Phenobarbital Increases Spontaneous Transmitter Release at the Frog Neuromuscular Junction

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Phenobarbital (1–2 × 10–4 M) markedly increases the frequency of miniature end-plate potentials at the neuromuscular synapse of the frog. This effect was seen in calcium free media containing EGTA. The drug probably acts presynaptically at an intracellular locus to increase the presynaptic free calcium concentration.

Phenobarbital is known to increase the quantal content of the end plate potential at the frog neuromuscular junction [1, 2, 3]. Thompson and Turkanis reported that the action potential recorded extracellularly from frog motor nerve terminals was increased in duration by phenobarbital [1]. As it has been shown that an increase in either the amplitude or duration of a depolarizing pulse applied to frog nerve terminals causes a larger amount of transmitter to be released [4, 5], this mechanism could explain the phenobarbital-induced enhancement of quantal content. However, Weakly and Proctor [3] have recently shown that phenobarbital can significantly increase quantal content without having an observable effect upon the configuration of the presynaptic action potential. This suggests that the drug acts in some other manner to increase evoked release than by increasing the size or duration of the action potential at the nerve terminal.

The experiments reported here were performed in the expectation that phenobarbital might act intracellularly to increase the concentration of free calcium at the nerve terminal. This would increase the rate at which transmitter packets are spontaneously released and could result in increased quantal content. Initial results have supported the view that phenobarbital mobilizes calcium at an intracellular locus.

METHODS

Experiments were done at room temperature on the isolated sartorius neuromuscular preparation of the frog (Rana pipiens) which was bathed in standard Ringer's solution of the following composition: 115 mM NaCl; 2.0 mM KCl; 1.8 mM CaCl2; sodium phosphate buffer 0.25 mM at pH 7.1–7.4.

The effect of phenobarbital upon miniature end plate potential (mepp) frequency and amplitude was determined. As the drug reduced the mepp amplitude toward noise level, experiments were also performed in the presence of neostigmine chloride 4 μg/ml to increase the size of mepps in order that the assessment of frequency be more accurate. The effect of phenobarbital upon mepp frequency and amplitude was

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also assayed in calcium-free media containing EGTA 1.0 mM and MgCl₂ 2.0 mM. The Na, K and PO₄ ion concentrations and pH in these experiments were identical to standard Ringer. As in standard Ringer, neostigmine 4 ug/ml was added to some experiments.

After an equilibration period of at least one hour in the control medium followed by a control reading period of 10–20 min, the preparation was perfused with an identical solution but containing either 1 × 10⁻⁴ M or 2 × 10⁻⁴ M phenobarbital. Drug perfusion was continued for 5 to 15 min. and followed by a wash of up to 30 min. in experiments in which more than one concentration was used initially.

The methods of changing solutions and electrical recording from superficial muscle fibers were carried out by conventional techniques. A single KC13M-filled micropipette was used for the intracellular recording of the membrane potential (resistances 10–20 megohms).

RESULTS

Phenobarbital induced an increase in mepp frequency during perfusion in 8 of 9 experiments (Fig. 1). The time course of this change is indicated in Fig. 2. The drug caused a rise in frequency which varied from 50 to 300 percent of control values within 5 minutes and a dose-related response was shown (Fig. 3) such that increasing the dose caused an increase in the response. The increase bore no relationship to the external calcium concentration and was seen at 1.8 mM and in the absence of external Ca in solutions containing EGTA. The average resting membrane potential was -80.8mV during the control period and -79.7mV after perfusion with phenobarbital and -76.0mV at the end of the wash.

Because phenobarbital reduced mepp amplitude through a post-synaptic mechanism, the effect of phenobarbital on mepp frequency could only be appreciated in the presence of neostigmine as the reduction in mepp amplitude caused by phenobarbital made it difficult otherwise to distinguish mepps from noise. The average amplitude of mepps in the control solution was 1.03mV ± 0.5 (SD) and in phenobarbital 0.67 mV ± 0.4 p < 0.01).

![CONTROL](image1)

![PHENOBARBITAL](image2)

![WASH](image3)

FIG. 1. Miniature end plate potential frequency increased after 15 min. exposure to phenobarbital 2 × 10⁻⁴ M in 0 Ca Ringer containing 1 mM EGTA and neostigmine 4 ug/ml.
DISCUSSION

Barbiturates are well known blockers of skeletal neuromuscular transmission [1]. Thesleff [6], on the basis of experiments performed with intracellular recording techniques, concluded that the primary action of barbiturates was to prevent the normal depolarizing action of acetylcholine on the post-junctional membrane. Other experiments have indicated that barbiturates may also influence presynaptic events. This was first suggested by experiments [7,8] which indicated that barbiturates, at concentrations which markedly reduced the depolarization produced by iontophoretically-applied acetylcholine, had a much smaller effect upon the amplitude of the end-plate potential (epp). This suggested that in addition to depressing the

FIG. 2. Miniature end plate potentials frequency recorded in 0 Ca Ringer containing 1 mM EGTA and neostigmine 4 ug/ml. The phenobarbital concentration was $2 \times 10^{-4}$M.

FIG. 3. Miniature end plate potential frequency in 0 Ca Ringer containing neostigmine 4 ug/ml and 1 mM EGTA. The white bar represents exposure to phenobarbital $1 \times 10^{-4}$M and the black bar exposure to phenobarbital $2 \times 10^{-4}$M in the same cell following a 30 min. wash.
post-synaptic membrane, barbiturates may enhance the presynaptic release of transmitter by nerve impulses.

Thompson and Turkanis [1] studied the influence of phenobarbital upon neuromuscular transmission. They found that phenobarbital increased the mean quantal content of the end-plate potentials and decreased the mean amplitude of miniature end-plate potentials (mepps). These authors noted only a slight increase in mepp frequency in preparations bathed in phenobarbital, but did not consider it significant. They attributed the phenobarbital-induced increase in quantal content to prolongation of the presynaptic action potential.

In a recent publication, Weakly and Proctor [3], using a similar preparation, demonstrated that phenobarbital can increase quantal content without having any effect on the size or duration of the action potential at the nerve terminal. This suggests that the increase in quantal content induced by phenobarbital does not necessarily reflect an increased influx of calcium at the presynaptic region during depolarization.

The results reported here confirm previous reports that phenobarbital acts postsynaptically, reducing the size of miniature end-plate potentials. In addition, phenobarbital markedly increases mepp frequency. This increase in mepp frequency was initially difficult to detect as the post-synaptic effect of phenobarbital reduced the size of mepps toward noise level. However, in the presence of neostigmine, the initial amplitude of the mepps was large enough so that even after the depression caused by phenobarbital, they could still be clearly distinguished from noise. It was found that on the average there was approximately a doubling of mepp frequency after 10 minutes of exposure to phenobarbital, $2 \times 10^{-4} \text{M}$. A dose-response effect was seen.

It seems reasonable to assume that the increase in the spontaneous release of transmitter caused by phenobarbital reflects an increase in the intracellular calcium concentration at the nerve terminal. This could arise from a disturbance in the balance between influx and efflux across the presynaptic membrane, a disturbance of uptake by the mitochondria, or calcium leakage from the mitochondria or other calcium-binding intracellular structures. These processes cannot be separately assessed with present methods at the neuromuscular junction, but the fact that an increase in the spontaneous release of transmitter could be observed, even in the absence of appreciable amounts of calcium in the extracellular fluid, indicates that the main source of calcium for the elevated spontaneous release is intracellular.

**SUMMARY**

It was found that phenobarbital, in solutions containing neostigmine, markedly increased mepp frequency and this effect was seen in calcium-free media which contained EGTA. These findings are consistent with the theory that phenobarbital causes an increase in the intracellular free calcium concentration of the nerve terminal and that it does this by acting at an intracellular locus.

The experiments reported here confirm the findings that phenobarbital acts postsynaptically as well to reduce the amplitude of mepps. So potent is the drug in reducing mepp amplitude that within a few minutes following exposure, it is difficult to distinguish mepps from noise. In the presence of neostigmine, however, mepp amplitude is sufficiently increased so that even after the depression in their amplitude caused by phenobarbital, they can be clearly seen and the effect of phenobarbital on mepp frequency assayed.
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