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Effects of pentobarbitone on acetylcholine-activated channels in mammalian muscle

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1 Acetylcholine-activated single channel currents were recorded from the extrajunctional region of chronically denervated skeletal muscle of the rat by the patch clamp technique.

2 In control experiments, the cumulative open-time, closed-time and burst length distributions could be well described by the sum of two exponentials.

3 Pentobarbitone decreased the mean open time and increased the time constant of the fast component of the closed time distribution. These effects increased with drug concentration.

4 The mean burst length was relatively independent of pentobarbitone concentration over the range of concentrations used (10–500 μM).

5 These observations are inconsistent with a simple sequential blocking model and it is suggested that pentobarbitone has an allosteric site of action on receptor-channel complexes that makes the open state less stable.

Introduction

A wide variety of drugs that change the decay of endplate currents are thought to do so by blocking endplate channels. For example, barbiturates have been known for some time to increase the rate of decay of endplate currents (Adams, 1974; Seyama & Narahashi, 1975; Torda & Gage 1976) and it has been suggested that barbiturate molecules might reduce channel open time by entering and blocking endplate channels (Adams, 1974; 1977). This mechanism is kinetically equivalent to the sequential blocking model proposed to explain the actions of local anaesthetics on endplate currents (Steinbach, 1968; Adams, 1977; Neher & Steinbach, 1978).

The sequential blocking mechanism can be described by the following reaction scheme.

$$\text{closed} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{open} \underset{k_{-2}}{\overset{x_B k_2}{\rightleftharpoons}} \text{blocked}$$

where, x_B is the concentration of the 'blocking' drug and k_1, k_{-1}, k_2 and k_{-2} are reaction rate constants.

Three predictions from the model (see e.g. Neher & Steinbach, 1978) are:

(i) Mean open time should decrease with drug concentration.

$$\text{mean open time} = 1/(k_{-1} + x_B k_2) \quad (1)$$

(ii) Mean blocked time should be independent of drug concentration.

$$\text{mean blocked time} = 1/k_{-2} \quad (2)$$

(iii) Mean burst length should increase linearly with drug concentration.

$$\text{mean burst length} = (1 + x_B k_2/k_{-2})/k_{-1} \quad (3)$$

The development of the giga-ohm patch clamp technique (Hamill *et al.*, 1981) has made it possible to record the currents flowing through single channels with a much greater resolution than before and this allows more detailed kinetic analysis of ion channel gating than could be achieved previously. The purpose of this study was to examine the effects of pentobarbitone on the properties of single acetylcholine-activated channels and to determine whether changes in channel behaviour are in accord with the predictions of the sequential blocking model. It will be shown that pentobarbitone produces flickering behaviour in open channels but that neither prediction (ii) nor (iii) is obeyed.

Methods

Experiments were performed on denervated (12 to 16 days) extensor digitorum longus muscles of 300–400 g male Wistar rats. To prepare the muscle for patch clamp recording, it was first necessary to remove the collagenous matrix surrounding the individual fibres. The whole muscle was pinned out in a perspex bath

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and two small perspex guides were introduced to isolate the central region of the muscle from the tendinous attachments. The gap under the guides above the surface of the muscle was sealed with vaseline. Collagenase (0.3% Sigma type 1A) was added to the standard extracellular solution which contained): NaCl 145 mM, KCl 2.5mM, $CaCl_2$ 2.5mM, $MgCl_2$ 1.0 mM; tetrodotoxin 30 nM and Na-Hepes 2 mM and was adjusted to a pH of 7.3. This solution was then introduced into the central well and the preparation was warmed to approximately 35°C for 1.0–1.5 h. The perspex guides and excess vaseline were then removed from the bath and the muscle was rinsed in fresh saline. Following enzyme treatment, single muscle fibres could be isolated with a small glass hook (Neher *et al.*, 1978). The bath temperature was maintained at 16°C for all experiments by means of a feedback-controlled Peltier device.

Patch pipettes were prepared from micro-haematocrit tubes (Clay Adams 1021) according to the procedure of Hamill *et al.* (1981). Pipettes were given a hydrophobic coating by dipping them in a 2% solution of liquid silicone (Dow Corning 200 fluid) before heat polishing the tip on a microforge. Pipettes contained the standard solution plus 200–400 nM acetylcholine chloride. Sodium pentobarbitone was included in both the pipette and bath solutions.

Single channel currents were recorded as described by Hamill *et al.* (1981) using a List Electronics (L/M EPC-5) I-V converter headstage and amplifier. The membrane potential of isolated fibres was measured with an independent intracellular electrode. The potential across the isolated patch of membrane was clamped at − 100 mV for all experiments by holding the potential inside the pipette at a voltage equal to the difference between the intracellular potential and a command potential. Any change in the resting membrane potential of the muscle fibre during the course of recording was automatically compensated for by an equal and opposite change of potential within the pipette. Current recordings were low-pass filtered (− 3dB at 1.3 kHz, 4 pole Bessel) and then sampled at 10 kHz using a microcomputer (Biosystems). Data were taken in blocks of 1 s duration separated by 3 second intervals during which the data were stored on magnetic discs.

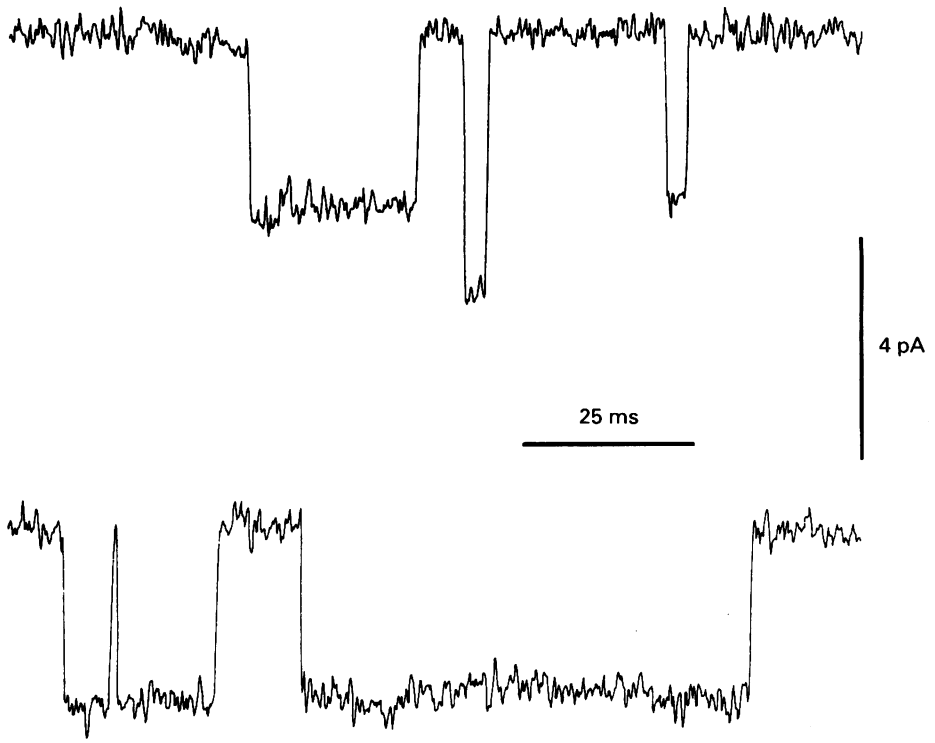

Figure 1 Single channel currents recorded from a denervated muscle showing both junctional and extrajunctional channels. Signals were filtered at 1.3 kHz (− 3dB point, Bessel response). Holding potential, − 100 mV. Temperature, 16°C.

To analyse the data, a programme was used that automatically identified and subtracted the baseline current level from individual records. On a second pass of the corrected data, a mid-level threshold crossing routine was used to identify single channel events and the duration of channel open and closed times within each record was stored in sequential order. The cumulative distribution of open and closed times could be calculated directly from the measured transition times. To calculate the burst length distribution, a critical closed time was calculated using a procedure similar to that of Magleby & Pallotta (1983). Burst length was defined as the sum of open and closed times (within a single record) that were not separated by a closed time greater than the calculated critical value which typically fell within the range 3 to 5 times the time constant of the fast component of the closed time distribution. The cumulative distributions were then fitted with single or double exponential functions using a non-linear least squares procedure. The dead time for the midlevel threshold detection of events was 155 μs for a 1.3 kHz (− 3dB point) setting on the filter. For this reason, curve fitting was limited to events greater than or equal to 200 μs duration. The mean open and closed times calculated from the curve fitting were then corrected to take into account the limited frequency response of the recording system following filtering (Neher, 1983).

Results

Single channel currents recorded from a denervated muscle are shown in Figure 1. Very few rapid fluctuations in current during the open state of a channel were seen. There were clearly two types of channel with different conductances in the denervated muscle; an 'extrajunctional' channel with conductance of 32 ± 0.5 pS and a 'junctional' channel with conductance of 46 ± 0.5 pS (mean ± s.e.mean). Both types can be seen in the upper trace in Figure 1. The relative numbers of each channel type observed in individual patches were variable. Some patches appeared to have only junctional channels whereas others had only extrajunctional channels. Most commonly, however, a mixture of channels was observed with extrajunctional channels (32 pS) being most common. For the purpose of this study, only patches in which there was a predominance (greater than 95%) of extrajunctional channels were used. Any currents from junctional channels in these patches were not included in the analysis. Cumulative distributions of open times of

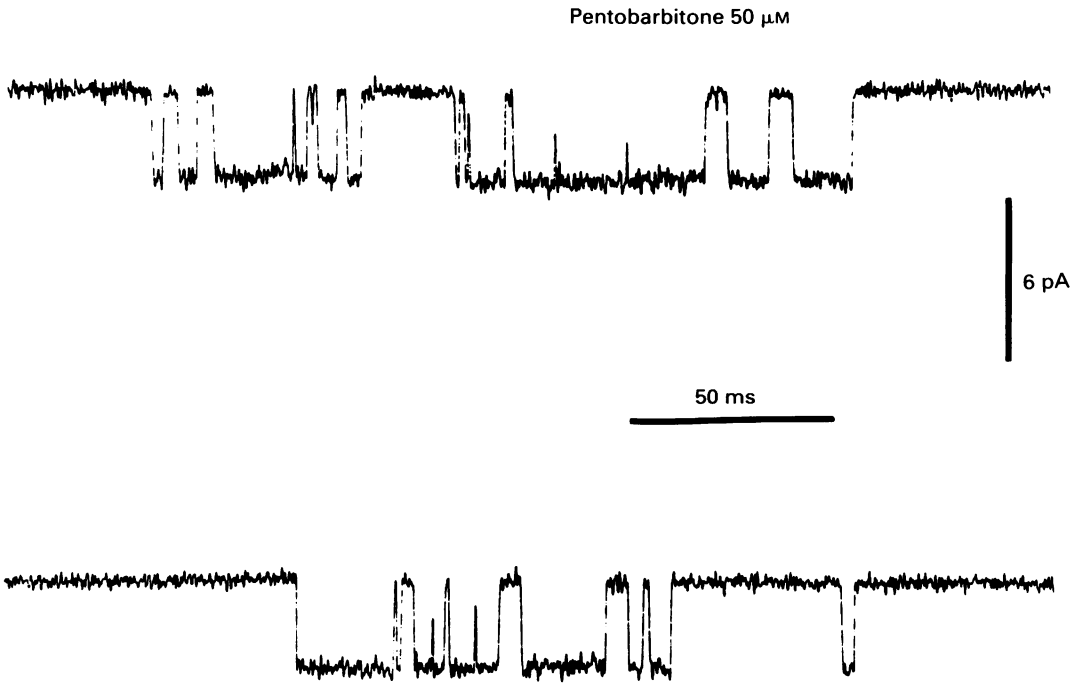

Figure 2 Single channel currents in the presence of sodium pentobarbitone 50 μM showing flickering between open and closed states during a burst. Membrane potential − 100 mV. Temperature 16°C.

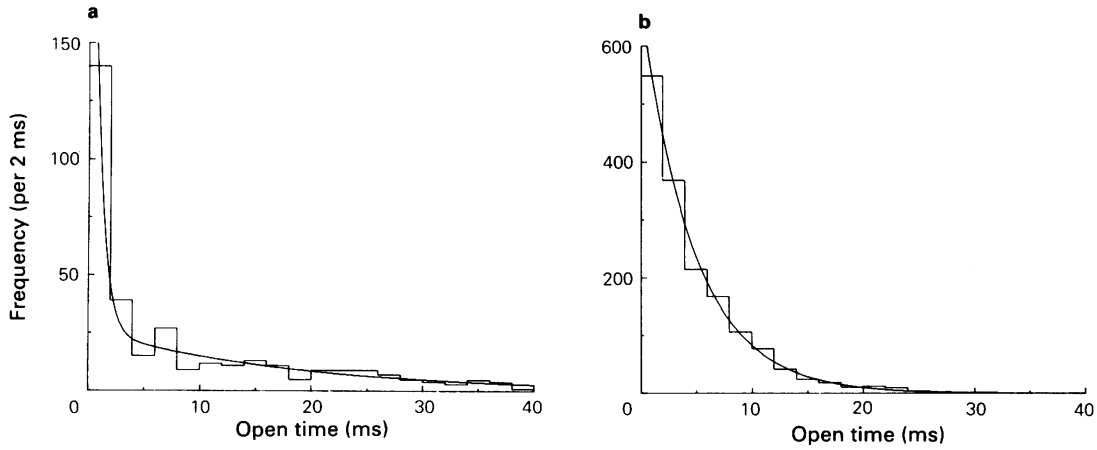

Figure 3 (a) Distribution of channel open times in a patch of normal denervated muscle. The fitted curve is the sum of two exponentials with time constants of 700 μs and 17.9 ms. The proportions of the components were 38% and 62%, respectively. (b) Distribution of channel open times in a patch of denervated muscle following exposure to sodium pentobarbitone 50 μM. The fitted curve is a single exponential with time constant of 4.8 ms. A faster component would not have been seen because of the frequency limitations of the recording system.

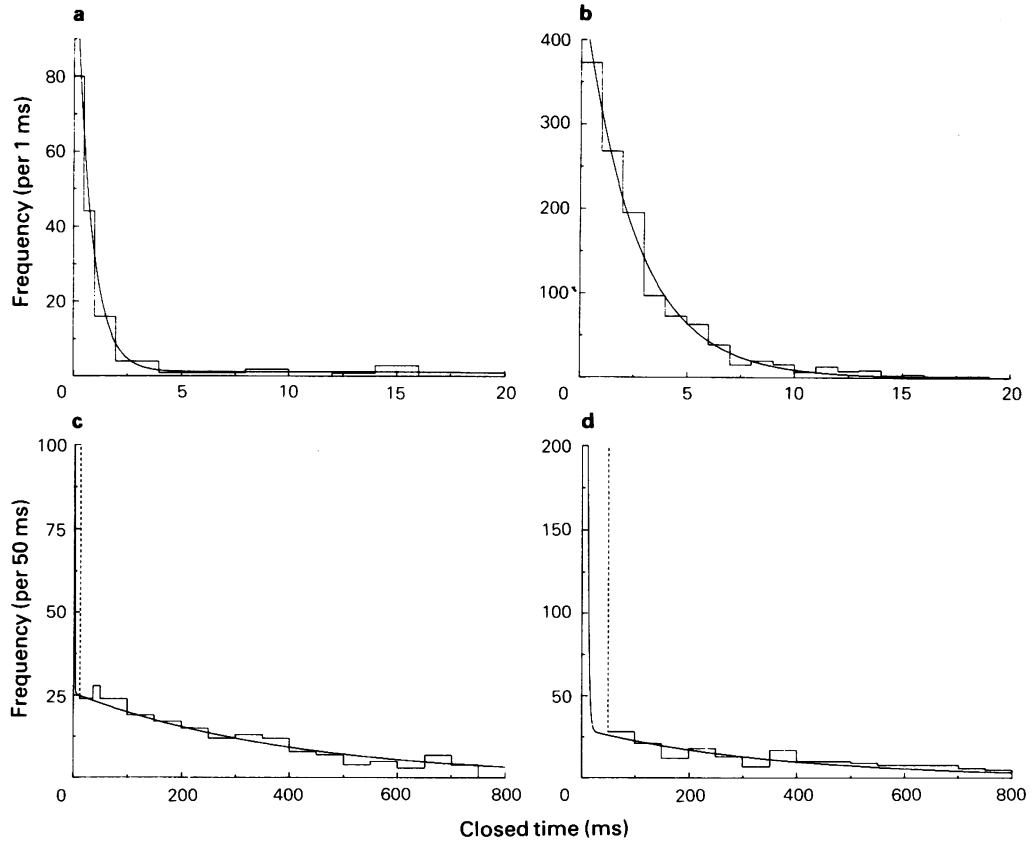

Figure 4 (a) Distribution of channel closed times in a denervated muscle (same patch as in Figure 3a). The fitted curve is the sum of two exponentials with time constants of 710 μs and 390 ms. The proportions of the components were 19% and 81%, respectively. (b) Distribution of channel closed times in the presence of sodium pentobarbitone 50 μM (same patch as in Figure 3b). The time constants of the fitted curve were 2.5 ms and 360 ms. The proportions of the components were 85% and 15%, respectively. (c) Same data as in Figure 4a replotted over the time interval 0 to 800 ms to illustrate the distribution of long closed times. (d) Same data as in Figure 4b replotted over the time interval 0 to 800 ms to illustrate the distribution of long closed times.

extrajunctional channels in a patch contained two exponential components as illustrated in Figure 3a.

The obvious effect of pentobarbitone was to produce rapid fluctuations between open and closed states of channels (see Figure 2). In these records, obtained in the presence of pentobarbitone 50 μM, the channel openings were unusually long and clearly show the 'bursting' character of openings in the presence of this drug. Only a single time constant could now be resolved in the open time distribution (Figure 3b). It was assumed that any fast component comparable to the fast component seen before exposure to the drug was lost because of the high frequency limitations of the recording system. For this reason, the slow component of the control open time distribution (Figure 3a) was compared with the single component seen in the presence of pentobarbitone (Figure 3b).

The closed time distribution in both the presence and absence of pentobarbitone was composed of two clearly separate components (Figure 4). The proportion of the total number of closed times included in the fast component of the distribution increased from approximately 20% in the control recordings to

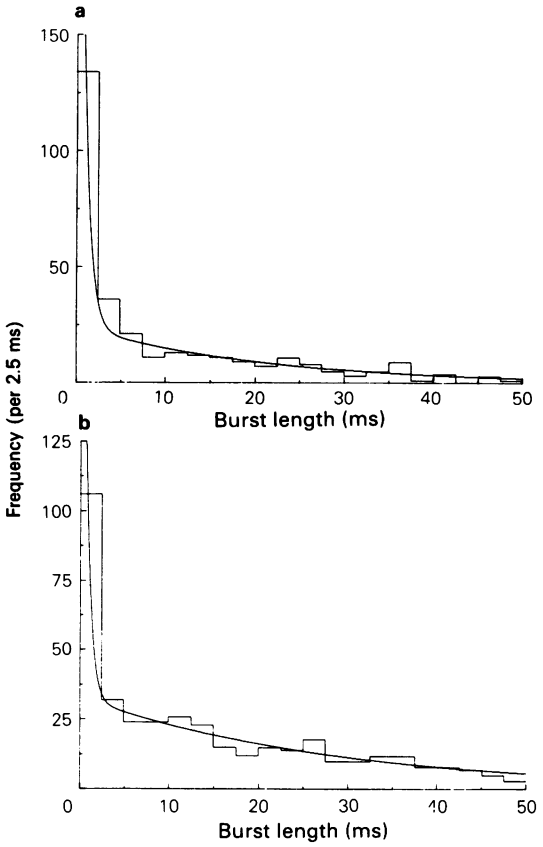

Figure 5 (a) Burst length distribution in a patch from a denervated muscle fibre (same patch as in Figure 3a). The fitted curve is the sum of two exponentials with time constants of 700 μs and 20.3 ms. The proportions of components were 41% and 59%, respectively. The distribution of burst lengths was calculated using a gap of 2.6 ms calculated from the distribution of closed intervals shown in Figure 4a. (b) Burst length distribution in the presence of pentobarbitone 50 μM (same patch as in Figure 3b). The time constants of the fitted curve were 540 μs and 28.3 ms. The proportions of components were 19% and 81%, respectively. Distribution of burst lengths was calculated using a gap of 12.5 ms calculated from the distribution of closed intervals shown in Figure 4b.

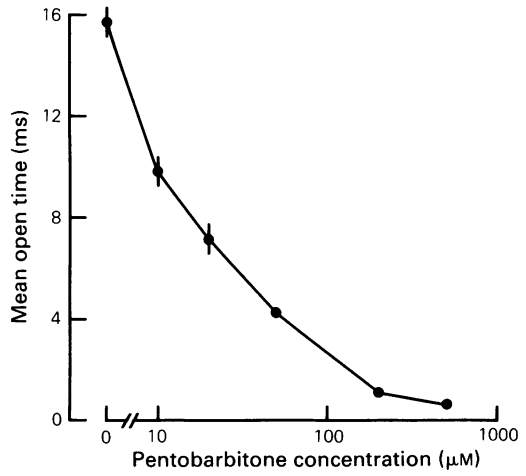

Figure 6 Plot of the average time constant of cumulative open time distributions against pentobarbitone concentration. Each point shows the average of results from 3–5 patches and vertical bars denote ± s.e.mean where larger than the symbols.

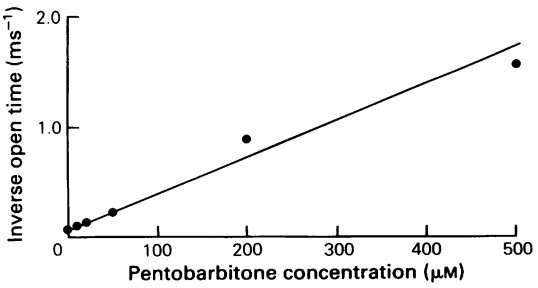

Figure 7 Plot of inverse mean open time against pentobarbitone concentration. The fitted line coresponds to the equation: inverse open time = $(k_{-1} + x_B k_2)$, where $k_{-1} = 67\,s^{-1}$, $k_2 = 3.4 \times 10^6\ M^{-1}s^{-1}$ and x_B is the barbiturate concentration.

approximately 80% in the presence of pentobarbitone at concentrations of 50 μM or more.

The burst distribution in both the presence and absence of pentobarbitone was consistently composed of two separable components (Figure 5). Because the fast component was often poorly resolved in the presence of pentobarbitone, only the time constant of the slow component was used for comparison.

From the average values of the time constants fitted to the cumulative distributions of open and closed times and the mean number of openings per burst in the presence of pentobarbitone 50 μM, the following values for the rate constants for the sequential blocking model were calculated.

$$k_{-1} = 67\,s^{-1}$$
$$k_2 = 3.4 \times 10^6\,M^{-1}s^{-1}$$
$$k_{-2} = 450\,s^{-1}$$

As predicted by the blocking model, the mean open time decreased with increasing pentobarbitone concentration (Figure 6) from 15.7 ± 0.6 ms before exposure to the drug to 0.63 ± 0.03 ms in the presence of pentobarbitone 500 μM. The relationship between inverse open time and pentobarbitone concentration is shown in Figure 7. The line through the points shows the prediction from equation (1) using rate constants ($k_{-1} = 67\,s^{-1}$ and $k_2 = 3.4 \times 10^6\,M^{-1}s^{-1}$) calculated from the mean open time in the presence of 50 μM pentobarbitone. This result is consistent with the sequential blocking model in which the reduction of open times is due to a simple bimolecular association reaction between the barbiturate molecule and the receptor-channel complex.

The effect of pentobarbitone on the time constant of the fast component of the closed time distribution is shown in Figure 8. The time constant of the fast component shows a steady increase with increasing pentobarbitone concentration. This result is inconsistent with the sequential blocking mechanism which predicts that the average blocked time should be independent of blocking drug concentration.

The average time constant of the slow major component of the burst distribution showed only a small increase over the concentration range used in these experiments (Figure 9). This observation is also at variance with the sequential blocking model which predicts a strong dependence of mean burst length on blocking drug concentration. For example, the rate constants calculated in the presence of pentobarbitone 50 μM predict a slow time constant for the burst distribution of 39 ms and 73 ms in the presence of 200 μM and 500 μM pentobarbitone respectively, whereas the actual experimental values were 24 ms and 28 ms.

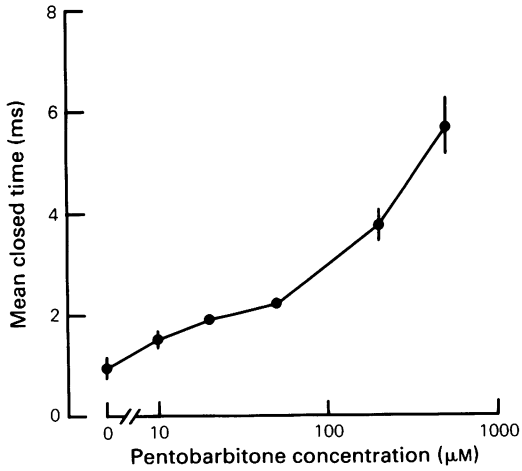

Figure 8 Plot of the average fast time constant of the cumulative closed time distribution against pentobarbitone concentration.

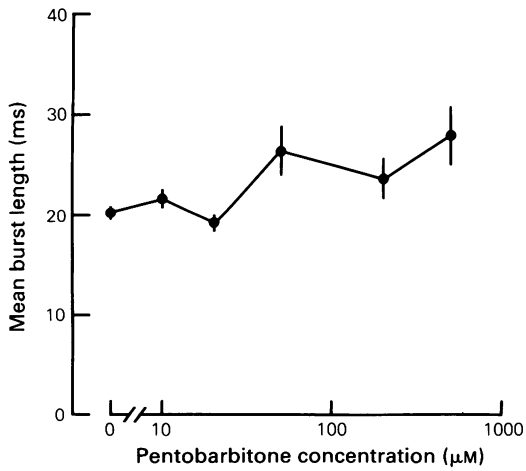

Figure 9 Plot of the average slow component of the burst length distribution against pentobarbitone concentration.

Discussion

The results do not support a mechanism of drug action in which a barbiturate molecule simply enters and blocks an open endplate channel without having any other effects. Whether it is meaningful to talk of 'channel block' by drugs such as the barbiturates (or any drug that acts primarily in neutral form) is questionable. It seems more likely that such drugs modify channel gating by binding to allosteric sites on

the receptor-channel complex rather than by physically blocking channels. Several other drugs thought to have been simple channel blockers have also been found not to act in this way (Gage & Sah, 1982; Gage *et al.*, 1983; Neher, 1983; Gage & Wachtel, 1984).

It has been suggested (Franks & Lieb, 1984) that general anaesthetics such as halothane and aliphatic alcohols act by competing directly with endogenous ligands for binding sites on receptors. This hypothesis seems plausible only in those cases where the endogenous ligand is hydrophobic in nature. Most neurotransmitters do not fall into this category and it would seem that some other explanation is necessary to explain the effects of the various anaesthetics on receptors. One possibility is that anaesthetics compete with the lipids of the membrane for binding sites on the protein channel-receptor complex and that disruption of the normal interactions between lipid molecules and the integral membrane protein could cause changes in the affinity between the agonist and receptor and/or change the rates of transition between the open and closed states of the complex.

In order to gain any further insight into the mode of action of drugs such as pentobarbitone, it may first be necessary to obtain a more complete understanding of the mechanism by which acetylcholine itself activates the receptor-channel complex. It is now widely accepted (Colquhoun & Sakmann, 1981; Sine & Steinbach, 1983) that gating of the acetylcholine receptor requires transitions through a number of closed states before the open state is reached. It is possible that the binding of a barbiturate molecule to the receptor-channel complex increases the lifetime of any one of a number of postulated intermediary non-conducting states other than the resting closed state. Allosteric modification of the rates of transition to and from these transient closed states would give the effects we have observed.

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