Molecular actions of pentobarbitone on sodium channels in lipid bilayers: role of channel structure†

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SUMMARY
The molecular mechanisms by which anaesthetics interfere with neuronal function are controversial. We have examined the effects of pentobarbitone on muscle-derived (eel electroplax) sodium channels incorporated into planar bilayers under exactly the same experimental conditions that we used previously to study the anaesthetic modification of human brain channels. This technique allows examination of protein-mediated similarities and differences. Sodium channels from the electroplax (muscle-derived) of the electric eel were purified and reconstituted into planar lipid bilayers containing 4:1 phosphatidylylethanolamine:phosphatidylycholine in the presence of batrachotoxin, a sodium channel activator. Pentobarbitone had similar voltage-independent blocking effects on sodium channels from eel electroplax and human brain, as demonstrated by similar dose-response curves (IC50 = 613 μmol litre−1). However, activation of sodium channels from eel electroplax, in contrast with human brain, was relatively insensitive to the concentration of pentobarbitone. The only significant effect was a −5.8-mV shift in the activation midpoint with pentobarbitone 680 μmol litre−1. Therefore, differences in primary structures played no role in the observed voltage-independent block of channels by pentobarbitone, whereas subunits or other structural differences between sodium channels from eel electroplax and human brain must be responsible for the minimal effect of pentobarbitone on activation of muscle-derived sodium channels. (Br. J. Anaesth. 1994; 72: 668-673)

KEY WORDS
Anaesthetics, i.v.: pentobarbitone. Ions: ion channels. Membrane, cell.

The molecular mechanisms that cause the anaesthetic state are unknown. It is believed generally that anaesthetics alter the excitability of neuronal membranes and that excitable channels are the likely sites of action [1]. However, the primary target(s) of these agents and their mechanisms of action remain controversial.

It has been proposed variously that anaesthetics alter neuronal function either by direct binding to specific sites on ligand-operated or voltage-gated channel proteins, or both, or by non-specific alterations in ion channel function, caused by hydrophobic interactions with either the biological membrane or hydrophobic regions of ion channel proteins themselves [1]. Biological membranes are heterogeneous structures of varying lipid, protein and carbohydrate composition. In situ membrane composition is virtually impossible to control experimentally, although its composition in cell cultures can be modified to some extent [2]. Additionally, there are a large number of ion channel subtypes whose structure and function exhibit marked heterogeneity both between and within the same tissue [3, 4], even for channels of the same ionic class (i.e. sodium channels, potassium channels, etc.). It is unclear how or if these structural differences affect protein-anaesthetic interactions.

We have examined anaesthetic modification of structurally distinct sodium channels in identical lipid and aqueous environments using planar lipid bilayer methodology [5, 6]. This technique allows channels from different tissues and species to be placed in identical, experimentally controlled surroundings for examination of basic function and drug interactions [7].

Previous results with human brain sodium channels indicated distinct effects of anaesthetics on two channel functions, single channel open time and activation, depending on the properties of the anaesthetic [8]. To study the effects of altered channel structure on this anaesthetic interaction, we have examined barbiturate modification of a muscle-derived sodium channel placed in the same experimental milieu as the human brain sodium channels studied previously. This muscle-derived channel, purified from the electroplax of Electrophorus electricus, consists of a single polypeptide [9, 10], while brain channels generally have additional subunits [11]. The subunit composition of human muscle sodium channels has not yet been determined.

and they have not been incorporated successfully into lipid bilayers.

We report here the effects of pentobarbitone on fractional open time and activation gating properties of highly purified, batrachotoxin-modified sodium channels from electric eel incorporated into planar lipid bilayers. These results are compared with previous data obtained with human brain sodium channels and their implications for understanding anaesthetic mechanisms are discussed. A preliminary account of this work was presented at the Annual Meeting of the American Society of Anesthesiologists [12].

MATERIALS AND METHODS

Preparation of sodium channels

Sodium channels from *Electrophorus electricus* were solubilized with CHAPS (3-[3-cholamidopropyl]-dimethylammonio-1-propane sulphonate; Calbiochem-Behring Corp., La Jolla, CA, U.S.A.) and purified using the standard techniques of ion exchange and exclusion chromatography described previously [10, 13]. Analysis indicated the presence of a single polypeptide and it was estimated that more than 80% of this reconstituted protein was functional [10]. The channels were reconstituted into lipid vesicles by dialysis removal of CHAPS and incorporated into planar bilayers as described below [6, 14].

Planar bilayer measurements

The planar bilayer consists of a bimolecular leaflet of phospholipid molecules, analogous to a cellular membrane, which separates two symmetrical aqueous compartments which correspond to intra- and extracellular solutions. The purified sodium channel preparation is bubbled over the bilayer to facilitate channel incorporation [14]. Channels are incorporated into the bilayer, probably by fusion of the reconstituted vesicles with the planar bilayer lipids.

Planar bilayers were formed from neutral phospholipid solutions containing (4:1) 1-palmitoyl-2-oleoyl-phosphatidyl-ethanolamine and 1-palmitoyl-2-oleoyl-phosphatidylcholine (Avanti Polar Lipids, Birmingham, AL, U.S.A.) in decane (5% weight volume, 99.9% pure) (Wiley Organics, Columbus, OH, U.S.A.). All experiments were conducted at room temperature (mean 24 °C) in symmetrical NaCl 50 mmol litre⁻¹ (United States Biochemical). Teflon chambers were prepared and used as described previously [14]; the chambers were divided into a cis compartment to which the reconstituted preparation was added and a trans compartment. Silver–silver chloride electrodes made direct contact with both chambers. Membrane currents were recorded under voltage-clamp conditions and filtered at 50 Hz [14].

Purified, reconstituted eel sodium channels were incorporated into the bilayers in the presence of batrachotoxin 1 μmol litre⁻¹ (batrachotoxin was a gift from Dr J. Daly). As described previously [6], batrachotoxin is used commonly in bilayer experiments to study the steady-state properties of sodium channels by removing inactivation and increasing open times. (More detailed consideration of the effects of batrachotoxin in these experiments is given in the Discussion.) After incorporation, the orientation of the channel in the membrane was determined by measuring the potentials where the channel underwent activation gating [6]. When channels were incorporated asymmetrically in the bilayer (i.e. some channels with their extracellular sides facing the cis chamber and others facing the trans chamber) tetrodotoxin (TTX) 25–50 μmol litre⁻¹, a specific sodium channel blocker, was added to one of the chambers to block all channels facing one direction [15]; the electrophysiological sign convention was used in the presentation of all results [14]. Pentobarbitone (acid form) was purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.); purified pentobarbitone isomers were obtained from the NIDA drug supply system as acid. Pentobarbitone was dissolved in ethanol and added to the extracellular compartment in aliquots [6].

To measure the probability of sodium channels being open (fractional open time), current traces were recorded by computer, time-averaged and the membrane capacitative transient at each potential was subtracted. After converting currents to conductances, the fractional open time, \( f_o \), was calculated as follows: the background conductance of the lipid bilayer was subtracted from the time-averaged conductance and this corrected conductance was divided by the conductance of the fully open channels.

Voltage-dependent, steady-state activation was examined by measuring the fractional open time as a function of membrane potential. Membrane potential was held initially at +50 mV and sequentially hyperpolarized in −10-mV increments until the channel closed completely [6]. The membrane was then depolarized in a reverse sequence of +10-mV steps back to the original holding potential. Fractional open times were plotted as a function of membrane potential and fitted to a two-level Boltzmann distribution by a least squares fitting procedure as described [6]:

\[
\frac{f_o(V)}{f_{\text{max}}}=\frac{1}{1+\exp\left(-\frac{V-V_e}{RT}\right)}
\]

where \( F = \) Faraday constant, \( V = \) membrane potential, \( R = \) gas constant and \( T = \) absolute temperature. This distribution is characterized by three variables: (i) maximal value for fractional open time, \( f_{\text{max}} \); (ii) steady-state activation midpoint potential, \( V_e \), at which fractional open time reaches its half-maximal value; and (iii) the slope of the distribution at this potential, determined by \( z_e \), the effective gating charge. \( z_e \) is the effective charge that has to move across the membrane in order for the sodium channel to switch between open and closed conformations. Control and anaesthetic modified data were compared with a paired Student’s \( t \) test. \( P < 0.05 \) was considered statistically significant.

RESULTS

In the presence of batrachotoxin, eel sodium channels remained open for the most part at potentials more positive than −50 mV (fig. 1, top trace),
and a fully closed state ("flickering") (fig. 1, middle trace), the channel undergoes more frequent transitions to a non-conducting state, resulting in a decrease in the time spent in the open state. At a larger dose of pentobarbitone (680 μmol litre⁻¹, bottom trace), the transitions between the open and non-conducting state became too frequent for full resolution. O and (s)-(−) pentobarbitone; slope conductances were 24 pS (r² = 0.999, untreated channel), 17 pS (r² = 0.999, pentobarbitone 340 μmol litre⁻¹) and 11.2 pS (r² = 0.999, pentobarbitone 680 μmol litre⁻¹).

This was observed also in our bilayer system. In the presence of pentobarbitone 340 nmol litre⁻¹, the channel undergoes more frequent transitions to a non-conducting state, resulting in a decrease in the time spent in the open state. At a larger dose of pentobarbitone (680 μmol litre⁻¹, bottom trace), the transitions between the open and non-conducting state became too frequent for full resolution. O and (s)-(−) pentobarbitone; slope conductances were 24 pS (r² = 0.999, untreated channel), 17 pS (r² = 0.999, pentobarbitone 340 μmol litre⁻¹) and 11.2 pS (r² = 0.999, pentobarbitone 680 μmol litre⁻¹).

exhibiting brief and infrequent closures. After addition of pentobarbitone, sodium channels underwent more frequent transitions between a fully open and a fully closed state ("flickering") (fig. 1, middle trace), but became too rapid for full resolution at higher concentrations (fig. 1, bottom trace). Therefore, this block was quantified by averaging the current over time; from these data, fractional open times (the fraction of time that the channel conduct ions) were calculated.

The stereospecificity of this pentobarbitone-mediated block was examined with purified pentobarbitone (s)-(−) and (s)-(−) isomers. During the control period, channels had a fractional open time of mean 0.96 (SEM 0.02) which was independent of membrane potential between +50 and −50 mV (fig. 2), in agreement with previous reports [14]. Both isomers induced dose-dependent block of the channels, which was independent of membrane potential between +50 and −50 mV (fig. 2A, B). The isomers exhibited no significant differences in voltage-independent block of purified sodium channels (fig. 3), in agreement with previous results obtained with human brain sodium channels [6]. In addition, there were no differences when a racemic mixture of the isomers was used for the same measurements [Wartenberg, unpublished data]. The combined data for the individual isomers were fitted to a rectangular hyperbola and an IC₅₀ value of pentobarbitone 583 μmol litre⁻¹ with maximum effective suppression of 102%. Inset = Scatchard analysis of the averaged isomer data. Data were transformed as shown and fitted by linear regression.

FIG. 1. Original current traces from a membrane containing a single sodium channel at +80 mV. The upper trace shows the untreated control channel. The channel is open almost all the time. In the presence of pentobarbitone 340 μmol litre⁻¹ (middle trace), the channel undergoes more frequent transitions to a non-conducting state, resulting in a decrease in the time spent in the open state. At a larger dose of pentobarbitone (680 μmol litre⁻¹, bottom trace), the transitions between the open and non-conducting state became too frequent for full resolution. O and (s)-(−) pentobarbitone; slope conductances were 24 pS (r² = 0.999, untreated channel), 17 pS (r² = 0.999, pentobarbitone 340 μmol litre⁻¹) and 11.2 pS (r² = 0.999, pentobarbitone 680 μmol litre⁻¹).
PENTOBARBITONE MODIFICATION OF SODIUM CHANNELS

Fig 4. Steady-state activation response of a channel to (−)-pentobarbitone doses. A = Control; B = pentobarbitone 340 µmol litre⁻¹; C = pentobarbitone 500 µmol litre⁻¹; D = pentobarbitone 680 µmol litre⁻¹. Single channel current traces were time-averaged, converted into conductances (SCC) and plotted as a function of membrane potential. ○ = Channel conductance at potentials more positive than activation potentials, ● = conductances during activation gating. The curves show the data fitted to a two-level Boltzmann distribution as described previously [14]. The mid-point of activation (where the channel conductance was 50% of the conductance at non-gating potentials) was −79 mV for the control and −86 mV, −89 mV and −89 mV for increasing doses of pentobarbitone, respectively.

Fig 5. Average effect of doses of pentobarbitone (PB) on channel activation mid-point (Vₐ). The midpoints of channel activation were calculated from Boltzmann distributions, as in figure 4, and averaged for each concentration of pentobarbitone; each point is the average of four to seven membranes (mean, SEM). The results were compared with control data using Student’s paired t test; no significant difference from control was found, except at a concentration of 680 µmol litre⁻¹ where the activation midpoints of channel activation were significantly different from control (P < 0.05, n = 6). For demonstration only, data were computer-fitted with a rectangular hyperbola using a least squares fit (dashed curve); this fit yielded an IC₅₀ value of pentobarbitone 1803.9 µmol litre⁻¹ and a maximal shift of −19.2 mV.

−100 mV, the channel underwent transitions between open and resting (closed) states, with more negative potentials favouring the resting state [14, 16]. This decrease in the ratio of channel occupation of the open state to the closed state resulted in a corresponding decrease in fractional open time, which was determined experimentally from the time-averaged conductance of the channel (fig. 4A). At approximately −100 mV, the channel was closed almost all of the time. The voltage-dependence of this activation gating could be adequately fitted with a two-level (open and closed state) Boltzmann distribution to yield the midpoint of channel activation (Vₐ) and the effective gating charge (zₑ), the latter being calculated from the steepness of the activation curve (see Methods). Previously it was found that the midpoint of channel activation was approximately 20 mV more positive for eel sodium channels [14] than for human brain [17] sodium channels.

The interaction of pentobarbitone isomers with eel channel activation was examined as described previously [5, 6]. Pentobarbitone caused only minor changes in channel activation gating variables (figs 4, 5). Figure 4 shows an example from a membrane containing a single channel with a conductance of about 25 pS, which was open most of the time at potentials more positive than −50 mV (fig. 4A). When pentobarbitone was added (fig. 4B, C and D), the channel exhibited potential-independent block, described above, at all potentials more positive than −50 mV. At more negative potentials, the channel again “gated” between the partially blocked open and the resting state in a voltage-dependent manner; the midpoint of activation was shifted slightly to more negative potentials with increasing concentrations of pentobarbitone, although these shifts were not statistically significant at most concentrations of pentobarbitone. The results with both
isomers were similar and therefore were averaged for further analysis.

A summary of the change in activation midpoint of 27 channels from 16 membranes is shown in figure 5. It may be seen that although there is a slight shift in the activation midpoint to more hyperpolarized potentials, this shift is not significant except for pentobarbitone 680 μmol litre⁻¹. At pentobarbitone 1 mmol litre⁻¹, activation shifted, on average, less than —7 mV, but this shift was not significant (P > 0.05). In contrast, a much stronger interaction was found previously with human brain sodium channels [5, 6]. No change was found in E, before or after addition of pentobarbitone.

**DISCUSSION**

The results of this study support two general conclusions: (a) in identical lipid environments and under identical experimental conditions, voltage-independent pentobarbitone block of the single polypeptide comprising the eel sodium channel is indistinguishable from pentobarbitone block of human brain sodium channels containing additional subunits; (b) in contrast, the effect of pentobarbitone on sodium channel activation differed between the two channel structures.

Our observation that sodium channels with different structures respond similarly to anaesthetic modification suggests strongly that structural differences in the primary sequences of the large polypeptide comprising the main functional component of the sodium channels (and the presence or absence of the beta subunits), do not affect voltage-independent blocking interactions of pentobarbitone with the channel. This is a remarkable result in that the functional purposes of these channels are quite different and have evolved to operate at different temperatures and in different environment. Further, their primary sequence homology is only about 60% [18, 19]. This suggests that the site or sites of pentobarbitone interaction with sodium channels are well conserved channel structures. In addition, any differences in the anaesthetic response of sodium channels from these or other tissues in situ are most likely the result of either other anaesthetic interactions with the channels, such as those described on activation, or result from differences in the lipid or cellular milieu of the tissues.

Although the exact mechanism of pentobarbitone block of the channels is unknown, it is significant that the block is independent of potential. Even in the presence of batrachotoxin, sodium channels undergo voltage-dependent conformational changes. Thus most other sodium channel blockers, such as TTX, saxitoxin (STX) and some local anaesthetics [16, 20], exhibit voltage-dependent block of the sodium channel. Veratridine and grayanotoxin, other sodium channel activators which bind to the same site as batrachotoxin, also exhibit voltage-dependent interactions with the channel [21]. The voltage-dependent binding of TTX, STX and the activating toxins to the modified channel are caused primarily by conformational changes in the channel structure dependent on potential [21, 22]. Thus it appears that pentobarbitone alters a conserved sodium channel structure which does not undergo conformational change with potential, yet which affects channel function. Furthermore, the lack of voltage-dependent block with pentobarbitone indicates either that the neutral (uncharged) species is responsible for block or, if the charged form of the anaesthetic is responsible for block, it interacts with a site on the channel surface and not within the pore where membrane potential would be sensed.

However, it is important to bear in mind the role played by batrachotoxin in the anaesthetic response of these channels. So far it has not been possible to conduct pharmacological studies with sodium channels in bilayers in the absence of activating alkaloid toxins. Of the alkaloid toxins, batrachotoxin is the most commonly used. It partially removes sodium channel inactivation, allowing the channel to be open for longer recording times needed for channel resolution in this system. However, batrachotoxin modifies most or all sodium channel functions [23]. Nevertheless, this modification differs among various sodium channels examined (see table V in [14]). It appears unlikely therefore that the identical dose responses of both channels to pentobarbitone block result primarily from batrachotoxin modification. The advantage of using batrachotoxin in bilayers over any other single channel technique is that it functions as a label, allowing us to observe a single sodium channel for hours, knowing that the same channel is being observed.

Another factor which needs to be considered is the effective dose range of pentobarbitone in these experiments. Although the anaesthetic concentrations used are close to the clinically relevant dose range [6], the IC₅₀ of pentobarbitone block of the sodium channel is about 10-fold greater than that needed to achieve clinical anaesthesia [24]. However, without knowing the neuronal pathways involved in anaesthesia, it is not possible to predict how clinical doses of pentobarbitone and IC₅₀ values of sodium channel suppression in bilayers should be related. Nonetheless, there is experimental evidence that sodium channel function is altered at doses of pentobarbitone which induce anaesthesia [25], and which range from 25 to 50% of our experimental IC₅₀ value.

In contrast with the similarities of pentobarbitone interaction with the two channels, the interaction of pentobarbitone with the activation gate of the sodium channel apparently is dependent on protein structure. Because these experiments were conducted with identical electrolyte and lipid compositions, the most likely variable responsible for this anaesthetic variation was channel structure. It is unclear, however, if this difference results from the presence of other pentobarbitone binding sites on the brain channel, differences in the primary structures of the sodium channels from the two species or is related to the presence of the beta subunits in humans. Nonetheless, this result does indicate that different sodium channel subtypes (at least three in human brain, and more in other tissues [4]) may be affected differentially at the same anaesthetic concentration. These differential effects of pentobarbitone on
distinct channels indicate that it is not possible to generalize the measured effects of anaesthetics on sodium channels (or other ion channels) from one tissue or cell to another.

ACKNOWLEDGEMENTS

We thank Dr S. R. Levinson, University of Colorado Health Sciences Center, Denver, CO, for the supply of purified eel sodium channels; and Drs T. J. J. Blanck and C. Frenkel for reading of the manuscript and Ms Allison Hernandez for technical assistance. Supported by NIH grant GM41102 (D.S.D.).

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