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The voltage-dependent action of pentobarbital on batrachotoxin-modified human brain sodium channels

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Abstract

The voltage-dependent action of the intravenous anesthetic pentobarbital on human brain sodium channels activated by batrachotoxin was examined using planar lipid bilayer methods. Fractional open time-data were fitted by Boltzmann functions to yield simple parameters characterizing the voltage-dependence of the fractional open time. Pentobarbital caused a dose-dependent reduction of the maximum fractional open time of the sodium channel and a shift of the potential of half-maximal open time towards hyperpolarized potentials, whereas the slope parameter of the Boltzmann-fits was unaffected. A statistically significant increase of the variability of these parameters was found only in the case of the maximum fractional open time, indicating a random fluctuation of pentobarbital-induced suppression of the sodium channels over time. The voltage-dependent action of pentobarbital probably results from either a pentobarbital-modification of channel activation gating and/or a modification of the pentobarbital action by the gating process itself.

Key words: Sodium channel; Anesthetic; Pentobarbital; Channel activation gating

1. Introduction

A large variety of anesthetics suppress sodium currents in neurons [1–3]. The interactions of anesthetics with this ion channel are complex, and macroscopically result in multiple changes in sodium channel activation and inactivation parameters [4–6]. To analyze the molecular mechanisms which underlie these macroscopic changes in sodium channel behavior, we have been examining the interactions of a variety of anesthetics with batrachotoxin-modified human brain sodium channels in planar lipid bilayers [7].

Our previous studies with pentobarbital showed that human brain sodium channel functionality appeared to be altered by at least two mechanisms [8,9]. First, at depolarized potentials, pentobarbital caused a voltage-independent reduction of the fractional open time (the fraction of time the channel spends in the open state) of the sodium channel. Second, pentobarbital also appeared to shift the voltage-dependence of sodium channel activation to more negative membrane potentials and to make it more variable [9]. However, because of this large (intrinsic) scatter of the data, the statistical significance of the shift had not been demonstrated. As voltage-activation is a critical parameter of sodium channel function with respect to neuronal excitability, a further examination of anesthetic effects on channel activation is important for understanding the mechanisms of anesthetic action on neuronal function. To investigate these interactions, we examined the parameters of voltage-activation, focusing on both the mechanism(s) underlying the change in the voltage-dependence of channel fractional open time, as well as investigating the parameters which might be responsible for the experimentally observed variability.

The results presented in this paper demonstrate that pentobarbital has a statistically significant, dose-dependent and voltage-dependent effect on human brain sodium channels in the membrane potential range where steady-state activation occurs. Furthermore, the increased variability of channel responses results mainly from a variable suppression of sodium channels despite constant drug concentrations.

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2. Materials and methods

A detailed description of the methods used to examine human brain sodium channels in planar bilayers has been given before [9,10]. Here we present a summary of the method and its modifications.

Human brain samples were obtained as surgical waste with the approval of the Cornell Committee on Human Rights in Research. The tissue was immediately frozen at −80°C and processed to yield synaptosomal fractions (88N14 and 89203) which were stored at −80°C [11].

Standard Teflon chambers contained two 4 ml-compartments separated by a Teflon partition [12]. Lipid bilayers were formed across a hole (100–300 μm diameter) in the Teflon partition from a lipid solution containing a 4:1 mixture of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine and 1-palmitoyl-2-oleoyl-phosphatidylcholine (Avanti Polar Lipids, Birmingham, AL) in decane (0.05 mg lipid/μl decane).

All experiments were conducted in symmetrical 500 mM NaCl buffered at a pH of 7.35 and at room temperature (22.0–27.4°C, mean 23.5°C). The alkaloid batrachotoxin (0.25 μM, gift from Dr. J. Daly, National Institutes of Health, Bethesda, MD) was added to the trans (back) chamber [13]. After formation of a lipid membrane the background conductance through the bilayer was measured at all potentials used for channel characterization. Synaptosomal preparation was then added to the cis (front) chamber by blowing it across the bilayer in small aliquots of less than 1 μl. Incorporation occurred in 100 out of 1110 membranes. If no incorporation occurred a new membrane was formed after 20–60 min. Membranes with more than two channels were not used for these experiments.

Most membranes used in this study contained only single channels (18 membranes); 2 membranes contained 2 channels. Channel sidedness was determined by channel activation gating characteristics and the electrophysiological sign convention was used. Racemic pentobarbital (Sigma, St. Louis, MO) was added to the 'extracellular' side of the channel (the side facing the extracellular space in situ) from an ethanol stock solution. The concentrations of ethanol reached in these experiments (< 100 mM) have no effect on channel behavior in this system (Schmitz, S. and Urban, B.W., unpublished data).

Currents were amplified using a standard patch-clamp amplifier (Axopatch 200, Axon Instruments), filtered at 50–200 Hz (depending on background current noise) and recorded on hard disk using commercially available software (pClamp and Axotape, Axon Instruments; digitizing frequencies were 125 and 1000 Hz for the respective programs). Voltage-clamp protocols were controlled by the same software. To examine pentobarbital reduction of channel fractional open time at depolarized potentials, voltages from −45 to +45 mV were applied in depolarizing and hyperpolarizing sequences using 15-mV steps with a duration of 30 s each. Time-averaged channel conductances were calculated by integrating the current over time and dividing by the potential; measurements of the amplitudes of open-closed-open current transitions with cursors on a computer screen were used to construct current–voltage plots. Single-channel conductances were determined as the slope of the current–voltage plots. Dividing the time-averaged channel conductance (after subtraction of the background conductance) by the single-channel conductance and the number of channels present yielded the channel fractional open time (f_o).

The voltage-dependent channel fractional open time ($f_o(V)$) in the voltage region of channel activation was measured using a hyperpolarizing sequence from +50 to −110 mV in 10-mV steps each lasting 4 s. Compared to single-channel conductance and channel fractional open time determinations outside the gating region, however, measurements at potentials at which channel activation gating occurs are intrinsically more variable [9,10,14]. This variability made it necessary to record many of these voltage sequences on the same channel in order to arrive at statistically significant conclusions regarding anesthetic interactions with voltage-dependent channel fractional open time. Therefore, 4–11 (mean of 6) voltage sequences were recorded under control conditions before pentobarbital was added, followed by another set of 3–12 (mean of 5) voltage sequences after the addition of the anesthetic. At least 2 min elapsed between sequences to ensure residual transients did not interfere with data acquisition. Because of a greatly increased risk of incorporating more channels and/or breaking membranes at the high potentials required to fully deactivate (close) the channel, a large number of experiments were needed to complete this study.

Capacitative transients were eliminated by subtraction of the current trace at 0 mV from all other traces. Background (membrane) conductance (5–15 pS) was measured at each potential during long channel closures (> 1 s) and subtracted from the total measured conductance (membrane and channel) at each potential. Additionally, nonspecific noise sometimes occurred at large hyperpolarized potentials; all determinations where such noise occurred were excluded from the analysis. For each membrane, individual determinations of control and anesthetic fractional open time data were fitted to a two-level Boltzmann distribution as described [15], using the Marquardt-Levenberg algorithm (Sigmaplot, Jandel Scientific, Corte Madera, CA). The function $f_o(V) = f_{max}/(1 + \exp(-z_v \cdot F \cdot (V - V_{\frac{1}{2}}))/RT)$ is characterized by three parameters, with f_{max} being the maximum fractional open time; $V_{\frac{1}{2}}$, the midpoint potential at which the function assumes its

half maximal value and the slope parameter, z_v, being proportional to the slope of the curve at this potential [16]. F is the Faraday constant, R the gas constant and T the absolute temperature.

To determine overall averages before and after pentobarbital addition, and the variability between membranes, the fractional open time data of all voltage sequences for each membrane were averaged (for any given membrane potential); the averaged data from each membrane were then averaged with the data from all other membranes. The overall averages were then fitted with a Boltzmann-function to determine the average $V_{\frac{1}{2}}$ and z_v in the presence and absence of pentobarbital.

To examine the effect of pentobarbital on the variability of the voltage-dependent fractional open time a different way of averaging the data was used. Here the data of each voltage sequence were fitted with a Boltzmann-function and the parameters of the fit were averaged with those from all fits of the same membrane and standard deviations for f_{max}, $V_{\frac{1}{2}}$ and z_v calculated. The standard deviations were then averaged among membranes and the averages before and after pentobarbital addition compared.

Pentobarbital data were compared with control data for the same channels using paired t-tests ($P < 0.05$) for statistical analysis. Data are represented as means ± S.E. unless otherwise noted.

3. Results

The results of this study were obtained from 20 membranes containing a total of 22 sodium channels. In the absence of pentobarbital these channels had all the expected characteristics of batrachotoxin-modified human brain sodium channels [10]: at depolarized potentials the average fractional open time was 0.94 ± 0.01 (±S.E.) and the single-channel conductance was 26.0 ± 0.41 pS. The averaged midpoint potential of the Boltzmann function in the activation gating region, $V_{\frac{1}{2}}$, was −81.3 ± 3.7 mV ($n = 20$ membranes) with a slope parameter of 3.4 ± 0.44 electronic charges. Using only the data from single-channel membranes ($n = 18$), the slope parameter was determined as 3.5 ± 0.44 electronic charges.

Addition of racemic pentobarbital led to a 'flickering' suppression of the sodium channel (Fig. 1) as first described by Frenkel et al. [8,9]. The rapid open-closed transitions elicited by racemic pentobarbital resulted in a dose-dependent reduction of the channel fractional open time. This suppression of the channel (measured as the reduction of the time-averaged conductance) was voltage-independent at potentials more positive than −45 mV, i.e., outside the region of channel activation gating. The dose-dependence of this suppression was well fit by a simple rectangular hyperbola, indicating that the channel could be completely suppressed (103.2 ± 6.9% maximum suppression) and having a half-suppression concentration of 847 ± 141 μM. These values are close to those found by Frenkel et al. [8,9] for the pentobarbital isomers.

3.1. Single-channel conductance is largely unaffected by pentobarbital

The frequency response of the recording system allowed only partial resolution of the pentobarbital-induced 'flickering' suppression (Fig. 1). Openings and closures lasting longer than 10 ms were used to measure single-channel current amplitudes (see traces). Fig. 2 shows the current–voltage relationship of a single sodium channel before and after the addition of 340 and 680 μM pentobarbital. Slope conductances were 27.5 ± 0.1 pS for control data, 27.5 ± 0.1 pS for 340 μM and 26.7 ± 0.1 pS for 680 μM. These differences were not statistically significant. It is particularly important to note that in the gating region (at poten-

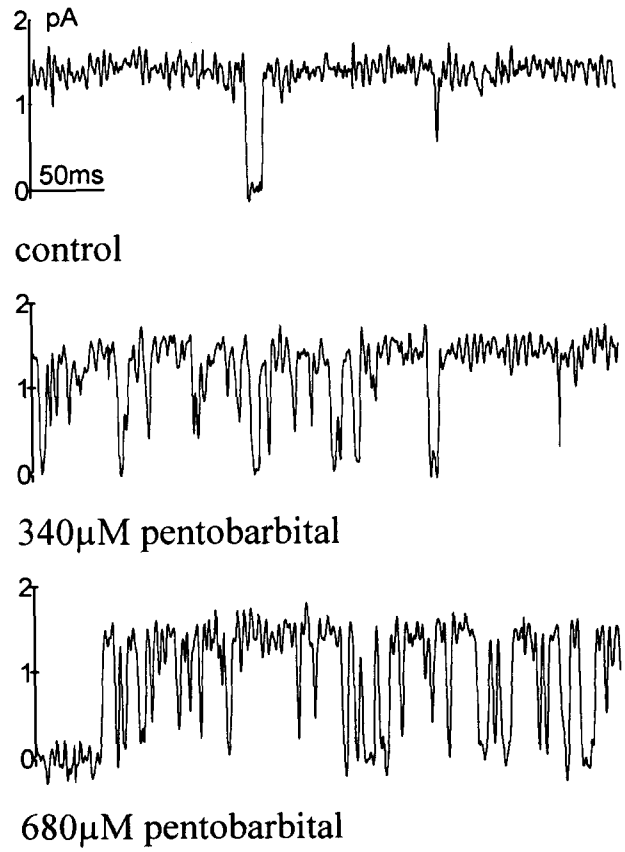


Fig. 1. Current traces of a single sodium channel recorded at a membrane potential of +50 mV under control conditions and after addition of 340 and 680 μM pentobarbital. The traces were recorded at 1000 Hz, the signal was filtered at 200 Hz (membrane capacity 190 pF).

tials more hyperpolarized than −45 mV) the single-channel conductances still remain constant, i.e., any voltage-dependent effect in this region cannot be ascribed to a reduction in single-channel conductance.

3.2. Concentration-dependence of the parameters of the voltage-dependent fractional open time

Compared to single-channel conductance, the fractional open times in the activation gating region proved to be much more variable both before (Fig. 3A) and after (Fig. 3B) pentobarbital addition. It therefore became necessary to record many voltage sequences on the same channel in order to arrive at statistically significant conclusions. To compare the voltage-dependence of channel fractional open time in the absence and presence of pentobarbital, the data were fitted with a two-level Boltzmann function (see Materials and methods). This is necessarily a simplification of the molecular processes involved in this voltage-dependent behavior, since in the presence of pentobarbital the sodium channel might have more than one closed state at these potentials. Nonetheless, the Boltzmann function provides an adequate fit of the experimental data; we imply no molecular mechanism with this fit.

The individual and averaged Boltzmann fits for the membrane in Fig. 3A and B are superimposed in the last panels of Fig. 3A and B, demonstrating the variability increase. In this experiment, the difference in the midpoint potentials of the Boltzmann functions of the averaged data before and after pentobarbital addition is statistically significant.

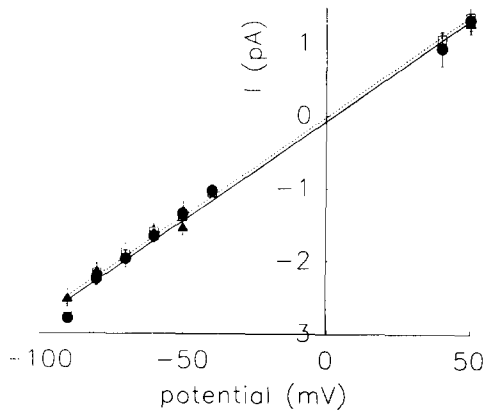


Fig. 2. Current–voltage data and linear regression lines for a single sodium channel before addition of pentobarbital (filled circles, solid line), with 340 μM pentobarbital (open squares, dashed line) and with 680 μM pentobarbital (filled triangles, dotted line). Data were obtained as averages from at least 10 clearly distinguishable open-closed-open transitions at each potential. Underlying current traces were filtered at 200 Hz and recorded at 500 Hz. Slope conductances determined by linear regression were 27.5 ± 0.1 pS for control data, 27.5 ± 0.1 pS for 340 μM and 26.7 ± 0.1 pS for 680 μM. Within our experimental uncertainty those values are not significantly different ($p = 0.85$ for 340 μM and 0.68 for 680 μM). Error bars indicate S.E.

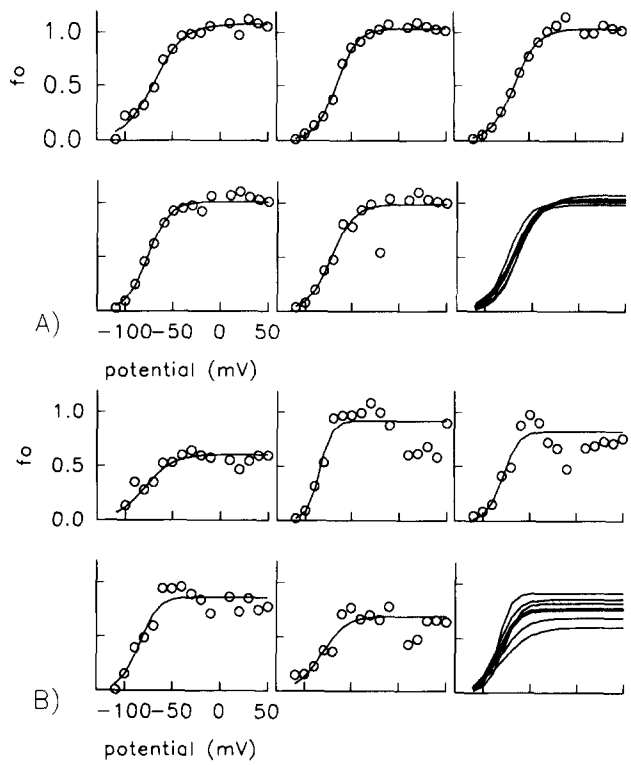


Fig. 3. Consecutive determinations of the voltage-dependent fractional open time (open circles) from a single sodium channel (A) before and (B) after addition of 680 μM pentobarbital. Lines are least-squares fits of a Boltzmann function to the data. The last panel in each sequence shows the fits of all determinations (thin lines) together with the fit through the averaged data (thick line) of the five determinations. The fits yielded the following potentials of half-maximal fractional open time: −69.4, −66.1, −65.6, −76.6 and −72.4 mV for the controls without pentobarbital and −79.0, −84.8, −78.6, −84.9 and −81.5 mV after addition of pentobarbital. For the averaged data the midpoint potentials are −69.9 mV for control and −83.1 mV with 680 μM pentobarbital.

The averaged fractional open times from different experiments were themselves averaged and are displayed in Fig. 4. Comparing the data in the presence and absence of pentobarbital, there is an apparent voltage-dependent decrease in the suppression of the fractional open time at potentials where the channel undergoes activation gating. The data can be adequately fit with two-level Boltzmann distributions, and the parameters resulting from these fits were plotted as a function of pentobarbital concentration (Fig. 5).

The potential at which half-maximal fractional open time is reached, $V_{\frac{1}{2}}$, became more negative in the presence of pentobarbital as first described by Frenkel et al. [9] (Fig. 5A). The dose-response curve can be approximately fit with a rectangular hyperbola, yielding a maximal shift of 32.7 mV and a half-maximal shift at 862 μM pentobarbital.

The slope parameter of the Boltzmann function was determined only in single-channel membranes, since the presence of more than one channel may result in an aggregate Boltzmann curve from which the slopes of the individual Boltzmann curves are not extractable [17]. As can be seen in Fig. 5B there is a large variation of z_v values even among the controls. No significant differences were found in z_v at any pentobarbital concentration from the corresponding controls in a one- or two-tailed t-test.

The decrease in maximum fractional open time obtained as a parameter of the Boltzmann fits (Fig. 5C) has a very similar dose-dependence to the suppression of the averaged fractional open time measured at depolarized potentials. It is noticeable that this parameter shows much less variability between different channels than the midpoint potential and the slope parameter.

3.3. Variability of the parameters of the voltage-dependent fractional open time in the presence and absence of pentobarbital

Apart from variability between channels (Fig. 5), each single-channel displayed variability in the voltage-dependent fractional open time during consecutive determinations, as indicated in Fig. 3. Frenkel et al. [9] observed that pentobarbital seemed to increase this intrinsic variability of the voltage-dependent fractional open time in the activation gating region. To examine whether this variability was significantly altered by pentobarbital, we compared the standard deviations of the parameters of the Boltzmann functions describing the voltage-dependent fractional open time within single-channel experiments before and after the addition of pentobarbital (Fig. 6).

The variability of the midpoint of the fractional

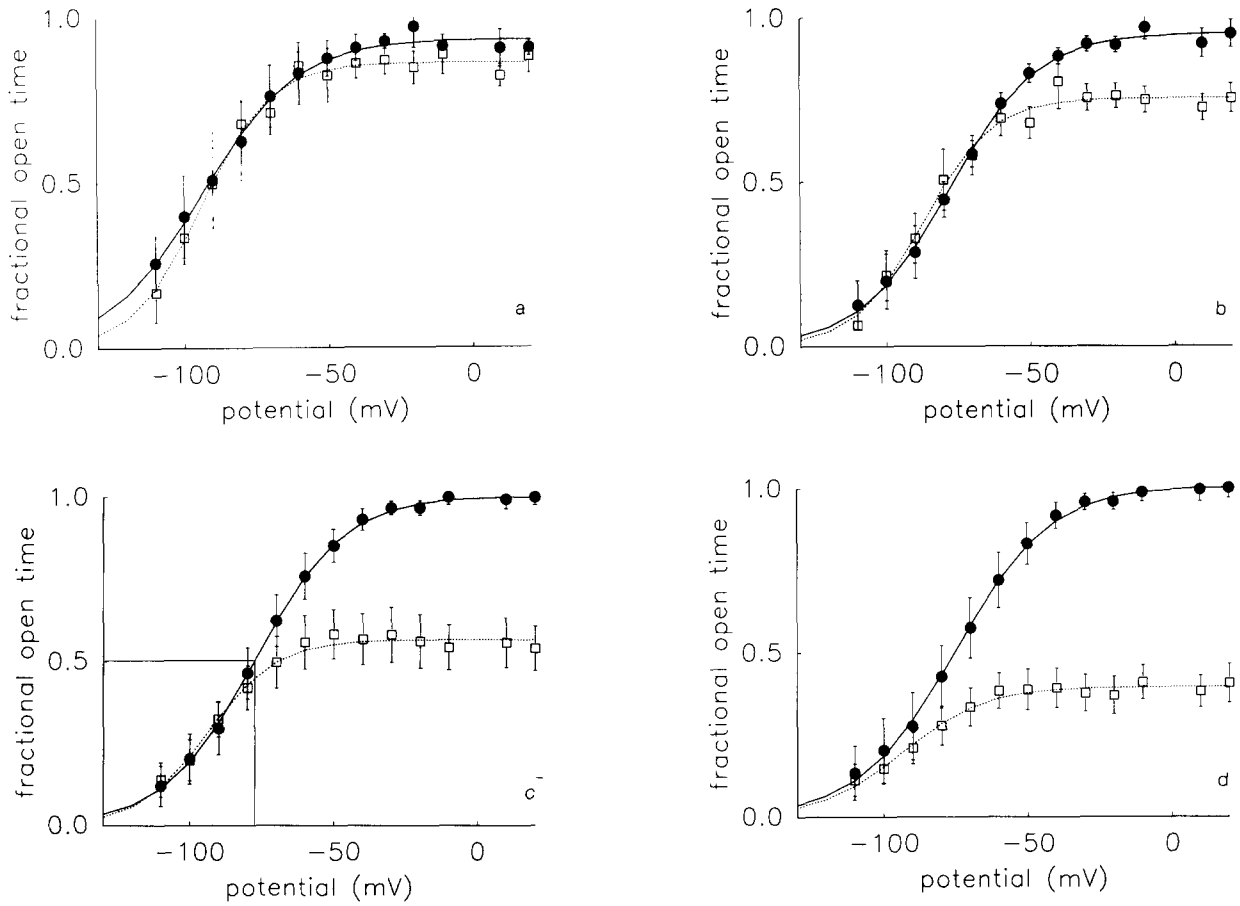
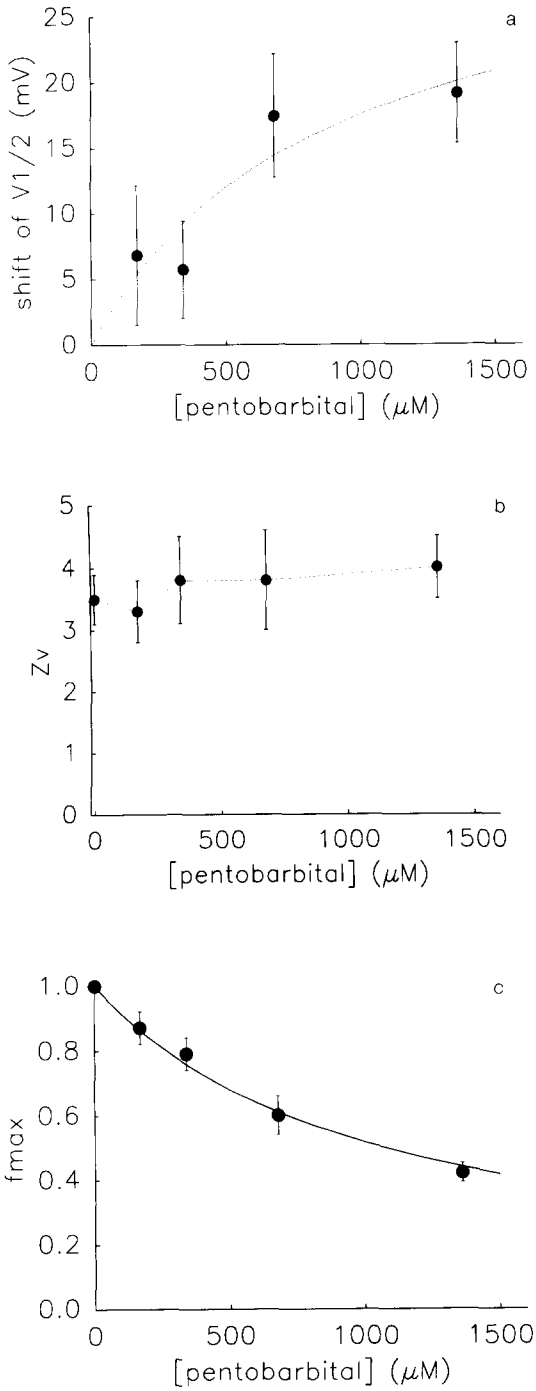


Fig. 4. Averaged fractional open time data of all experiments at different pentobarbital concentrations and control data from the same channels. Filled circles denote data before, open squares data after addition of pentobarbital. Curves represent least-squares fits of a Boltzmann function to the data (solid line, controls; dotted line, pentobarbital). Data were calculated by averaging the averaged data from each membrane. (A) 170 μM pentobarbital; 4 experiments (3 single-channel membranes, 1 membrane contained 2 channels). (B) 340 μM pentobarbital; 10 experiments (all single-channel membranes). (C) 680 μM pentobarbital; 9 experiments (7 single-channel membranes, 2 membranes contained 2 channels). (D) 1360 μM pentobarbital; 7 experiments (5 single-channel membranes, 2 membranes contained 2 channels). Error bars indicate S.E. The rectangular lines in (C) show the determination of the potential at which the fractional open time reaches a half-maximal value; maximum fractional open time is 1.00 for the control data and 0.60 for 680 μM pentobarbital. The potential of half-maximal open probability is thus shifted from −77.7 mV for controls to −91.8 mV for 680 μM.

open time function was neither significantly different from controls at any pentobarbital concentration (Fig. 6A) nor did it display any obvious trend. The variability of the slope parameter was also not significantly different from controls, although it appeared to be increasing with pentobarbital concentration (Fig. 6B). Differences in the variability of the third fit parameter, the maximum fractional open time, became significant as shown in Fig. 6C. Despite an apparent increase in variability at all pentobarbital concentrations, this increase was significant only at 680 μM pentobarbital.



4. Discussion

The work presented here extends our investigation of the molecular modifications of voltage-gated sodium channels by pentobarbital. In this paper we examined the effects of pentobarbital on the single-channel conductance as well as on the voltage-dependent sodium channel fractional open time in the range of membrane potentials where activation gating occurs.

Previously we have shown that pentobarbital reduces the time-averaged conductance of single sodium channels [9]. However, it could not be determined whether this reduction resulted from changes in single-channel conductance or a decrease in channel open-time. The results presented here indicate that the single-channel conductances, as measured from the slope of the current–voltage relationship of current transition steps (Fig. 2), were unchanged by pentobarbital at all examined potentials and concentrations. Therefore, pentobarbital increased the time that the channel remained in a non-conducting state. This parallels findings of pentobarbital action on acetylcholine-activated channels [18] and suggests that the conformation of the pore conducting region of the channel molecule is not affected much during pentobarbital suppression. Pentobarbital most likely reduces the ion conductance of the channel by either physically obstructing ion flow through the pore of the channel ('plugging' the pore), by allosterically closing one of the channel gates (activation or inactivation), or by allosterically 'closing' the channel via a process not related to normal activation or inactivation gating. In either case the mechanism is voltage-independent at depolarized potentials (positive to −50 mV).

Fig. 5. Concentration-dependence of the Boltzmann-fit parameters. Error bars (S.E.) indicate variability in between experiments. (A) Concentration-dependent shift of the potentials of half-maximal fractional open time, obtained as differences of the potentials of half-maximal fractional open time with and without pentobarbital at 170, 340, 680 and 1360 μM. Data are averages of the shifts of individual experiments, error bars indicate the S.E. of the variability between membranes. A hyperbolic fit, weighted for sample size, at each data point, yielded a maximum shift of 32.7 ± 16.2 mV and a half-maximal shift at a pentobarbital-concentration of 862 ± 834 μM. The midpoint potential was significantly shifted at all pentobarbital concentrations except 170 μM (p-values for a paired one-tailed t-test are 0.16, 0.03, 0.01 and 0.01 for 170, 340, 680 and 1360 μM). (B) Slope parameter (z_v) vs. pentobarbital concentration. Data were obtained from 3, 10, 7 and 5 membranes containing only single channels at the pentobarbital concentrations of 170, 340, 680 and 1360 μM, respectively and from 18 control membranes. No significant differences from control were found (P-values for a paired two-tailed t-test are 0.64, 0.47, 0.45 and 0.27 for 170, 340, 680 and 1360 μM). Curve is drawn point to point. (C) Concentration-dependence of the maximum fractional open time f_{max}. A weighted least-squares fit to a hyperbolic function yielded a half-maximum suppression of f_{max} at a pentobarbital concentration of 1062 ± 65 μM. Same n as in Fig. 4.

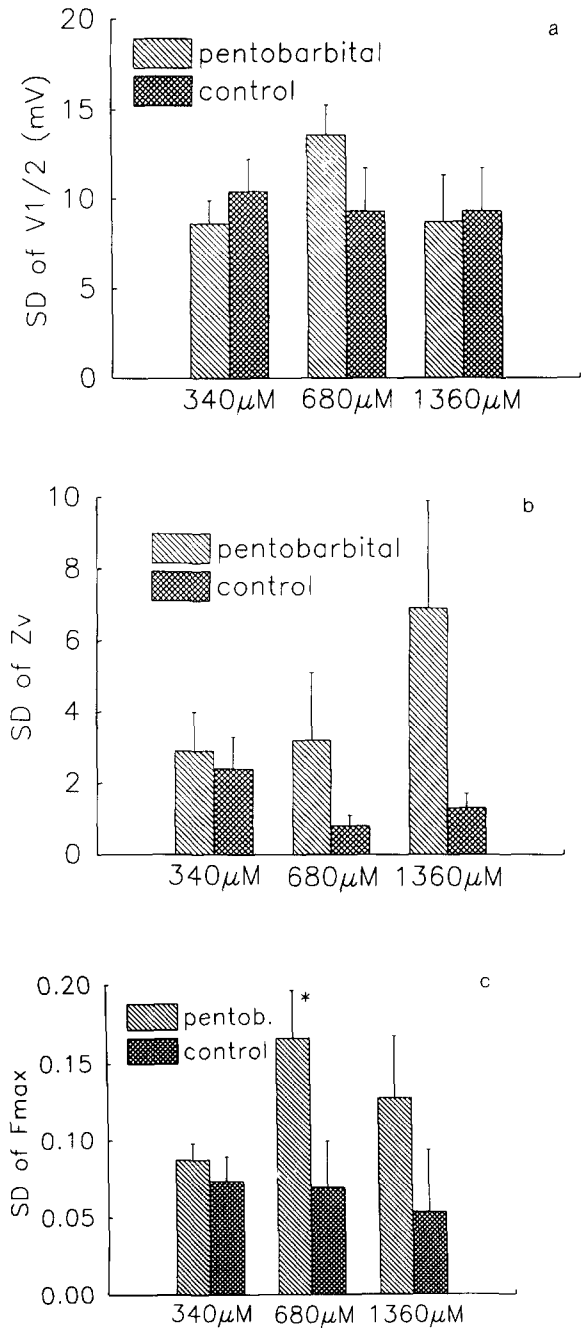


Fig. 6. Variability of the parameters of the Boltzmann fits before and after addition of pentobarbital. The averages of the standard deviations (S.D.) of the midpoint potential within each experiment (hatched columns, with pentobarbital; crosshatched columns, controls) are plotted. Only single-channel experiments with at least 3 determinations for each pentobarbital concentration were included ($n = 10$, 7 and 5 experiments for 340, 680 and 1360 μM pentobarbital, respectively, at 170 μM data were not sufficient for variability analysis). (A) Variability of the potential of half-maximal fractional open time ($V_{\frac{1}{2}}$), P-values for a paired one-tailed t-test are 0.21, 0.05 and 0.4 for 340, 680 and 1360 μM, respectively), (B) variability of the slope parameter (z_v), P-values are 0.26, 0.14 and 0.13, (C) variability of the maximum fractional open time (f_{max}) before and after addition of pentobarbital, P-values are 0.21, 0.02 and 0.10. Error bars indicate S.E. The star (*) indicates a statistically significant difference in a paired one-tailed t-test.

Pentobarbital was also found to cause a significant shift in the midpoint of the Boltzmann function describing the voltage-dependence of the fractional open time of the batrachotoxin-modified sodium channels. The dose-response curve for this shift falls within a similar range of pentobarbital concentrations as that measured for the suppression of the voltage-independent fractional open time (half-maximal effect occurring at 1.1 mM pentobarbital as compared with 670 μM [9] to 850 μM (this study)). The curve-fit contains a high degree of uncertainty so that the estimated concentrations for the half-maximal effect cannot be considered significantly different from each other. The explanation for this similarity, if not coincidence, could be either that binding of pentobarbital to a single site causes more than one effect, or that pentobarbital is binding to different, e.g., lipophilic, sites with similar affinities. In particular, lipophilic sites would not be expected to vary greatly in their binding affinities for lipophilic drugs.

The interpretation of this shift in the midpoint potential of the Boltzmann function must be taken with care, however, because it contains the superposition of two different processes. In the absence of pentobarbital, this midpoint is interpreted as the midpoint of channel activation [19]. In the presence of pentobarbital, the additional process of pentobarbital suppression is superimposed on the activation gating. The measured shift of the midpoint potential could result from a voltage-dependent shift in either, or both, of these processes.

If pentobarbital were to reduce sodium channel

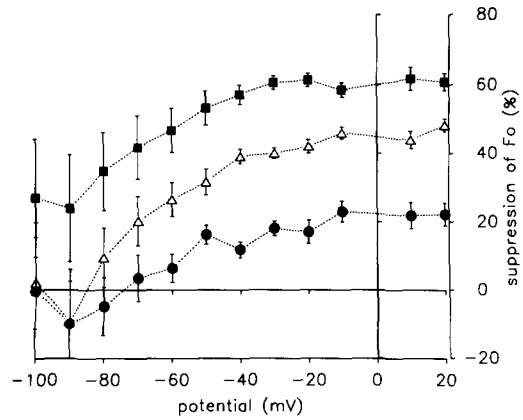


Fig. 7. Voltage-dependent decrease in the suppression of the fractional open time by pentobarbital in the voltage region of activation gating. The suppression was determined as the difference in channel fractional open time before addition of pentobarbital and with 340 μM (circles), 680 μM (triangles) and 1360 μM pentobarbital (squares), divided by the control fractional open time (100% indicates complete suppression of the channel). Data are same as in Fig. 4, lines are drawn point-to-point. At hyperpolarized potentials the fractional open times become very small, therefore the errors increase (error bars indicate S.E.).

fractional open time independent of potential in this potential range, too, then this effect could be separated out simply as an additional multiplying factor. The Boltzmann function would be the steady-state activation curve multiplied by that factor. In this case, the shift in the midpoint potential would reflect a pentobarbital-induced shift in the activation gating of the sodium channel towards hyperpolarized potentials.

On the other hand, the data could also be interpreted as a voltage-dependent decrease in the suppression of the fractional open time by pentobarbital at potentials where the channel undergoes activation gating (Fig. 7). In this case, voltage-dependent conformational changes in the sodium channel might reduce pentobarbital affinity. For example, pentobarbital may not interact with the resting (closed) state of the channel and the pentobarbital-induced reduction of fractional open time would decrease at potentials where activation gating occurs. Conformation-dependent changes in agonist/antagonist binding have been found for other channel modifiers [20–24]. If such voltage-dependent unbinding occurs it must take place in such a way that the combination of voltage-dependent reduced suppression and activation gating can still be described by a two-level Boltzmann function. Higher resolution recordings are necessary to distinguish between pentobarbital-modified activation gating and gating-modified pentobarbital interaction by separating the time constants for activation gating and the pentobarbital-suppression.

An increase in intra-channel variability of the voltage-dependent fractional open time of pentobarbital-modified channels, previously suggested, was confirmed and shown to be statistically relevant, at least at a pentobarbital concentration corresponding to a half-maximal suppression. Of the three parameters determining the Boltzmann function only the standard deviation of the maximal fractional open time could be demonstrated to be significantly increased by pentobarbital at this concentration. Although other increases were also observed, their statistical significance could not be proven despite considerable experimental effort. As previously observed [9], the pentobarbital-induced suppression of the fractional open time outside the activation gating region proved to be randomly variable with time (Fig. 3), despite a constant drug concentration. Whether this is an important feature of drug interaction with large membrane proteins at the molecular level remains to be verified in future experiments in other, solvent-free (i.e., without decane), model systems, using different drugs and channels.

In summary we were able to demonstrate a statistically significant voltage-dependent action of pentobarbital in the activation gating region. This action can be formally described as a shift in the midpoint of a two-level Boltzmann curve that is used to fit the voltage-dependent fractional open time in the steady-state activation region. This shift could reflect either pentobarbital-modified activation gating or gating-modified pentobarbital action, or both. Despite a constant drug concentration, the pentobarbital-induced suppression fluctuated randomly with time.

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