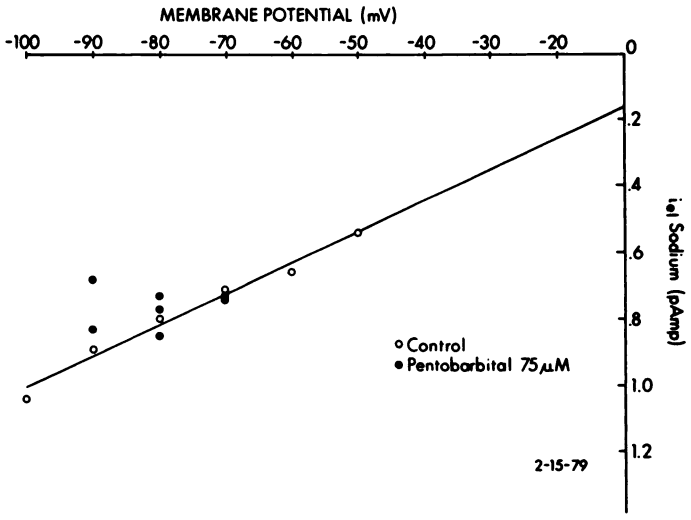


FIG. 1. *Effect of PB on elementary conductance*

i_{el} is plotted as a function of voltage, and the extrapolated equilibrium potential is +8 mV; γ = 9.4 pS. The line is a least-squares fit (room temperature).

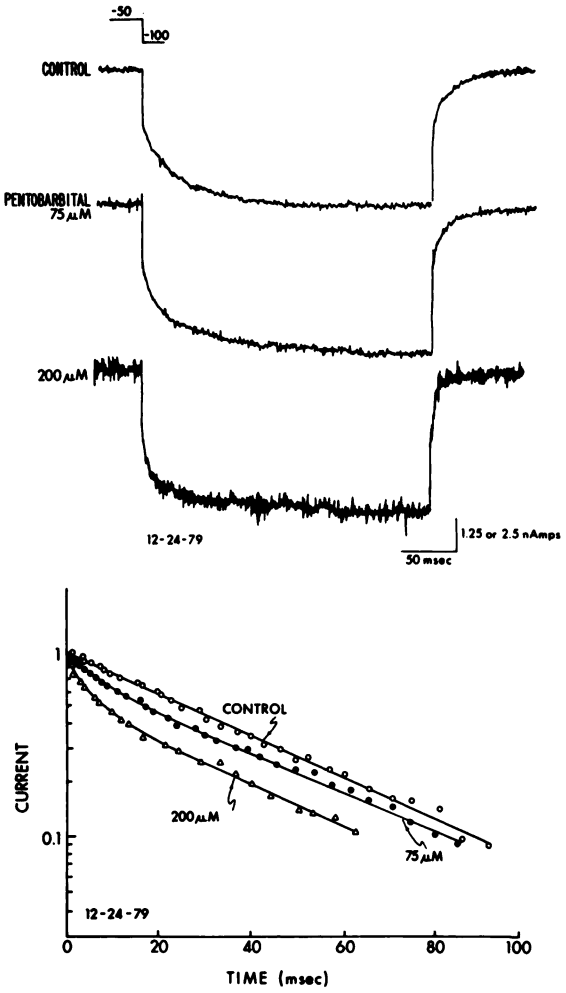


FIG. 2. *ACh-induced current relaxations and semilogarithmic plots*

A. ACh-induced current relaxations measured in the absence and presence of PB (temperature 11°).

B. Semilogarithmic plots of relaxations at −100 mV. *Solid lines* represent computer fits consisting of a single exponential in control, or the sum of two exponential components for the barbiturate relaxations.

tions could be described by a single exponential with a time constant τ_{con}. In the presence of PB concentrations >50 μM, current relaxations occur much faster than in control (Fig. 2). Semilogarithmic plots show that PB relaxations measured during the first 100 msec following the voltage jump are not single exponentials, but can be represented by the sum of two exponential components with time constants τ_f and τ_s ($\tau_f < \tau_s$) and amplitudes A_f and A_s. PB relaxations always take the same direction as control relaxations. No "inverse" or outward components were ever observed in response to hyperpolarization, even during pulses lasting several minutes. Changes in the relaxations were apparent 10 min after application of PB, and were complete after 20 min. Below 50 μM, two separate components could not be isolated because $A_f \ll A_s$. The time constants did not depend on the dose of ACh or the amplitude of the steady-state current response.

Beginning about 100 msec after the voltage jump, a third component was often visible (see relaxations illustrated in ref. 20). This component is much slower than the slow component of the relaxations and does not appear to be related to single-channel kinetics. Even in the absence of ACh, the baseline holding current often became more and more inward when cells exposed to the higher concentrations of barbiturate were maintained at hyperpolarized potentials. Application of ACh enhanced this large increase in inward current, and a slowly increasing inward current was therefore seen superimposed on ACh-induced relaxations at hyperpolarized potentials. This current probably arises from a membrane conductance that is activated directly by barbiturates (7, 21), which may be both voltage- and ACh-sensitive. Alternatively, the inward current may be the result of nonspecific leakage effects arising from stresses imposed on the membrane when it is hyperpolarized during states of high conductance, such as during the application of ACh. This extremely slow current was not considered when examining the effects of barbiturates on ACh-induced current relaxations, and all semilogarithmic plots are based on data obtained during the first 100 msec following the voltage jump. Relaxations approached zero-current asymptotes drawn to current values measured at 150–200 msec, where the size of the slow current was always less than 10% of the total relaxation amplitude.

The fast component. As the concentration of PB is increased, τ_f becomes faster and the ratio A_f/A_s increases. If this fast component of the relaxations were due to blockade of open channels, then assuming $fd \gg \alpha$ (16, 17),

$$\frac{1}{\tau_f} = fd + b$$

and $1/\tau_f$ should be a linear function of the drug concentration. As shown in Fig. 3, a plot of $1/\tau_f$ as a function of PB concentration is approximately linear. If this model were applicable, then the slope f would be 1.2 sec^{-1} μM^{-1}, and the intercept b would be 24/sec at −100 mV.

Figure 4 illustrates the voltage dependence of τ_f at 75

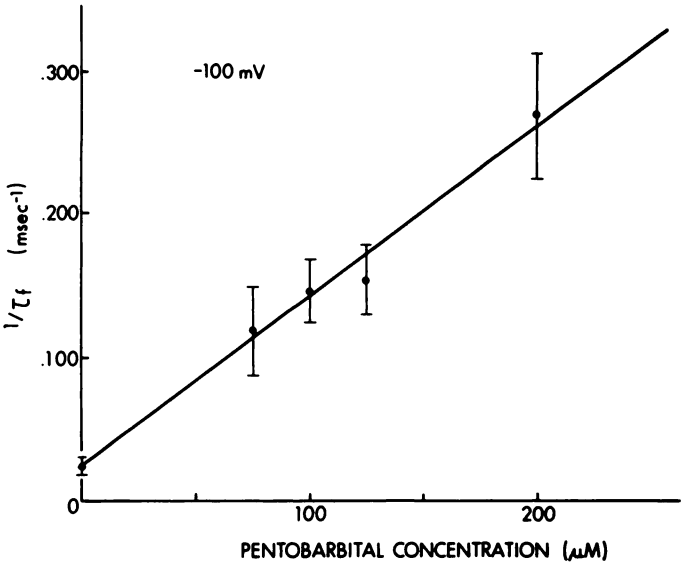


FIG. 3. *Linear relationship between $1/\tau_f$ and PB concentration*

Values shown were measured at −100 mV. Error bars indicate ± standard deviation (temperature 10–12°).

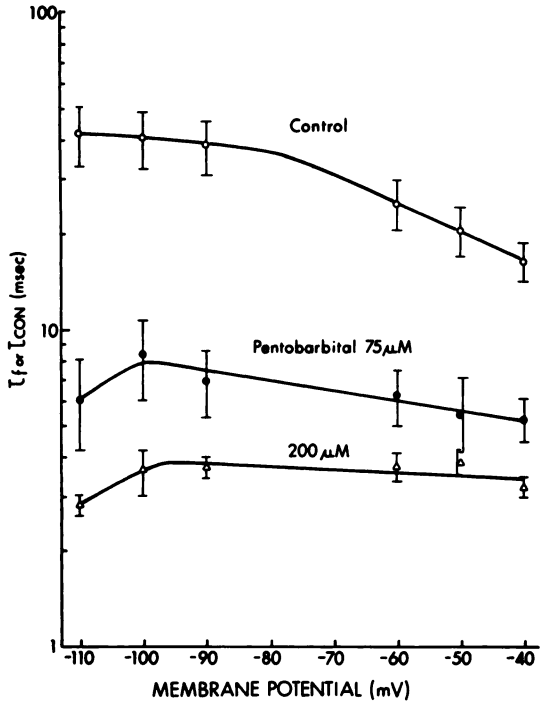


FIG. 4. *Voltage dependence of τ_f*

Control relaxations could be described by only one time constant. Error bars indicate ± standard deviation (temperature 10–12°). Lines have been drawn by eye to fit the data points.

μM and 200 μM PB. A relative decrease in τ_f at −110 mV was observed consistently, and may possibly reflect a voltage-dependent enhancement of barbiturate action. In the range of −40 to −100 mV, however, the effects of PB were not markedly influenced by voltage.

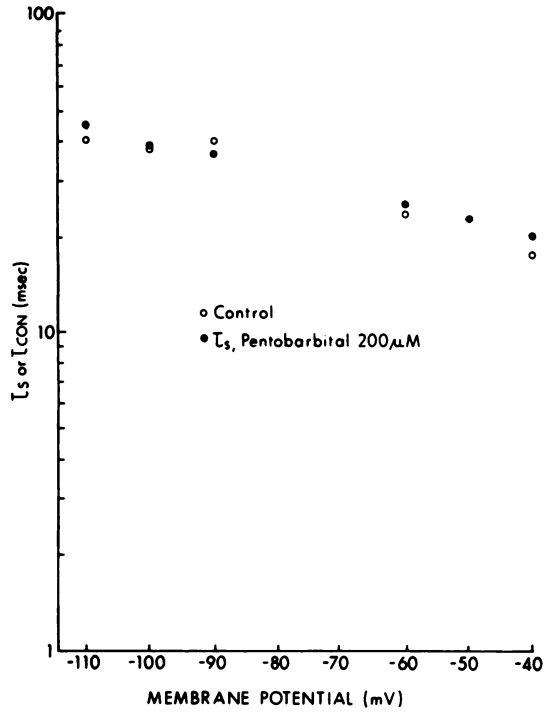
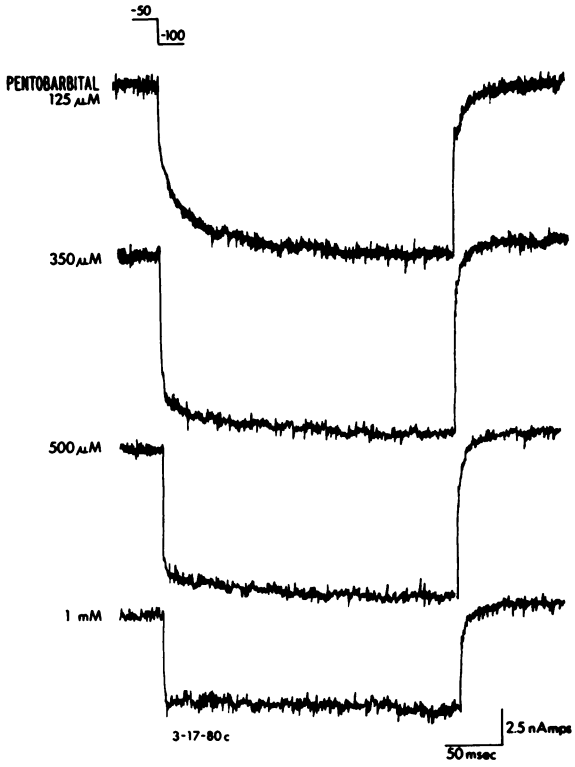


FIG. 5. *Voltage dependence of τ_s*

τ_s is indistinguishable from τ_{con} at all voltages tested. Each point represents the mean of three to five independent measurements (temperature 10–12°).



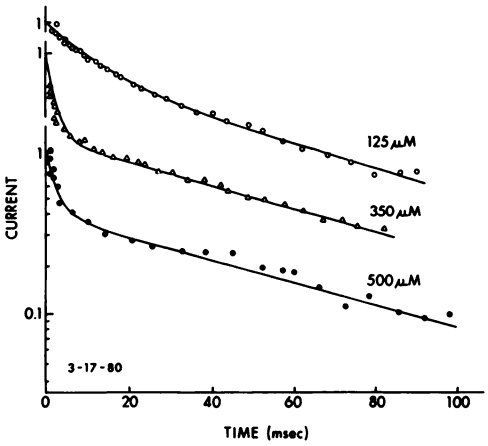


FIG. 6. *Effects of high concentrations of PB on current relaxations*

A. Current relaxations in response to voltage jumps from −50 to −100 mV (temperature 10°).

B. Semilogarithmic plot of relaxations at −100 mV. τ_f cannot be measured accurately at PB concentrations 200 μM, but τ_s still has the same value as τ_{con}.

The slow component. τ_s always has about the same value as τ_{con}, and does not become slower at higher drug concentrations. At 200 μM PB (Fig. 5), τ_s is indistinguishable from τ_{con} at all voltages tested ($p > 0.85$ by paired *t*-test). This finding is not consistent with the sequential model of blockade of open channels, in which τ_s should become even slower at higher drug concentrations (16, 17):

$$\frac{1}{\tau_s} = \frac{\alpha b}{fd + b} + \beta$$

Figure 6 illustrates current relaxations obtained during exposure to extremely high concentrations of PB. The slow component is still visible at 500 μM, although $A_s \ll A_f$. τ_s is not appreciably slowed, even though τ_f is extremely fast. At 1 mM PB, the slow component has disappeared completely. The fast component is virtually indistinguishable from the jump, and no current relaxation is visible at −100 mV. The apparent "off" relaxation observed upon depolarization to −50 mV is due to a K-current that is enhanced by ACh (10).

The finding that τ_s does not become slower than τ_{con} is consistent with the effects of PB on the decay of inhibitory postsynaptic currents in *Aplysia* (22). Currents recorded in the presence of PB exhibit biphasic decays, but the slower component is not slower than control. This observation is also not consistent with the sequential model.

pH dependence. Prior experiments were all conducted at pH 8.0, which is the pK_a of PB. At pH 8, equal concentrations of unchanged and anionic forms of the drug are present in the solution. To determine whether the charged or uncharged form of PB is more potent, each neuron tested was exposed to several solutions of 200 μM PB adjusted to pH values between 6 and 9. Control relaxations were not influenced by pH in the range 6–9.

When 200 μM PB is applied at pH 9.0, it has no effect on the current relaxations (Fig. 7). Relaxations show a single-exponential time course that is indistinguishable from control. However, changing the pH of the bathing solution from 9.0 to 7.0 produces rapid alterations in the relaxations, and they then resemble those described previously for PB. At pH 7.0, τ_f increases with higher PB concentrations (not shown), while τ_s is the same as τ_{con}.

A return to pH 9.0, while still at 200 μM, results in reversal of the effects of PB, and relaxations again are indistinguishable from control. If the pH is now lowered to 6.0, PB is extremely effective. τ_f becomes quite fast, and the ratio A_f/A_s is high.

This experiment was also performed in the reverse order, starting at pH 7 instead of 9. Similar results were observed, and the effects of changes in pH were completely reversible.

These experiments suggest that PB has little activity in its ionized state, and that the uncharged form is active. However, one may argue that PB may be acting at a site located in the interior of the cell, or at a relatively inaccessible location within the membrane. PB would then have to diffuse through the lipid phase of the membrane to reach its active site. Biological membranes are essentially impermeable to charged molecules, and the anionic form of PB may be unable to reach the active site. Since only the free acid will be able to penetrate the membrane, the apparent ineffectiveness of anionic PB may be due to poor accessibility of the active site.

The effects of lowering the pH were relatively rapid for this preparation (10–15 min), and had about the same time course as PB action during its initial application. Reversal of PB action upon raising the pH was also quite rapid, and was complete in 15 min. The fast time course of PB action suggests that PB is acting at a site that is readily accessible from the outside of the membrane. If the uncharged form of PB were diffusing to a remote site, where either the charged or uncharged forms might be active, then increasing the pH of the external solution would not be expected to produce such an immediate drop in barbiturate potency. PB must therefore be more active in its unionized form, in agreement with previous findings for axons (23, 24) and at the end plate (15).

When the pH is lowered from 7.0 to 6.0, the effects of PB are greatly enhanced. Although the ratio of uncharged to anionic forms changes from 10:1 to 100:1, the concentration of the uncharged form is not appreciably different at pH 6.0 from that at 7.0. The increased efficacy of PB at low pH values cannot be explained entirely by

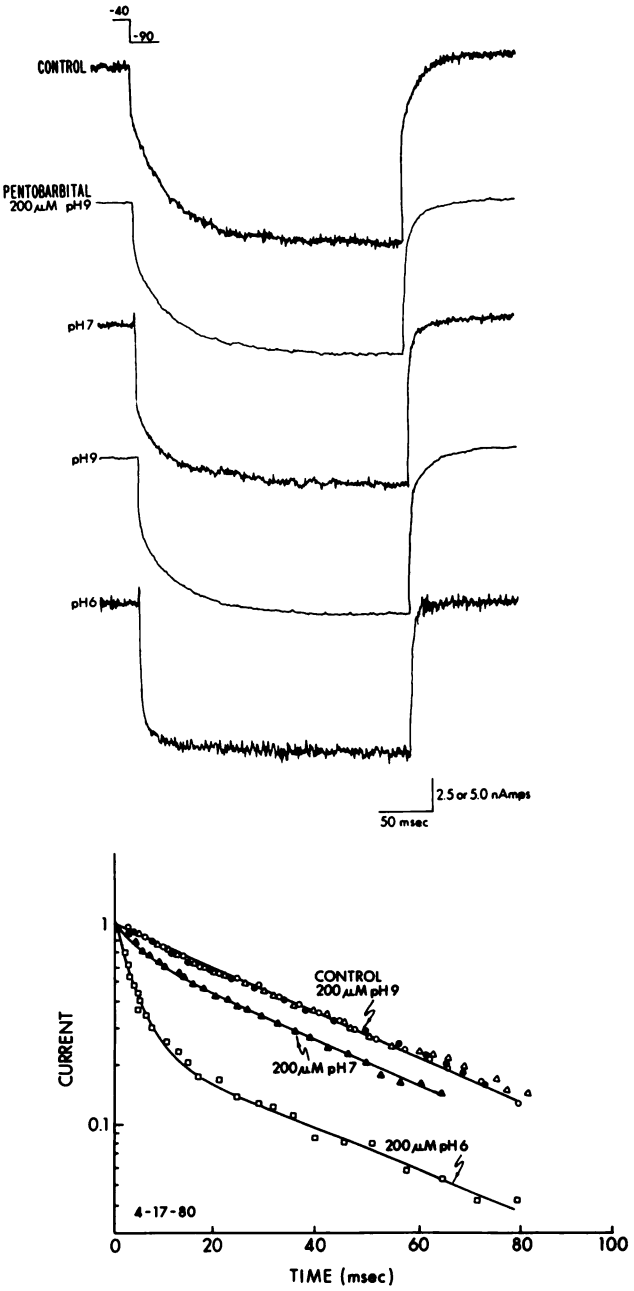


FIG. 7. *Effects of pH on the potency of PB*

A single cell was exposed to four solutions of 200 μM PB at different pH values. Solutions were applied in the order shown.

A. Current relaxations in response to voltage jumps from −40 to −90 mV (temperature 11°).

B. Semilogarithmic plots of relaxations at −90 mV. PB was more potent at low pH, and the effects of changes in pH were rapidly reversible.

a greater activity of the uncharged form unless the interfacial pH is significantly different from the pH of the bulk solution. Under these conditions, lowering the pH from 7.0 to 6.0 could produce a substantial change in the concentration of the uncharged form at the membrane surface. Alternatively, the apparent potency of PB may possibly be affected by the state of ionization of membrane proteins associated with its site of action.

PhB. PhB also attenuates iontophoretic responses to ACh (8), although it is slightly less potent than PB. In contrast to the effects of PB, however, PhB did not modify current relaxations at concentrations as high as 1 mM.

Because we were concerned that the apparent ineffectiveness of PhB might be due to a diminished sensitivity of some cells to the effects of barbiturates in general, PB was also applied to cells that appeared to be resistant to PhB. A concentration of 500 μM PB, which would normally produce an extremely fast component that would dominate the relaxations, appeared to be relatively ineffective when applied together with 1 mM PhB. If PB were applied first to demonstrate the sensitivity of the cell to barbiturates, subsequent application of PhB, followed by high concentrations of PB, failed to produce any further change in the relaxations. PB thus is ineffective when applied following PhB, suggesting that PhB antagonizes the actions of PB. These results were observed in a total of seven independent experiments with three different manufacturing lots of PhB.

AmoB. AmoB is a sedative/hypnotic barbiturate which is clinically effective at approximately the same dosages as PB. The effects of AmoB on current relaxations were qualitatively similar to those of PB, although AmoB appears to be somewhat less potent.

DPB. DPB is an anticonvulsant barbiturate which is approximately equipotent with PhB (25). At a pH equal to its pK_a of 9.4, DPB had no effect on current relaxations at a concentration of 250 μM. Even when the pH was lowered to 8.0 so that DPB was almost entirely in its unionized form, DPB had only minimal effects on current relaxations.

DISCUSSION

Current relaxations measured in the presence of PB consist of two components. The fast component becomes faster and relatively larger as the concentration of PB is increased. The slow component always has the same time constant as control, and does not become slower at higher drug concentrations. These observations have been considered in terms of several possible theories of drug action.

Blockade of Open Channels

If the fast component of PB relaxations were due to blockade of open channels and the slow component represented dissociation of the drug out of the channels and a return to a conducting state, then assuming $fd \gg \alpha$ (16, 17),

$$\frac{1}{\tau_f} \cong fd + b$$

$$\frac{1}{\tau_s} = \frac{\alpha b}{fd + b} + \beta$$

This model correctly predicts that the fast component should become faster as d is increased at higher drug concentrations. However, it also predicts that the slow component should become slower as the concentration of PB is increased. In these experiments, τ_s did not depend on PB concentration.

Concentration effects on the slow component may not be apparent if drug dissociation from the channel is very rapid compared with the normal channel closing rate. If $b \gg \alpha$, then τ_s will not be appreciably slower than control at drug concentrations much less than the equilibrium constant for the blocking reaction, $K = b/f$. A plot of τ_f at −100 mV as a function of PB concentration (see Fig. 3) yields a straight line with slope $f = 1.2$ sec^{-1} μM^{-1} and intercept $b = 24$/sec. The equilibrium constant for the proposed blocking reaction would therefore be about 20 μM. Since the concentrations of PB used in these experiments are much greater than 20 μM, the behavior of τ_s cannot be explained by this model.

Changes in α

At the neuromuscular junction, anesthetics and long-chain alcohols decrease channel lifetime and shift power spectra to higher frequencies (14, 26–28). They increase the decay rate of end-plate currents, but do not alter the single-exponential nature of the decay process or change its voltage or temperature sensitivity. These drugs appear to increase the rate at which open channels close by increasing α, but do not seem to alter the basic mechanisms by which channel closure occurs.

If PB were acting in a similar fashion in *Aplysia*, this would explain the presence of a fast component in the relaxations which becomes faster at higher drug concentrations. However, the slow component would then have to represent a fraction of channels that were resistant to the effects of PB, in which case $\tau_s = 1/\alpha$. This idea is not consistent with the finding that the ratio A_s/A_f decreases at higher PB concentrations. At 1 mM PB, only the fast component is visible (see Fig. 6), and the slow component is no longer apparent. A decrease in the amplitude of the slow component at higher drug concentrations would not be predicted if a portion of the channels were resistant to PB.

A population of channels may be less susceptible to changes in α, but not totally resistant to the effects of PB. τ_f and τ_s would then represent different populations of channels having different affinities for PB, and both τ_f and τ_s should decrease with increasing drug concentrations. The invariance of τ_s with changes in PB concentration cannot be explained by a model involving a simple increase in the rate of channel closure.

Binding to a Modulatory Site

PB may conceivably be acting at a specific modulatory site which regulates the lifetime of transmitter-activated channels. Barbiturate binding to this site may alter the affinity of ACh for its receptors, or change the coupling mechanism which opens an ionic channel in response to receptor binding. In effect, occupation of this site may change the agonist properties of ACh, and receptor binding would result in the opening of channels with altered lifetimes (29).

If this were the case, current relaxations should show two components, corresponding to two populations of channels: normal channels and channels whose lifetimes have been decreased by the presence of PB at their associated regulatory sites. As the concentration of PB is increased, a larger fraction of the regulatory sites would become occupied, and the ratio A_f/A_s should increase. However, this model does not predict that τ_f should become faster at higher PB concentrations. Although more channels may be affected at higher drug concentrations, the lifetime of those channels that are affected should not change.

Antagonism by a Stereoisomer

Another possible explanation for the effects of PB is suggested by PhB antagonism of PB action. PhB prevents the reduction in channel lifetime which is normally produced by PB; in the presence of PhB, channels appear to be resistant to the effects of PB. The slow component seen in relaxation experiments may therefore represent a population of channels that are "spared" the effects of PB due to similar antagonism by some portion of the PB molecule.

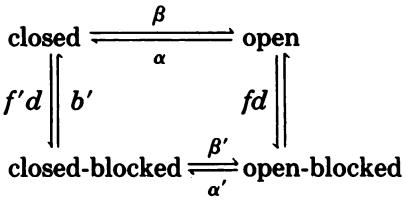
Solutions of PB actually contain four different structures; charged and uncharged forms, and (+)- and (−)-isomers. All forms of the molecule may not be active, and one form may possibly antagonize the actions of the other forms. Experiments at low pH have shown that PB is much more potent in its uncharged form. Even at pH 6, however, where the ratio of uncharged to charged forms is 100:1, the double-exponential time course of the relaxations is not abolished. This experiment indicates that the charged form is not acting as an antagonist of the uncharged form.

Huang and Barker (30) have demonstrated that (+)-PB is less potent than (−)-PB in potentiating responses to γ-aminobutyric acid in cultured spinal neurons. In *Aplysia*, one isomer may possibly be much less active than the other, and may even act to protect channels from the effects of the more active isomer. Under these conditions, some of the channels might appear to be less susceptible to changes in channel lifetime, and relaxations would show two components. Since the ratio of (+)- and (−)-isomers remains constant at all PB concentrations, however, antagonism by a stereoisomer would not account for the decrease in τ_f and the increase in the ratio A_f/A_s at higher drug concentrations. For an agonist and competitive antagonist at equilibrium, only the ratio between the two concentrations is important. The effects of the agonist should not be enhanced when the concentrations of both forms are raised in parallel. Unfortunately, samples of (+)- and (−)-PB are not available at this time, and this particular theory could not be tested directly.

Alternative Models

The actions of PB are not totally consistent with any one of these models. PB does not resemble any other pharmacological agent that has been studied. At present, two alternative theories may be posed to account for the observed results.

Cyclic model.



A cyclic model has been invoked previously by Adams (16) to explain procaine depression of steady-state responses to carbachol. However, the values of α' and β' were assumed to be very small, so that this model was not kinetically different from the sequential model.

The cyclic model is quite different from the sequential model, however, if channels that are blocked by molecules of drug are allowed to close without first returning to the open state. Assuming $fd \gg \alpha$, then (16)

$$\frac{1}{\tau_f} \cong fd + b$$

$$\frac{1}{\tau_s} \cong \frac{f'd\beta' + \beta b'}{b' + f'd} + \frac{fd\alpha' + \alpha b}{b + fd}$$

If $f'd, \beta', \beta, b \ll b', \alpha, \alpha', fd$, then

$$\frac{1}{\tau_s} \cong \alpha \quad \text{when} \quad \alpha' = \alpha$$

α' would equal α if the blocking reaction did not alter the stability of the open conformation and the closing reaction were not affected by the presence of a drug molecule in the channel. Under these conditions, the relaxation would not be slowed by the trapping of open channels in a blocked open state, since blocked channels would have the same probability of closing as nonblocked channels.

Two-site model. The effects of PB might also be explained by postulating that PB binds with a high degree of cooperativity to specific sites within the membrane. Channel lifetime may be altered by the presence of PB molecules dissolved in the membrane near the channel, and the reduction in τ might then depend on the number of drug molecules surrounding each channel. If these binding sites possessed a high degree of cooperativity, then initial entry of a molecule of PB into the membrane would be followed by the further entry of many more molecules. Under these conditions, a large number of channels may remain unaffected by PB, whereas a few would be highly affected and contribute an extremely fast component to the relaxations. As the concentration of PB is raised, more channels would be affected, and the speed of the fast component would be further increased as more molecules of PB surrounded each channel. This theory would explain the behavior of both the fast and slow components, but it does not account for the linear dependence of $1/\tau_f$ on PB concentration. The dose-response curve for this model should be fairly flat at low concentrations, but rise steeply at higher doses.

A unique type of cooperative binding model may be more compatible with the data. All of the observed results can be explained by postulating the existence of two types of PB binding sites. Occupation of one site

would facilitate binding of PB to a large number of other sites within the membrane. All sites in this latter category would be identical, and PB binding would not exhibit further cooperativity once the first site was occupied. Binding to the first site would regulate the number of channels that were susceptible to PB, while occupation of the second site would determine the extent of changes in channel lifetime.

For example, suppose PB bound to a relatively inaccessible site (Type 1) near the receptor-channel complex, and thus exposed several other binding sites (Type 2). Once exposed, these other sites would then be titrated as the concentration of PB was increased, and changes in channel lifetime would be determined by the number of such sites occupied. At low PB concentrations, only a few of the Type 2 binding sites would be uncovered by occupation of Type 1 sites, and the majority of channels would be unaffected by PB. Of those channels that were affected, τ would be only minimally altered, since only a few of the available Type 2 sites would be occupied. At higher concentrations, more Type 1 sites would be occupied, more Type 2 sites would be exposed, and a larger fraction of exposed sites would be occupied. The fast relaxation would thus become relatively larger, and τ_f would also become faster. Small steady-state responses would be depressed more than larger ones if PB had a higher affinity for Type 1 sites associated with closed channels than with open channels.

This two-site model can also explain the apparent antagonism of PB by PhB. PhB could conceivably be a classical antagonist of PB at the Type 1 binding sites. Binding of PhB to Type 1 sites would then prevent further binding at the Type 2 sites. This model of drug action therefore accounts for all of the kinetic data.

CONCLUSIONS

The precise mechanism by which PB reduces channel lifetime in *Aplysia* has not been identified, since the results of these experiments cannot be explained in terms of any simple model of drug action. Although the effects of several barbiturates at the vertebrate neuromuscular junction are consistent with a sequential model of channel blockade, PB does not act in a similar fashion in *Aplysia*. The data are completely consistent with the concept of two types of binding sites associated with the channel, but such a model is rather unattractive because of its complexity. Additional experiments involving a variety of drugs and several different preparations may be required to formulate a new model of drug action.

Of the four barbiturates considered in this study, only PB and AmoB were effective in reducing channel lifetime. These two barbiturates are strong sedatives, whereas PhB and DPB, which were relatively ineffective in increasing the speed of current relaxations, are anticonvulsant at serum levels that produce little sedation. Although it would be attractive to speculate that alterations in channel lifetime are associated with sedative rather than anticonvulsant barbiturates, such a conclusion would be premature. Quantitative examination of a wide spectrum of barbiturates is required in order to reach any conclusions about the relationship between the ability of a barbiturate to alter channel properties and its clinical effects.

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