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Barbiturate-induced transmitter release at a frog neuromuscular junction

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Summary

1. The influence of barbiturates on neuromuscular transmission at end-plates of frog sartorius muscles was investigated electrophysiologically on preparations bathed in Ringer solution containing a low concentration of calcium and a high concentration of magnesium.

2. Effects of a convulsant barbiturate, 5-(2-cyclohexylideneëthyl)-5-ethyl barbituric acid (CHEB), were compared with those of phenobarbitone.

3. CHEB and phenobarbitone increased the mean quantum content of the end-plate potentials and decreased the mean amplitude of the miniature end-plate potentials.

4. Both barbiturates enhanced the duration of the nerve-terminal action potential and had little or no effect on the effective resistance of the skeletal muscle membrane.

Introduction

Most barbiturates produce mainly general depression, but it is well known that barbiturates can evoke preanaesthetic excitation ranging from muscular twitches to overt convulsions. Some barbiturates are predominantly excitatory. The convulsant barbiturate, 5-(2-cyclohexylideneëthyl)-5-ethyl barbituric acid (CHEB), exhibits many stimulatory effects, such as convulsions in mice (Downes, Perry, Ostlund & Karler, 1970), an increase in the spinal monosynaptic (2N) reflex (Downes & Williams, 1969), induced action potentials in dorsal root ganglion cells (Downes & Franz, 1971), and contraction in aortic strips (Hupka, Williams & Karler, 1969). CHEB can produce central nervous system excitation with few or no signs of drug-induced depression (Downes & Williams, 1969).

The major site of action of the barbiturates is generally ascribed to synapses (Sharpless, 1970). Since some aspects of barbiturate-induced excitation may be the result of enhanced transmitter release, the influence of CHEB and phenobarbitone on transmitter release at end-plates of the frog sartorius muscle was studied electrophysiologically. The frog neuromuscular junction provides an isolated synapse upon which to investigate direct actions of drugs on synaptic transmission. We have found that both barbiturates increase the mean quantum content of the end-plate potentials and depress the post-junctional response in the above preparation. A preliminary report of the findings of the present investigations was presented at the Fifth International Congress on Pharmacology (Turkanis & Thomson, 1972).

Methods

Sartorius muscles and attached nerves were excised from the frog *Rana pipiens*. Muscles were stretched to 1·1 times their resting length *in vivo* and pinned at fascia and tendon in a recording chamber. Preparations were bathed in Ringer solution of the following composition (mM): NaCl 95·0 ; KCl 2·0 ; $CaCl_2$ 0·6 ; $MgCl_2$ 10·0 ; Na_2HPO_4 5·0 ; NaH_2PO_4 1·0. Neostigmine methylsulphate (Roche Laboratories) 1 μg/ml was added to the Ringer solution to increase the amplitude of the miniature end-plate potentials (m.e.p.ps.) ; pH of the bathing solution ranged from 7·3 to 7·4. Bath temperature was maintained at approximately 19° C.

End-plate potentials (e.p.ps.) and m.e.p.ps. were recorded intracellularly with glass micro-electrodes filled with 3 M KCl. The e.p.ps. were evoked by stimulating the motor nerve supramaximally at intervals of 3 s for a total of 200 stimuli per test. The e.p.ps. were summed by an Enhancetron 1024 computer (Nuclear Data, Inc.) along with a constant calibration pulse to allow calculation of the mean amplitude of e.p.ps. A series of about 100 m.e.p.ps. was then recorded photographically for amplitude and frequency measurements. Frequency was calculated from the time required for occurrence of about 100 m.e.p.ps. Mean quantum content of the end-plate potential (m) was calculated from the ratio of the mean amplitude of the e.p.ps. to the mean amplitude of the m.e.p.ps. (del Castillo & Katz, 1954), subsequently designated as the 'mean amplitude' method. Large m values (>5) were corrected for non-linear summation of the unit potentials (Martin, 1955, 1966). The accuracy of the Enhancetron measurements of e.p.ps. was tested by measuring photographs of individual e.p.ps. Mean amplitudes of e.p.ps. obtained by both methods agreed favourably.

Extracellular recordings of e.p.ps. and of nerve-terminal action potentials were made with glass micro-electrodes filled with 2 M NaCl ; the technique for focal recording was described by Katz & Miledi (1965). The motor nerve was stimulated supramaximally at intervals of 2 s for a total of 100 or 300 stimuli per test. Nerve-terminal action potentials were also summed by the computer and photographed. The duration of action potentials was measured from enlarged photographs ; the width of the negative phase was measured at the level of the baseline (see dash line in Figure 5). In these experiments, m was calculated from the $\log_e$ of the ratio of the number of trials to the number of failures (del Castillo & Katz, 1954), subsequently designated as the 'failures' method. 'Failures' were determined from photographic records.

Effects of the barbiturates on m, amplitude and frequency of the m.e.p.ps., and amplitude and duration of the action potential at the nerve terminal were tested. After control measurements were completed (usually three sets of control measurements at 15-min intervals), the sodium barbiturate solution (10–100 μl) was added to the bathing solution (10 ml), and its effects were followed for 2 hours. Each experiment was conducted in a different muscle. Preliminary studies indicated that the various parameters of neuromuscular transmission described above were relatively stable for 2 to 3 hours.

The effective resistance of the skeletal muscle fibres was also measured before and after the addition of drug by methods described by Stefani & Steinbach (1969). Effective resistance was calculated from the amount of current required to produce small depolarizations ; each value stated in the **Results** is the mean of three determinations.

Results

Intracellular recording

The effects of two concentrations of CHEB and phenobarbitone on the amplitude and frequency of the miniature end-plate potentials (m.e.p.ps.) and the mean quantum content of the end-plate potentials (m) were tested with intracellular recording techniques. Changes in the responses obtained with a concentration of 20 μM of either drug were small; a concentration of 200 μM produced similar but more pronounced effects without abolishing the post-synaptic response. Thus, the differences in effects of the two concentrations of drug were only quantitative (Fig. 1). Therefore, only the findings of experiments performed with concentrations of 200 μM are described below.

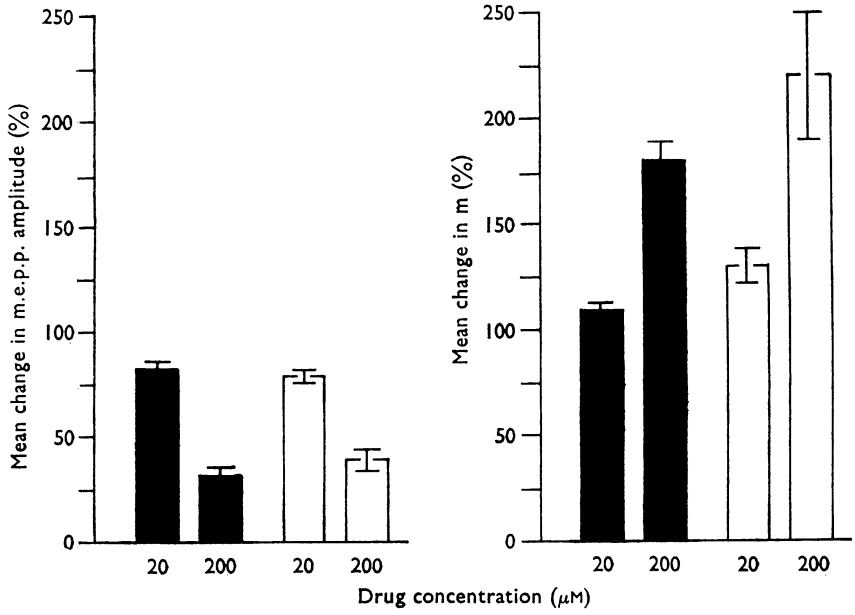

FIG. 1. Effects of 5-(2-cyclohexylideneëthyl)-5-ethyl barbituric acid (CHEB) and phenobarbitone on amplitude of miniature end-plate potentials (m.e.p.ps.) and mean quantum content of end-plate potentials (m). Bars with brackets represent mean and S.E.M. of maximum changes in five experiments expressed as % of control values. Drug effects were monitored for 120 min by intracellular micro-electrode techniques. Filled columns, CHEB; open columns, phenobarbitone.

Amplitude of m.e.p.ps. Post-junctional effects of the barbiturates were assessed by comparing the mean amplitudes of the m.e.p.ps. before and after drug. As shown in Figs. 1 to 3, CHEB and phenobarbitone greatly reduced the amplitude of the m.e.p.ps. After treatment with either agent, the amplitude decreased rapidly for 15 to 30 min, and then declined slowly or remained relatively constant during the remainder of the 120-min test period. Representative experiments are illustrated in Figures 2A and 2B.

The mean amplitudes of the control m.e.p.ps. were 0·54 and 0·45 mV, respectively, in the CHEB and phenobarbitone experiments. Maximum changes in mean amplitude after drug treatment are expressed as a per cent of the control value. On the average in five experiments each, CHEB reduced the m.e.p.p. amplitude to

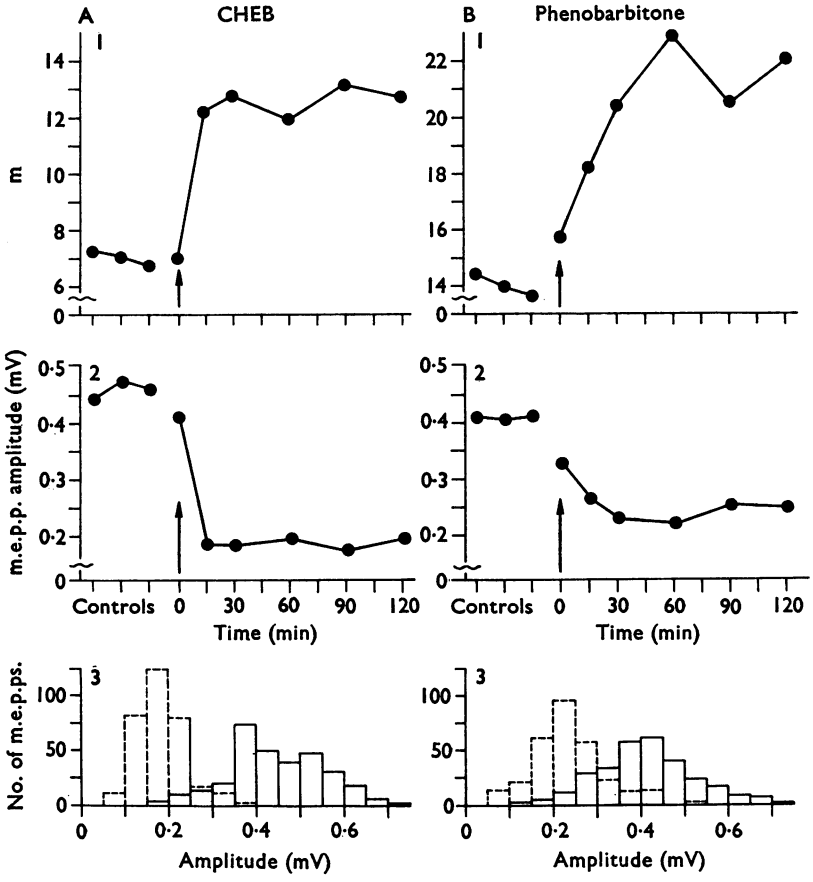

FIG. 2. Effects of 5-(2-cyclohexylideneëthyl)-5-ethyl barbituric acid (CHEB) and phenobarbitone on the amplitude of the miniature end-plate potentials (m.e.p.ps.) and mean quantum content of the end-plate potential (m). Concentration of drugs 200 μM; measurements made with intracellular micro-electrodes; for purposes of comparison, the m.e.p.p. amplitudes were adjusted for changes in membrane potential (Em). Histograms with solid lines represent control data; those with dashed lines represent data obtained after drug treatment. A. 1, 2 and 3, data obtained from one end-plate; CHEB solution added at arrow; control Em 91 mV; after drug Em gradually declined during the 120-min period to 84 mV. B. 1, 2 and 3, data obtained from one end-plate; phenobarbitone solution added at arrow; control Em 92 mV; after drug Em gradually declined during the 120-min period to 85 mV.

TABLE 1. *Effects of 5-(2-cyclohexylideneëthyl)-5-ethyl barbituric acid (CHEB) and phenobarbitone on the mean quantum content of end-plate potentials (m) and the mean amplitude of miniature end-plate potentials (m.e.p.ps.)**

Treatment	*n*‡	m$^{\Delta}$	m.e.p.p. amplitude$^{\Delta}$
CHEB†	5	180± 9	32±2
Phenobarbitone†	5	220±30	39±5

* Intracellular recording techniques employed. † Drug concentration, 200 μM. ‡ *n*, number of end-plates. $^{\Delta}$ Data represent means and S.E.M. of maximum changes expressed as % of control values in a 120-min period. From measurements obtained at each end-plate, both m and mean m.e.p.p. amplitude were calculated.

32% of control values, and phenobarbitone reduced the m.e.p.p. amplitude to 39% of control values (Table 1). In three experiments (one CHEB, two phenobarbitone) the drug was washed out with Ringer solution, and the mean amplitudes of m.e.p.ps. returned to near control values.

Racemic and (−)-pentobarbitone produced similar post-synaptic depression in five experiments. Thesleff (1956) also has reported that pentobarbitone diminished the amplitude of the m.e.p.ps. at neuromuscular junctions of the frog sartorius muscle. Methohexitone similarly reduced the amplitude of the m.e.p.ps. at end-plates of the rat diaphragm (Westmoreland, Ward & Johns, 1971).

For purposes of comparison, the amplitudes of the m.e.p.ps. were adjusted for changes in membrane potential (Katz & Thesleff, 1957). In many experiments, the membrane potentials gradually decreased by several mV after treatment with either phenobarbitone or CHEB. In some experiments, however, the membrane potential did not change, and in a few it increased. For example, typical results, such as 0 to 8 mV reduction in membrane potential, are indicated in the legends of Figures 2, 3 and 5. The findings are inconclusive but suggest that 200 μM concentrations of the drugs may have reduced the membrane potential. Thesleff (1956) reported that 403 μM and 1,208 μM concentrations of phenobarbitone decreased the membrane potential of the frog sartorius muscle to 95 and 87% of control values, respectively, yet he concluded that the membrane potential was not significantly altered by the drug. CHEB has been shown to depolarize dorsal root ganglion cells (Downes & Franz, 1971). It is important to note that the reduction in membrane

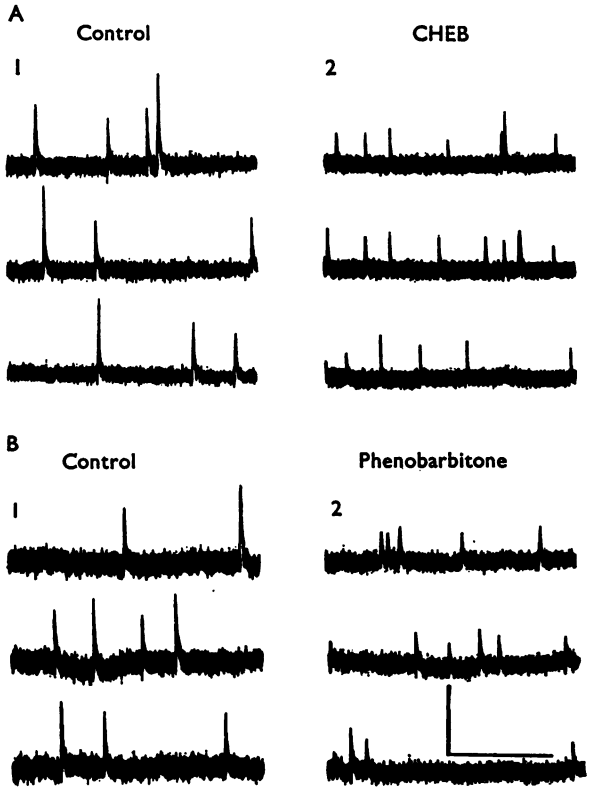

FIG. 3. Effects of 5-(2-cyclohexylideneëthyl)-5-ethyl barbituric acid (CHEB) and phenobarbitone on amplitude of the miniature end-plate potentials. Concentration of drugs, 200 μM. A. 1, controls, membrane potential (Em) 81 mV; 2, 60 min after CHEB, Em 79 mV. B. 1, controls, Em 94 mV; 2, 60 min after phenobarbitone, Em 86 mV. Records retouched for photographic purposes. Calibration, 0·5 mV and 1·0 second.

potential observed in the present study was usually less than 2 mV during any given series of measurements of end-plate potentials (e.p.ps.) and m.e.p.ps., and therefore it did not interfere with the accuracy of such measurements and estimates of m.

Frequency of miniature end-plate potentials. In the same series of experiments, the effects of the barbiturates on the frequency of m.e.p.ps. were also evaluated. Since the frequency of m.e.p.ps. can change markedly in 2 h even in the absence of drug (S. Turkanis, 1971, unpublished observations), measurements made after the 30-min test period were not considered. In studies with CHEB and phenobarbitone, the mean control frequencies were 1·3 and 2·2 s^{-1}, respectively. The mean m.e.p.p. frequencies in five experiments at 0, 15 and 30 min after phenobarbitone treatment were 110, 100 and 120% of control values, respectively. These observations suggest that phenobarbitone has little or no effect on the frequency during the 30-min period after the drug has been given. In contrast to the findings with phenobarbitone, the mean frequencies in five experiments at 0, 15 and 30 min after CHEB treatment were 120, 210 and 250% of control values, respectively. These results suggest that CHEB increases the frequency. Westmoreland *et al.* (1971) have reported that methohexitone also markedly augmented the m.e.p.p. frequency at end-plates of rat diaphragm.

Mean quantum content of the end-plate potentials (m). Effects of the barbiturates on the release of acetylcholine were determined by comparing m before and after drug. As illustrated in Figures 1, 2A and 2B, CHEB and phenobarbitone markedly enhanced m. After treatment with either drug, m increased rapidly for 30 to 60 min, then remained relatively constant, or increased slightly, or decreased during the balance of the 120-min test period. Barbiturates diminished the amplitude of the e.p.ps. as well as that of the m.e.p.ps. The mean amplitude of the e.p.ps. initially increased or did not change but decreased with time. Thus, release of transmitter was increasing when the amplitude of the e.p.ps. and m.e.p.ps. was decreasing.

The mean values of m during the control periods in the CHEB and phenobarbitone experiments were 8·1 and 7·2, respectively. Maximum changes in m after drug treatment are expressed as % of control values. On the average in five experiments each, CHEB increased m to 180% of control values, and phenobarbitone increased m to 220% of control values (Table 1). In three experiments (one CHEB, two phenobarbitone) the drug was washed out with Ringer solution, and m returned toward control values. Racemic and (−)-pentobarbitone also increased m at five end-plates tested.

Effective resistance of skeletal muscle membrane. Reduction of the amplitude of the m.e.p.ps. could be the result of a drug-evoked alteration in the conductance of the muscle membrane. Therefore, the effective resistance (V/I) of the skeletal muscle membrane adjacent to an end-plate was measured before, and 15, 30 and 60 min after CHEB and phenobarbitone (three experiments each) by means of intracellular recording methods. Effective resistance was calculated from the amount of current required to produce small depolarizations. The results indicate that CHEB and phenobarbitone have little or no effect on membrane resistance of the frog sartorius muscle. For example, in one muscle fibre the mean effective resistances before and 60 min after phenobarbitone were 0·48 and 0·46 MΩ, respectively; in another fibre the mean effective resistances before and 60 min after CHEB were 0·36 and 0·41 MΩ, respectively. Similar values were obtained 15 and

30 min after drug treatment. Thus, attenuation of the post-junctional response does not appear to be related to a drug-induced increase in membrane conductance. Typical current-voltage relationships are illustrated in Figure 4.

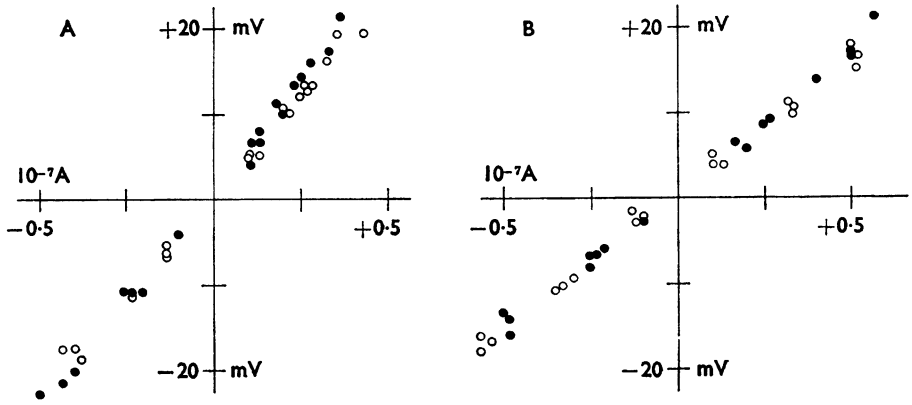

FIG. 4. Effects of phenobarbitone (A) and 5-(2-cyclohexylideneëthyl)-5-ethyl barbituric acid (CHEB) (B) on the effective resistance of skeletal muscle membranes. Effective resistance (V/I) calculated from the amount of current required to produce small depolarizations. Concentration of drugs, 200 μM. A. controls, filled circles, membrane potential (Em) 86 mV, mean effective resistance 0·48 MΩ; 60 min after phenobarbitone, open circles, Em 86 mV, 0·46 MΩ. B. controls, filled circles, Em 80 mV, 0·36 MΩ; 60 min after CHEB, open circles, Em 80 mV, 0·41 MΩ. Similar results were obtained 15, 30 and 60 min after drug treatment.

Thesleff (1956) found that pentobarbitone slightly increased the effective resistance of frog sartorius muscles. Weakly (1969) showed that pentobarbitone and thiopentone had no effect on resistance of spinal cord motoneurones of the cat. In contrast, Downes & Franz (1971) demonstrated that CHEB produced a marked decrease in resistance of dorsal root ganglion cells in the cat; this particular diversity in results probably reflects differences in inherent membrane properties of excitable tissues.

Another possible but unlikely explanation for the diminution in m.e.p.p. amplitude is that the barbiturates reduce the quantum size presynaptically; however, barbiturates are known to decrease the depolarization of frog sartorius end-plates caused by electrophoretically applied acetylcholine (Thesleff, 1956; Adams, Cash & Quilliam, 1970), which would be sufficient to explain the effect on m.e.p.p. size. In other words, barbiturates render the end-plate receptors less sensitive to the depolarizing actions of acetylcholine.

Extracellular recording

Nerve-terminal action potential. Effects of barbiturates on the action potential at the nerve terminal could provide a possible mechanism for the increase in m, since a change in the action potential can be expected to alter the release of transmitter (Katz & Miledi, 1967a, b). Therefore, the time course of the effects of barbiturates on the nerve-terminal action potential was studied in five experiments (two CHEB, three phenobarbitone) by extracellular recording techniques described by Katz & Miledi (1965). One hundred action potentials were summed at 5- and 10-min intervals for at least 60 min after drug treatment. The duration of the

action potential was increased initially within 15 min after drug and, in general, continued to increase progressively for the rest of the hour.

In additional experiments, 300 nerve-terminal action potentials were summed and the number of failures were counted 60, 90 and 120 min after drug treatment, when the barbiturates, as indicated by the results of intracellular studies, had attained their peak effects. The following data obtained 60 min after drug are qualitatively similar to those obtained after 90 and 120 minutes. In seven experiments with CHEB, the mean m and the mean duration and amplitude of the action potential were 140, 130 and 82% of control values, respectively. Thus, CHEB increased both m and the duration of the action potentials but decreased the amplitude. In six experiments with phenobarbitone, the comparable means were 150, 120 and 100% of control values, respectively. Therefore, phenobarbitone also increased m and the duration of the action potential but failed to alter the amplitude. However, in the experiments in which the time course of barbiturate effects was studied, both drugs decreased the amplitude of the action potential. The findings support the observations made with intracellular micro-electrodes that CHEB and phenobarbitone enhance m. In addition, prolongation of action-potential duration may provide a possible mechanism for the barbiturate-induced increase in m (Fig. 5). Barbiturates have been shown previously to increase the duration and reduce the amplitude of the action potentials of peripheral nerve and skeletal muscle (Heinbecker & Bartley, 1940 ; Thesleff, 1956).

In contrast to the experiments with intracellular micro-electrodes, the increases in m were not as profound, and in some drug tests m was not enhanced. The limited

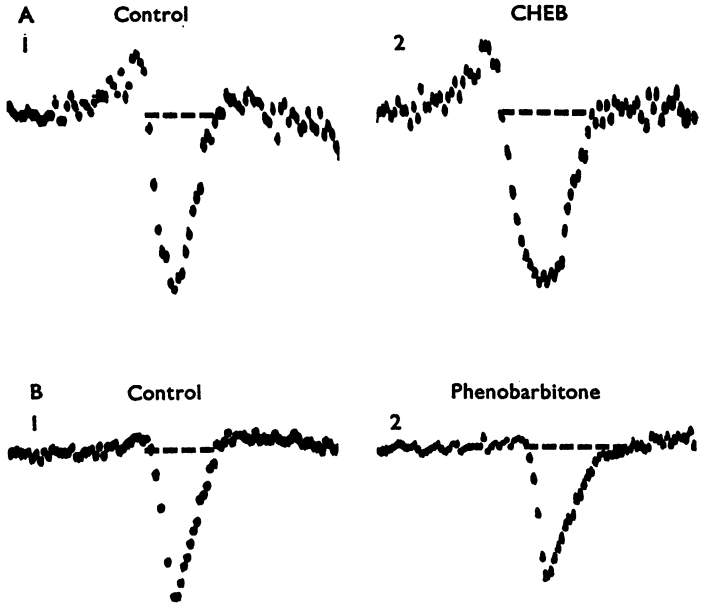

FIG. 5. Effects of 5-(2-cyclohexylideneëthyl)-5-ethyl barbituric acid (CHEB) and phenobarbitone on the nerve-terminal action potential. Concentration of drugs, 200 μM. Potentials recorded extracellularly with glass micro-electrodes. Each trace represents the sum of 300 action potentials. The dashed line indicates the width of the negative phase of the action potential measured at the level of the baseline. A. 1, controls ; 2, 120 min after CHEB. B. 1, controls ; 2, 120 min after phenobarbitone. Records retouched for photographic purposes.

changes in m seen with the extracellular recording methods are probably artifacts due to inaccuracies in the determination of the number of failures. With a marked depression of the post-junctional response, many small responses were probably overlooked and recorded as failures. Thus, m was presumably markedly underestimated. In spite of such technical difficulties, the data indicate that both drugs increase m.

Discussion

Barbiturates invariably increased the mean quantum content of the end-plate potential (m) in all experiments performed with intracellular micro-electrodes (Figs. 1 and 2). The ' mean amplitude ' method, which was employed in each test, yielded an accurate estimate of m because the amplitudes of the end-plate potentials (e.p.ps.) and miniature end-plate potentials (m.e.p.ps.) were easily measured even in the presence of extensive drug-induced post-junctional depression. Furthermore, the amplitudes of the m.e.p.ps. measured before and after drug, as indicated by m.e.p.p. amplitude histograms, were reasonable estimates of their population (Fig. 2A and 2B). If some of the small m.e.p.ps. were obscured in the baseline noise of the recording system as a result of drug treatment, the ' mean amplitude ' method could have underestimated m. In two preparations m was also calculated from the coefficient of variation of the e.p.p. amplitudes and by the ' failures ' method (del Castillo & Katz, 1954). A barbiturate-induced enhancement of m was obtained with all three procedures. Results obtained with extracellular electrodes also indicate that barbiturates augment m. D. Weinreich (personal communication) has found that thiopentone increased m, as determined by all three methods, decreased amplitude of the m.e.p.ps., and failed to alter the effective membrane resistance of frog sartorius muscle. In addition, T. Narahashi and his coworkers (personal communication), employing voltage clamp techniques at frog sartorius end-plates, have observed that pentobarbitone raised m.

Since Katz & Miledi (1967b) have shown that an increase in the amplitude or the duration of a depolarizing pulse applied to frog nerve terminals causes a larger amount of transmitter to be released, barbiturate-induced enhancement of m may be explained by prolongation of the nerve-terminal action potential (Fig. 5). Tetraethylammonium is also thought to increase the release of transmitter at frog end-plates by prolonging the presynaptic action potential (Koketsu, 1958 ; Benoit & Mambrini, 1970). The possibility that barbiturates affect some additional step in the chain of events leading to transmitter release has not been excluded.

The neuromuscular junction is not likely to be the site of action for barbiturate-induced preanaesthetic excitation or for CHEB-evoked convulsions, and it is not known whether the results of the present investigation can be applied to the central nervous system. However, an increase in mean quantum content or frequency of spontaneously released transmitter may provide a possible mechanism for central nervous system excitation produced by the barbiturates. Excitation in the central nervous system may reflect enhanced transmitter release, whereas according to the work of Weakly (1969) depression may be at least partially explained by a decrease in transmitter release. If all barbiturates are likely to have potential for both excitation and depression (Downes *et al.*, 1970), then the drugs may tend to enhance as well as decrease release of transmitter. The overt manifestations exhibited by a given agent, such as convulsions, excitation followed by depression, or

depression alone, may be dependent upon its predominant pharmacological action on transmitter release at a given dose and at a particular time.

In the present study, the effects of CHEB on m are indistinguishable from those of phenobarbitone. The enhancement of transmitter release probably represents a stimulatory effect that is common to most, if not all, barbiturates. The following reasons may explain why phenobarbitone produced only an increase in m. First, the maximal concentration of barbiturate employed *in vitro* was limited by the extensive decrease in the post-junctional response. Secondly, the 200 μM concentration of phenobarbitone used in the present study is below that attained in the brain by Waddell & Butler (1957) after intravenous administration of anaesthetic doses. Therefore, the concentration of phenobarbitone (200 μM) may have been sufficient only to augment m. Such a possibility may account for the two drugs, one a convulsant and the other a depressant, behaving in a similar manner. In contrast to their similar effects on m, CHEB and phenobarbitone appear to have different effects on m.e.p.p. frequency. The results suggest that within the period of time studied CHEB markedly increases m.e.p.p. frequency, whereas phenobarbitone does not.

There is no direct evidence that clinically employed barbiturates act on nerve terminals in the central nervous system to cause first an increase and then a decrease in transmitter release. However, diphasic effects of barbiturates on synaptic transmission have been noted. In studies on frog spinal cord *in vitro*, Richens (1969) has reported that methohexitone (352 μM) initially increased and then decreased the early spike discharge of the ventral root response evoked by stimulation of either dorsal roots or lateral columns. Weakly (1969, see Fig. 2) showed that 10 mg/kg i.v. of thiopentone at first increased then decreased the monosynaptic (2N) response in unanaesthetized spinal cats. He also found that both thiopentone and pentobarbitone decreased the mean quantum content of the monosynaptic excitatory post-synaptic potential in spinal motoneurones. However, the measurements related to transmitter release were only made during the period of maximal barbiturate-induced depression of the 2N response. Esplin (1963) reported that 2 to 4 mg/kg i.v. of phenobarbitone augmented and 8 to 18 mg/kg i.v. diminished the amplitude of the 2N spike recorded from ventral roots of unanaesthetized spinal cats; he attributed the enhanced response with the low doses of barbiturates to selective depression of inhibition. Downes & Williams (1969), using the same preparation, observed that 0·3 to 0·6 mg/kg i.v. of CHEB or 2 mg/kg i.v. of pentobarbitone enhanced the 2N response; they concluded that the augmentation of the 2N response was not the result of changes in presynaptic or postsynaptic inhibition. Such findings suggest that barbiturates can both stimulate and depress synaptic transmission.

Preanaesthetic excitation produced by the barbiturates may represent the combination of three effects in the central nervous system. Excitation may be explained by selective depression of inhibition (Esplin, 1963), by direct stimulation of neuronal membranes (Downes & Franz, 1971), and finally by enhanced release of transmitter. In view of the selectivity of the barbiturates for synapses and the results of the present study, the third proposal deserves further consideration at the level of the central nervous system.

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