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TETRODOTOXIN-INDUCED CENTRAL NERVOUS SYSTEM DEPRESSION

BY

G. B. FRANK* AND C. PINSKY

From the Department of Pharmacology and Therapeutics, University of Manitoba Faculty of Medicine, Winnipeg, Canada

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In recent years it has been shown that several non-volatile general anaesthetic drugs (Thesleff, 1956), some volatile anaesthetic drugs (Yamaguchi, 1961 ; Inoue & Frank, 1965) and local anaesthetic drugs (Shanes, Freygang, Grundfest & Amateniek, 1959 ; Taylor, 1959 ; Inoue & Frank, 1962) block production of action potentials in peripheral nerve or skeletal muscle by inhibiting the specific increase in membrane sodium conductivity which normally follows an adequate stimulus. Thesleff (1956) made observations also on the hypnotic behavioural effects of the general anaesthetics which he studied. These led him to suggest that general anaesthetic drugs might produce hypnosis and general anaesthesia by acting on the electrical excitability of cells in the central nervous system in a manner identical to that observed in skeletal muscle fibres.

The observations outlined above prompted Inoue & Frank (1962) to suggest that all anaesthetic drugs, whether local or general anaesthetics, acted by the same basic mechanism of action on excitable cells both peripherally and in the central nervous system. Local anaesthetics usually produce excitement and excessive activity when administered systemically in large doses, but it was suggested that this excitement represented an exaggerated form of Stage II general anaesthesia. In a subsequent study, the central effects of leptazol, general anaesthetics, and local anaesthetics were compared (Frank & Sanders, 1963). The results were consistent with the concept that local and general anaesthetics do have fundamentally similar actions on neurones in the central nervous system. Not only do all of the anaesthetics mentioned so far block the sodium-carrying mechanism, but also they suppress the secondary or delayed increase in potassium conductivity which normally follows an adequate stimulus. For this reason the recent finding that tetrodotoxin blocks the sodium-carrying mechanism without blocking the potassium-carrying mechanism (Narahashi, Moore & Scott, 1964) was of great interest. The present paper presents the results of experiments to determine the extent to which tetrodotoxin could reproduce the actions previously demonstrated for local and general anaesthetics in the central nervous system (Frank & Sanders, 1963) and from this to estimate the extent to which the suppression of the potassium-carrying mechanism might be responsible for the central actions of these anaesthetic drugs.

* Present address: Department of Pharmacology, University of Alberta, Edmonton, Canada.

METHODS

In the present study the central effects of tetrodotoxin are compared with those previously reported for local and general anaesthetics. The methods will be described briefly ; greater details are provided elsewhere (Frank & Sanders, 1963).

Effects of drugs on the motor activity of intact mice. Drugs in a volume of 0.1 to 0.5 ml. were injected intraperitoneally into Swiss Albino mice (Lemberger Co.) weighing between 20 and 30 g. Gross effects on motor activity were observed and noted ; loss of the righting reflex without prior convulsive activity was used as one criterion of central nervous depression. Two other criteria were used: one was the presence of certain spinal reflexes in the depressed animal as indicated by the withdrawal response to pinching the foot, the other was the eventual recovery of the righting reflex as the effects of the drugs wore off. Although not entirely specific in determining the presence of anaesthesia, these criteria did establish the existence or absence of a condition which was otherwise indistinguishable from general anaesthesia.

During tests of the response to each drug by itself, the animals were observed for 60 min after administration of tetrodotoxin (Sankyo Co.), and for 90 min after pentobarbitone sodium. For drug interaction studies the animals first were observed for 30 min after injections with pentobarbitone and then were injected with tetrodotoxin and observed further for 60 min. χ^2-Analysis was done on the lethality data and the dose/response curves of the drug interactions studies were compared by the method of Litchfield & Wilcoxin (1949).

Effects of tetrodotoxin on the cerebral cortex. Neuronally-isolated slabs of cerebral cortex *in situ* in unanaesthetized decerebrate cats were used as the test object in these experiments (Burns, 1951). The effects of tetrodotoxin on cortical excitability were assessed by measuring the threshold voltage for the production of surface-negative and surface-positive responses to direct electrical stimulation of the slab and by determining the threshold number of stimulating pulses for the production of epileptiform after-discharges (Frank & Sanders, 1963 ; Pinsky & Burns, 1962). Monopolar recordings were obtained from an active recording electrode on the surface of the slab, the reference electrode being on a killed area of cortex at one end of the isolated area. Bipolar platinum stimulating electrodes were placed on the slab 1 to 5 mm away from the active recording lead. Surface-negative and surface-positive burst responses were displayed on a Tektronix 502 oscilloscope and recorded photographically. Epileptiform after-discharges were recorded on an Offner dynograph. All amplifiers were direct-coupled.

Two different procedures were used for applying and testing the tetrodotoxin. In one a strip of filter paper (1 × 5 mm) moistened with saline was placed across the width of the slab between the stimulating electrodes and the recording electrode. A stimulus intensity was chosen which would produce a surface-negative response followed by a surface-positive burst (Burns, 1951) and the stimulus was applied every 30 sec throughout the test. Several control responses were obtained and then about 0.015 ml. of saline containing the tetrodotoxin in the specified concentration was delivered onto the filter paper. The drug-soaked paper strip was removed from the cortex after a few minutes and subsequent changes in the responses were recorded. In the other procedure, the animal was prepared as in the previous test but was immobilized with intravenous gallamine triethiodide (5 mg/kg) and artificially ventilated. Stimulus parameters were adjusted to determine the thresholds for the surface-negative response, the surface-positive burst response, and for epileptiform after-discharges. Intravenous tetrodotoxin was given evenly over a 2 min period and the thresholds were redetermined.

RESULTS

Tetrodotoxin effects on gross activity and lethality. Tetrodotoxin, over a narrow dose range (10 to 20 μg/kg), produced a remarkably consistent pattern of effects on gross motor activity when injected intraperitoneally into white mice. The first effect noted in all the animals was a marked decrease in spontaneous movements and motor activity. Each injected animal, although active when untreated, sat quietly in one corner of the

observation cage unless stimulated. With the exception of two animals, which showed signs of respiratory difficulty and were gasping, no other effects on motor activity were observed in mice that eventually recovered from the tetrodotoxin. The first sign of difficulty in those animals which died from the drug was the appearance of gasping. This was followed by excessive motor activity leading to convulsions, either tonic or clonic or both. The animals died within 1 to 2 min of the start of the convulsions. This series of effects, from gasping to death, took 4 to 5 min. All the animals tested with 20 μg/kg of the drug died, usually within 15 to 20 min after drug administration. With 15 μg/kg, which killed about 55%, survival times were from 30 to 35 min. No animals were killed by 10 μg/kg.

None of the animals which survived treatment with tetrodotoxin ever exhibited any signs of excitement. This suggested that the deaths, and the convulsions which invariably preceded them, resulted from tetrodotoxin acting to interfere with respiration at a site outside the central nervous system. To test this possibility, the mice were treated with phenobarbitone 30 min before injection of tetrodotoxin (15 μg/kg). Doses of phenobarbitone (70 and 80 mg/kg) were chosen which were known to have a definite depressant effect on the central nervous system (Frank & Sanders, 1963).

The rationale of this test is as follows: if tetrodotoxin causes convulsions and death by depression of the respiratory centres in the central nervous system, then previous treatment with phenobarbitone should enhance this depression and thus increase lethality. If, on the other hand, the deaths result from the convulsions, then phenobarbitone, by suppressing the convulsions, should decrease the lethality of the tetrodotoxin. Should the phenobarbitone have no effect on the lethality of the tetrodotoxin then some site other than the central nervous system can reasonably be implicated in the deaths.

The results of this test, presented in Table 1, show that previous treatment with phenobarbitone in no way modified the lethality of the tetrodotoxin. This result was obtained even though the phenobarbitone, in the doses used, in combination with the tetrodotoxin eliminated the righting reflex in most mice, and in all animals either greatly depressed

TABLE 1

EFFECTS OF ADMINISTRATION OF PHENOBARBITONE AND TETRODOTOXIN IN SWISS ALBINO MICE

Tetrodotoxin was given in a dose of 15 μg/kg. Pairs of numbers refer to the mice which lost the righting reflex or died, and the numbers of mice tested. For the lethality test, $\chi^2=0.77$, d.f.$=2$, $P>0.5$

Dose of phenobarbitone (mg/kg)	Loss of righting reflex	Deaths
0	0/22	12/22
70	6/8	5/10
80	11/11	8/12

the severity of the convulsions or eliminated them completely. These findings are consistent with the suggestion that depression of respiration at a peripheral site was responsible for the tetrodotoxin-induced convulsions and deaths in the animals tested in this study. This is supported by the work of Sakai, Sato & Uragachi (1961) who demonstrated that phrenic nerve potentials in the rat persist after diaphragmatic paralysis has been produced by parenteral administration of tetrodotoxin.

Drug interaction studies in intact white mice. As already mentioned, the only obvious effect of sublethal doses of tetrodotoxin given alone was a marked decrease in spontaneous movements, which made the mice appear to be sedated. This drug, however, could add to the central nervous system depression caused by phenobarbitone in various doses. This was quantitatively assessed from the loss of the righting reflex and the results are presented in Fig. 1. The graphs were plotted from the results for phenobarbitone alone

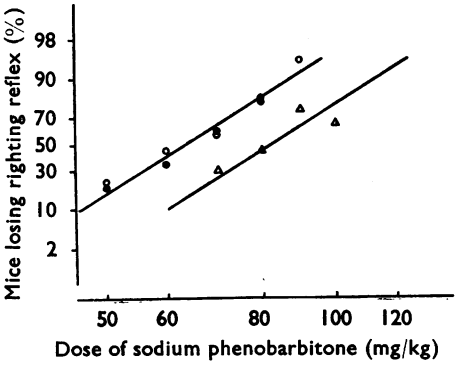

Fig. 1. Loss of righting reflex produced by tetrodotoxin given to white mice 30 min after various doses of phenobarbitone. Tetrodotoxin doses (in μg/kg): Δ, 0; ○, 10; ●, 12. Logarithmic probability plots. Each point is the mean for twenty mice.

and for phenobarbitone with tetrodotoxin (12 μg/kg). The lines do not deviate from parallel and there is a small but significant shift to the left. Results from other experiments with 10 μg/kg of tetrodotoxin have been included in Fig. 1 although they were not included in the statistical analysis. They do not appear to differ from the results obtained with 12 μg/kg.

A few drug interaction tests were conducted with 15 μg/kg of tetrodotoxin (Table 1). There was a further increase in the number of animals losing the righting reflex, but the high mortality discouraged further testing. The rapid lethal effect precluded any testing with tetrodotoxin in doses of 20 μg/kg or more.

Studies of drug effects on isolated slabs of cerebral cortex. It has been shown (Frank & Sanders, 1963) that topical application of general and local anaesthetics depresses both the surface-negative and the surface-positive burst response to direct electrical stimulation of isolated slabs of cat's cerebral cortex. Direct application of tetrodotoxin (0.5 μg/ml.) by means of a filter-paper strip produced the same effects (Figs. 2 and 3). Although the tetrodotoxin produced the same general pattern of effects as the general and local anaesthetics, there were certain differences worth noting. The effects of tetrodotoxin persisted after removal of the drug-containing strip of filter paper for longer than the effects of the local or general anaesthetics previously tested in this manner (Frank & Sanders, 1963). During this recovery period the duration of the surface-positive burst was greatly increased. In several experiments the latent period between the stimulus and the initial positive deflection of the response increased from a few msec to several hundred msec even though the recording electrode was only 1 to 2 mm from the stim-

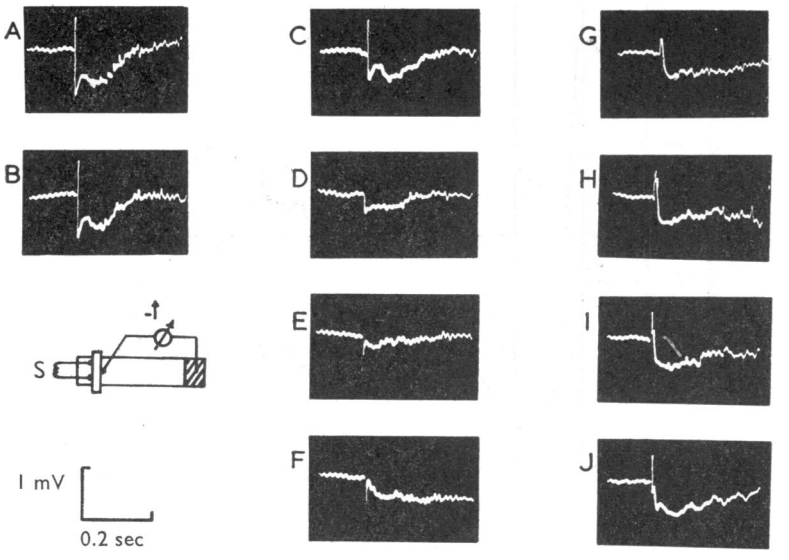

Fig. 2. Responses to direct electrical stimulation recorded from the surface of an isolated slab of cat's cerebral cortex before and after local application of tetrodotoxin (0.5 μg/ml.). A, and B, control responses. Responses after tetrodotoxin application: C, 15 sec; D, 45 sec; and E, 105 sec. The filter paper strip containing the tetrodotoxin was removed from the cortex 45 sec after E. Responses after removal of filter paper strip: F, 3.5 min; G, 13.5 min; H, 21.5 min; I, 22.5 min; and J, 24.5 min. At this sweep speed the stimulus artefact is concurrent with the start of the response and is apparent only in E and F. The cortex was stimulated every 30 sec during this test. Some measurements made from the responses recorded during this test are plotted in Fig. 3.

ulating electrodes and the isolation of the slab undoubtedly had severed the long subcortical conduction pathways.

Intravenously administered tetrodotoxin (5 μg/kg or more) always depressed cortical excitability. In four cats given 5 or 6 μg/kg the thresholds for the surface-negative and for the surface-positive burst responses were increased from 1.5 to 3.0 times their control values; this effect persisted for more than 30 min. In one cat the thresholds rose to 3.0 times their control values by 5 min after injection of tetrodotoxin and 75 min later had recovered only to about twice their control values.

Tetrodotoxin (5 μg/kg) in two other animals completely suppressed all signs of electrical activity, either evoked or spontaneous, in both the isolated cortical slab and in the intact cortex. The experiments with these animals were terminated soon after total suppression of activity was observed. Visual observation made at that time revealed an apparently healthy cortex with a good blood supply, and this suggested that electrical activity might return if sufficient time were allowed for recovery. This possibility was tested in a later experiment in which two doses of tetrodotoxin (5 μg/kg) were given 30 min apart. All electrical activity disappeared after the second dose. Small responses could be produced 1 hr later, however, by strong direct stimulation of the cortex. Recovery of the responses proceeded slowly and 3.5 hr elapsed after the second dose

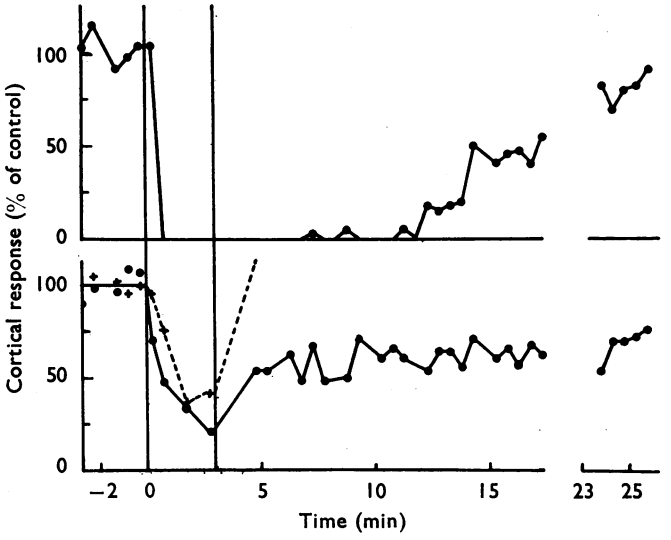

Fig. 3. Effects of local application of tetrodotoxin (0.5 μg/ml.) on the responses of a cat's isolated cerebral cortex to direct electrical stimulation with rectangular pulses. Upper graph, amplitude of surface negative response. Lower graphs: ●, amplitude of surface position response; +, duration of surface positive response. The cortex was exposed to tetrodotoxin during the time between the vertical lines starting at 0 min.

before the thresholds for the surface-negative and surface-positive responses returned approximately to their control values.

The effects of intravenously-administered tetrodotoxin on epileptiform after-discharges were tested in only two preparations. Tetrodotoxin (5 and 6.5 μg/kg) in both cases increased the threshold number of pulses required to initiate an after-discharge when the other parameters of stimulation were kept constant. The stimulus parameters were 2 msec (rectangular pulses), 40 to 45 V, and 30 shocks/sec. In one animal the threshold number of pulses rose from 30 to 48 and in the other from 15 to 30. The durations of the after-discharges were not altered by the drug.

A large-amplitude wave of surface-negative potential was recorded from the surface of the isolated cortical slab after intravenous administration of tetrodotoxin (5 μg/kg). This wave was similar in appearance to that seen during spreading cortical depression (Grafstein, 1956), but had a more rapid rising phase and a shorter duration lasting only a few minutes. Cortical excitability recovered during the falling phase of this wave. This surface negative wave might have been caused by the rapid, large fall in blood pressure which has been reported to occur during intravenous tetrodotoxin administration (Murtha, 1960).

DISCUSSION

The intent of the present study was to see to what extent tetrodotoxin, which can block the sodium-carrying mechanism but does not suppress the secondary or delayed increase in potassium conductivity which occurs during an action potential (Narahashi

et al., 1964), could produce the same effects on the central nervous system as do local and general anaesthetics which suppress both phases of the action potential. It was hoped in this way to determine if the suppression of the secondary increase in potassium conductivity is required for or contributes in any significant degree to the production of the state usually known as " general anaesthesia." Implicit in this approach is the assumption that general and local anaesthetics produce their effects on the central nervous system by virtue of their actions on membrane excitability. The present findings that tetrodotoxin could reproduce many of the effects on the central nervous system previously shown for local and general anaesthetics (Frank & Sanders, 1963) tend to support the suggestion that local and general anaesthetics depress the activity of the vertebrate central nervous system by a common mechanism of action at the cellular level (Thesleff, 1956 ; Inoue & Frank, 1962).

The severe toxic effects of tetrodotoxin, which kills the animals by an action on peripheral tissues, make it impossible to give a dose of this toxin large enough by itself to produce a state of " general anaesthesia " ; just as the lethal effects of high doses of local anaesthetics prevent the development of " general anaesthesia " when they are administered by themselves (Maykut & Kalow, 1955 ; Frank & Sanders, 1963). Nevertheless it was found that tetrodotoxin, when given after small doses of phenobarbitone, could produce a condition indistinguishable from general anaesthesia in many mice that would not have been so affected by the phenobarbitone alone. In experiments with cats, tetrodotoxin sometimes suppressed all electrical activity in the cerebral cortex for an hour or longer. In these animals cortical activity returned if the animal was supported with artificial ventilation ; animals so supported survived the tetrodotoxin. This observation suggests that the drug would be able to produce a state of reversible unconsciousness by its action on the central nervous system, if it were possible to give it in large enough doses to intact animals without their death resulting from its peripheral effects.

Tetrodotoxin, like the general and local anaesthetics, suppressed the electrical responses of isolated slabs of cerebral cortex when it was either applied directly to the cortex or given intravenously. The tetrodotoxin-induced increase in the threshold for epileptiform after-discharges, although not studied in detail, suggests that this drug might possess an anticonvulsive effect similar to that of the general and local anaesthetics (Bernhard & Bohm, 1955).

In only one important aspect were we unable to demonstrate an effect of tetrodotoxin comparable to one of those produced by both general and local anaesthetics. In none of the experiments was there any sign of central nervous system excitation which could be related to Stage II or the " excitement " stage of general anaesthesia. The convulsions which preceded death in the mice occurred only immediately before the death of the animals, and at no other time was there any sign of excitement. These convulsions, as previously mentioned, undoubtedly resulted from respiratory difficulties caused by an action of tetrodotoxin on the peripheral respiratory apparatus.

Our inability to demonstrate a stage of central excitation may simply be the result of a very narrow range between effective and lethal doses. This finding does, however, raise the interesting possibility that the suppression of the delayed increase in potassium conductivity might be the cause of, or at least might play an important role in, the production of the " excitement " stage of general anaesthesia. Thus, the suppression of

increased potassium conductivity might prolong the duration of depolarization during an action potential in the terminal branches of certain neurones in the central nervous system, thereby causing an increased release of their synaptic transmitter substance, and in this way produce excessive activity in postsynaptic neurones. However, without further evidence, this mechanism for the origin of the excitement stage of general anaesthesia remains merely an interesting possibility suggested by our results.

SUMMARY

1. Tests were conducted to determine the extent to which tetrodotoxin, which suppresses the increase in sodium conductivity but not the secondary increase in potassium conductivity after a stimulus of nerve fibres, could reproduce the central nervous system effects of general and local anaesthetics which suppress both these processes.

2. Given alone to intact white mice, tetrodotoxin produced only sedation or lethality, presumably by an effect on the peripheral respiratory apparatus.

3. When given 30 min after small doses of phenobarbitone, tetrodotoxin abolished the righting reflex in many mice that would not have been so affected by the phenobarbitone alone.

4. When applied directly to neuronally isolated slabs of cat's cerebral cortex, tetrodotoxin reduced the sizes of the surface-negative and surface-positive responses to direct electrical stimulation of the cortex.

5. When given systemically, tetrodotoxin increased the thresholds for surface negative and positive responses and for epileptiform after-discharges. High doses of tetrodotoxin could completely suppress all electrical activity, either spontaneous in intact cortex or responses to direct stimulation in isolated slabs, for 1 hr or longer.

6. No signs of direct central nervous system stimulation by tetrodotoxin were observed. This suggests the possibility that suppression of the increase in potassium conductivity in excitable cells in the central nervous system by local or general anaesthetics is involved in the production of Stage II (or the excitation stage) of general anaesthesia.

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