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TETRODOTOXIN AND THE CATION CONTENT, EXCITABILITY AND METABOLISM OF ISOLATED MAMMALIAN CEREBRAL TISSUES

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Abstract—Tetrodotoxin inhibited metabolic responses induced by electrical stimulation in isolated mammalian cerebral tissues; actions were not observed in unstimulated tissues. Half-maximal inhibition was given by 0·03 μM-tetrodotoxin (0·01 μg/ml).

The loss of tissue potassium induced on stimulation was also prevented by comparably small quantities of tetrodotoxin; it acted promptly, and the inhibitory effects could largely be removed by washing. The molar quantity of tetrodotoxin needed for inhibition was a fraction only of the quantity Na and K (6 mμ Equiv/g tissue) which was normally exchanged as the result of a single stimulating pulse. A number of other acidic guanidine derivatives showed less than 10^{-4} the potency of tetrodotoxin when tested under the same conditions.

TETRODOTOXIN, the poison of the puffer fish, includes in its actions central effects at impressively low dosage.[1–4] Systemic administration to cats of 1–5 μg/kg, or intraventricularly in total dose of 0·1–0·2 μg, affected brain-stem centres and some electroencephalographic characteristics. The compound is probably identical with tarichatoxin, in which central and other effects are reported by Kao and Fuhrman.[5] These authors, Fuhrman and Field[6] and Wagtendonk, Fuhrman, Tatum and Field[7] however found no action of tarichatoxin on cholinesterases or on oxidative metabolism in a number of neural systems, including frog sciatic nerve and mammalian brain.

Examination of the action of tetrodotoxin in peripheral systems has suggested its effects to be on specific ion movements associated with excitation.[8–9] Present experiments have therefore investigated electrically excited cerebral tissues, incubated under conditions permitting metabolic observations.[10] These have shown major actions of tetrodotoxin at quite low concentrations. Further, as the previous information regarding tetrodotoxin and ion movements has come by deductions from voltage–current relationships rather than by chemical determinations, evidence for action of tetrodotoxin on the preponderant cations of the incubated tissues has been sought by measurement of tissue composition.

EXPERIMENTAL

Tissue metabolism. Neocortex from the cerebral hemispheres of guinea pigs was obtained as described by McIlwain[11] and prepared as sheets 0·35 mm in thickness. Usually six slices from the two hemispheres were successively weighed, floated to incubation medium, mounted in electrodes and these placed in their vessels, which

already contained oxygenated medium. The mounted tissues were incubated at 37·5° within 15 min of the death of the animal.

Measurements of respiration were made manometrically, absorbing CO_2 with NaOH and taking pressure readings each 5 min; the incubation medium contained (mM): NaCl, 127; KCl, 5·1; KH_2PO_4, 1·3; $MgCl_2$, 1·3; $CaCl_2$, 2·7; glycylglycine (brought to pH7·4 by NaOH), 30 and glucose, 10. Bicarbonate medium contained NaCl, 120; KCl, 4·75; KH_2PO_4, 1·2; K_2SO_4, 1·2; $CaCl_2$, 2·6 $NaHCO_3$, 25 and glucose, 10; it was used in equilibrium with O_2 containing 5% CO_2.

Extraction and analysis. Incubation was terminated by releasing the tissues successively from their electrodes and quickly transferring them with a mounted bent wire to a dish containing 20 ml of 0·32 M sucrose at 0°. Within 1 sec each was further transferred to 4 ml of 6% (w/v) trichloroacetic acid in a test-tube homogenizer. The tissue was ground and after 15 min the suspension was centrifuged and measured portions of the supernatant taken for determination of Na and K, using an EEL flame photometer; other portions were taken for chloride determination as described by Keesey, Wallgren and McIlwain.[12]

Accumulation of lactate was measured by adding to $CuSO_4$ solution portions of the incubation media after tissue metabolism, and completing the determination according to Barker and Summersen.[13]

Electrical stimulation. Tissues were within silver-wire electrodes to which electrical pulses were applied during stated periods in the experiments. Pulses were of exponential time–voltage relationships obtained by charge and discharge of condensers, the stimulators and electrodes being described by McIlwain and Rodnight.[10]

Tetrodotoxin and other guanidines. An initial sample of tetrodotoxin was given by Dr. T. I. Shaw, and further supplies obtained from the Sankyo Co., Tokyo, as a mixture of the pure, crystalline solid with sodium citrate buffer. Portions containing 10–100 μg of tetrodotoxin were weighed using an electrobalance, dissolved in a few ml of water, and dispensed by micropipettes; when necessary, the solutions were kept in a deep-freeze for a few days. For addition during a manometic experiment, the aqueous solution was diluted in incubation medium and a measured volume of the solution pipetted to the side-arm of the manometric vessels. Data are given in terms of pure tetrodotoxin, mol. wt. 319.

The acidic guanidines of Table 2 were prepared as neutral solutions of 5–30mM, except in the instances noted; any necessary adjustment to pH 7·4 was made with NaOH or HCl.

RESULTS

Metabolic responses susceptible to tetrodotoxin

The characteristics of cerebral tissues first found susceptible to tetrodotoxin were metabolic, and these were affected under certain conditions only (Table 1). The large increase in respiration which ensued when the tissues were electrically stimulated in glucose-containing salines was greatly diminished by 0·4μM-tetrodotoxin. However, unstimulated tissues were not changed in respiratory rate when incubated with the same concentration of tetrodotoxin. Also, not all agents which increased the tissues' respiration made them sensitive to tetrodotoxin: in the presence of 50mM-KCl the respiration of normal tissues increased to an extent similar to the increase induced

electrically, but KCl-stimulated respiration was not comparably sensitive to tetrodotoxin (Table 1).

The formation of lactic acid from glucose by the cerebral cortical samples was also increased by electrical stimulation and by added KCl. Again, the increase induced electrically was diminished by tetrodotoxin while that following KCl was unaffected (Table 1; Fig. 1).

TABLE 1. SPECIFICITY AND REVERSIBILITY IN THE ACTION OF TETRODOTOXIN ON ELECTRICALLY STIMULATED CEREBRAL TISSUES

Tetrodotoxin (μM)	Additional procedures	Respiratory rate ± S.E. (μmole O_2/g tissue/hr)			Lactate accumulation (μmole/g tissue)		
		Normal conditions	Electrically stimulated	With KCl	Normal conditions	Electrically stimulated	With KCl
0	(i) None	63±3 (4)	102±6 (4)	100 (3)	29±1·5(5)	43±2·5(4)	38 (2)
0·4	(ii) None	61±2 (4)	67±4 (4)	99 (3)	29±2 (5)	31±2 (4)	37 (2)
0	(iii) Fresh saline after 15 min	55 (2)	84 (2)	—	—	41 (2)	—
0·4	(iv) As (iii); saline with tetrodotoxin	56 (2)	56 (2)	—	—	26 (2)	—
0·4	(v) As (iii); saline without tetrodotoxin	54 (2)	83 (2)	—	—	38 (2)	—

Guinea pig cerebral cortical tissues were incubated in glycylglycine-buffered glucose salines in manometric vessels with O_2. Some of the vessels carried the tissues within grid electrodes, and others contained M-KCl in a sidearm. After 30 min incubation the KCl was added and pulses (peak potential 10V, 0·4 msec. duration at 100/sec.) applied to specified vessels. Readings of oxygen pressure were taken each 5 min, incubation was continued for 60 min longer and the lactate content of the fluids determined.

The experiments which involved additional procedures (iii)–(v) were commenced in the same fashion but interrupted after 15 min incubation and the incubating fluids removed, leaving the tissues still mounted in their electrodes. Fresh media of the composition shown were added, the vessels replaced on their manometers and the respiratory rates measured before and after electrical stimulation of the characteristics quoted. The exchange of media resulted in tissues (iii)–(v) having lower respiratory rates than those of tissues (i) and (ii).

Small concentrations only were needed of tetrodotoxin for action on the electrically-stimulated tissue: Fig. 1 shows half-maximal inhibition of both respiration and the formation of lactate, to be caused on incubating tissues in media 0·03-μM in tetrodotoxin, or containing 0·01 μg/ml. As the experiments were carried out with 60–70 mg of tissue in 3·5 ml of fluid, these quantities correspond to 1·5 mμmole/g, or 0·5 μg tetrodotoxin/g cortical tissue.

Promptness and reversibility of tetrodotoxin action

When tetrodotoxin was added to tissues already incubating within electrodes in glucose-glycylglycine salines, at a time immediately before stimulation was commenced, tissue respiration and glycolysis did not undergo their normal increase. This implies an action of the tetrodotoxin (0·4 μM) within 2 min of its addition; the effect may be prompter without this being revealed in the experimental arrangement used.

When tissues were incubated in tetrodotoxin-containing media and these were removed and replaced by media without tetrodotoxin, the tissues were again excitable In the experiments of Table 1 tissues (v), which had undergone this procedure, are

seen to increase in respiration and glycolysis to the same extent as did tissues (iii), which were in glucose-saline throughout. When, however, the saline used in replacement also contained tetrodotoxin (tissues iv), respiration and glycolysis remained unstimulated by electrical pulses. In further experiments not quoted but analogous to (iii)–(v) of Table 1, the pre-incubation with 0·4 μM-tetrodotoxin was continued

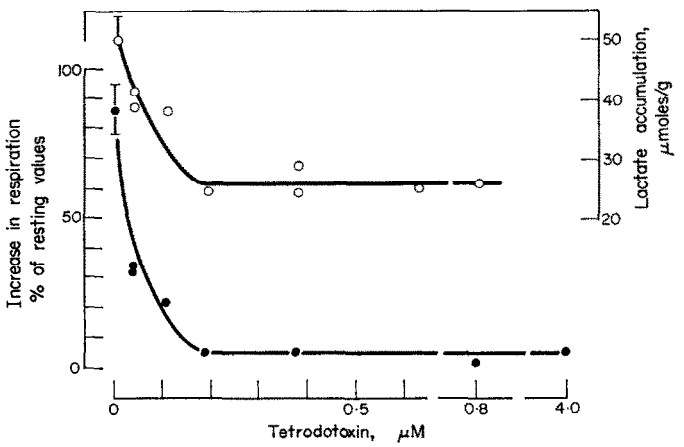


FIG 1. Diminution by tetrodotoxin of respiratory and glycolytic responses to electrical stimulation. Guinea-pig cerebral cortical tissues mounted in grid electrodes were incubated in glycylglycine-buffered glucose salines in manometric apparatus. Respiration was measured for 30–40 min before stimulation and during the subsequent 60 min during which condenser-pulses of peak potential 10V, 0·4 msec. in duration were applied to specific vessels at 100/sec. Respiratory rates before stimulation were 62–69 μmole O_2/g wet tissue/hr. At the end of the experiments the tissues were removed (see Fig. 2) and samples of the incubation fluid were taken promptly and their lactate content determined; in absence of stimulation, values were 29 μmole lactate/g tissue.

for 30 min and included 15 min during which tissues were exposed to electrical stimulation (pulses of 10 V peak potential, 0·4 msec. duration at 100/sec). During this initial period the tetrodotoxin prevented the increase in respiration which occurred in the other tissues. After exchange of media for one lacking tetrodotoxin, excitability was recovered: both respiration and glycolysis increased with stimulation. Thus, stimulation in the presence of tetrodotoxin was not demonstrated to increase the action of the toxin.

Tetrodotoxin and tissue constituents

After making the respiratory measurements summarized in Table 1, stimulation of the tissues was halted and they were released from their electrodes and extracted for determination of Na, K and Cl (Fig. 2, A–C). Of these constituents, K was greater in tissues exposed to tetrodotoxin, The results were variable, the major factor contributing to this being the portion of the cerebral hemispheres examined. Samples from the outer convexity of the brain and with only one cut surface were greater in K content than those cut subsequently. Appraising the two groups of tissues separately, however, showed tetrodotoxin to give greater K content in each group.

Tissue K was examined more fully under the alternative conditions of Fig. 2 D, chosen as optimal from a previous study of tissue electrolytes.[12] These experiments were briefer and were carried out in glucose bicarbonate salines. In the unstimulated tissues, tetrodotoxin caused little or no change in K content. The effect of tetrodotoxin was confined to the stimulated tissue, and allowed its K content to remain almost unchanged under conditions in which control tissues lost 20 μEquivK/g. The effect was shown equally by the two categories of samples examined.

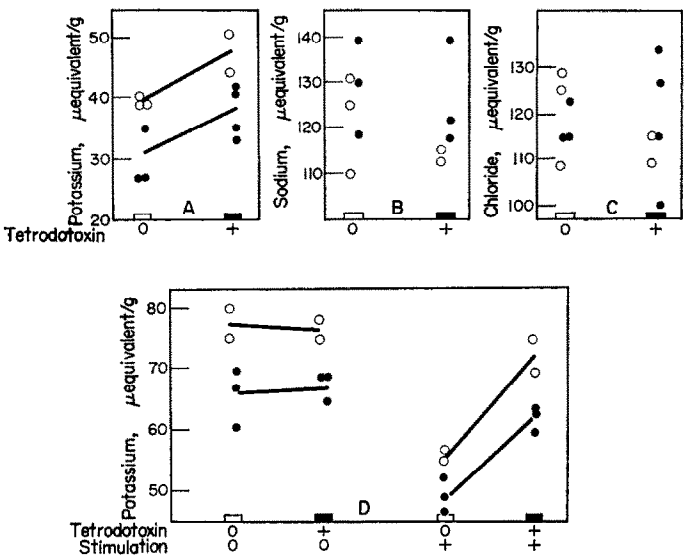


FIG 2. Tetrodotoxin and the Na, K and Cl of guinea pig cerebral cortex: ○, outer slices; ●, inner slices (see text). Upper diagrams: data from the tissues of the experiments of Fig. 1 (q.v.); the tissues were removed from their vessels, rinsed in ice-cold sucrose for 2 sec, and then ground in trichloroacetic acid for extraction; average period between terminating stimulation and extraction, 5 min.

Lower diagram: tissues were incubated in quick-transfer electrodes in bicarbonate-glucose saline with or without 0·25 μM-tetrodotoxin, for 30 min. Condenser pulses (characteristics, see Fig. 1) were then applied to specified electrodes for 12 min and were terminated by plunging the electrodes in ice-cold sucrose. Three seconds later the tissues were removed and ground in trichloroacetic acid

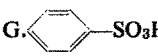
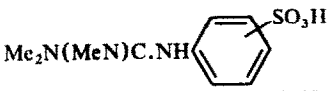
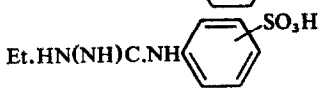
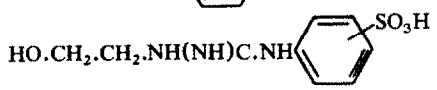
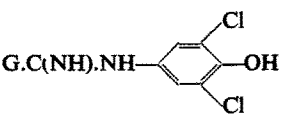
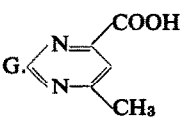
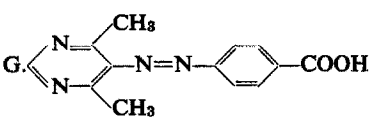
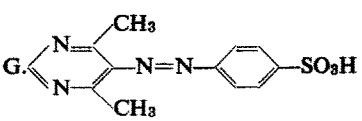
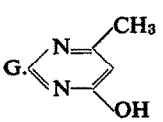
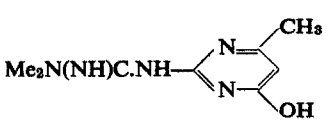
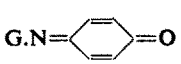
Other guanidines and tissue excitability

Tetrodotoxin[14] is a guanidine derivative which can exist as a zwitterion, and other compounds of these characteristics were examined for their effects on cerebral tissues under the conditions of Table 1. This permitted observation of any actions of the compounds on respiration, glycolysis, and responses to electrical excitation. The results summarized in Table 2, indicate that few of the compounds examined affected these characteristics at concentrations up to 3 or 6 mM.

The simplest compound examined, γ-guanidinobutyric acid (1, Table 2) occurs naturally in the brain and was without observed action; its sulphuric acid analogue (2, Table 2) was however inhibitory in high concentrations. A number of other acidic guanidines, carboxylic or sulphuric acids with aromatic or pyrimidine rings, were inactive. The diguanidine which was also a phenol (7, Table 2) inhibited, lowering

TABLE 2. GUANIDINE DERIVATIVES EXAMINED FOR ACTION ON ISOLATED CEREBRAL TISSUES

The compounds were included at the concentrations stated (parentheses: compound not completely dissolved), with glucose-salines of experiments conducted as described in Fig. 1. G = $NH_2(NH)C.NH$.

	Compound	Maximum concentration tested without observing action (mM)	Compound inhibitory at (mM)
1.	$G.CH_2.CH_2.CH_2.COOH$	3	—
2.	$G.CH_2.CH_2.CH_2.SO_3H$	1·5	6
3.		3	—
4.		3	—
5.		3	—
6.		1·5	—
7.		—	1·5 and 6
8.		(6)	—
9.		(3)	—
10.		3	—
11.		6	—
12.		3	—
13.		0·1	0·6 and 3
14.	Tetrodotoxin	3×10^{-5}	10^{-4}

respiration both in the presence and absence of stimulation; it also diminished tissue K to 20 μEquiv/g at the end of the experiments. A similar action was shown by the quinone (13, Table 2; final K, 22 μEquiv/g). The effect of these two compounds was therefore different from that of tetrodotoxin, which preserved tissue K (Fig. 2).

DISCUSSON

The sensitivity to tetrodotoxin now observed in isolated cerebral tissues appears adequate to give a basis for the central actions of the compound. The effects on the isolated tissue are moreover specific, in more than one sense. Thus they are associated with excitation, and the other guanidines examined are less active than tetrodotoxin by a factor of 10^4. Also, the analogues when they act affect the tissue K in a fashion which differs from that of tetrodotoxin, and which is not dependent on electrical stimulation.

Previous investigation of tetrodotoxin in other biological systems have also linked its action with processes of excitation, observed in these cases by measurement of current flow and potential rather than by the ion content and metabolic characteristics now observed. In the giant axons of the lobster and squid, Narahashi, Moore and Scott[15] and Nakamura, Nakajama and Grundfest[16] concluded that tetrodotoxin acted by blocking the increase in Na conductance which normally occurred on stimulation; the subsequent outward current, attributable to K, was not affected. A similar block by tetrodotoxin was found in the electroplaques of the electric eel[9], though in certain other respects the processes which followed excitation of the electroplaques differed from those in the giant axons. Blockage of excitation in a muscle fibre[8] and in Pacinian corpuscles[17] were also attributed to suppression of Na-carrying mechanisms.

In the present experiments, suppression of K movement was much more in evidence than alteration in Na movement, but the observations do not exclude an effect on Na, which for its adequate study requires further information obtained by measurement of fluid compartments or isotopically. The K loss observed on excitation in absence of tetrodotoxin[18] (Fig. 2) in other neural systems and presumably here also is permitted by the depolarization caused by Na entry.[19] If tetrodotoxin by suppressing Na entry prevents the depolarization, absence of subsequent change in K is understandable. To explain the K results in this way, however, the stimulating pulses in the present experiments must be concluded not have a direct depolarizing effect but to operate by initiating the sequence of permeability changes common to excitation in other neural systems. Other indications of this have been the large and approximately equivalent Na and K movements on stimulation of mammalian cerebral cortical specimens[12] and their susceptibility to local anaesthetics.[20] Similarities between the actions of local anaesthetics and tetrodotoxin have been noted.[4] In cerebral tissues as used in the present experiments, cocaine and procaine inhibited glycolytic and respiratory responses and diminished the depolarization associated with stimulation.[21] For these results they were required at 20μM, or concentrations 1000 times greater than those in which tetrodotoxin is now observed to act.

The minute concentration in which tetrodotoxin acts merits the following calculations (compare those of Moore, Narahashi and Shaw[24] in relation to lobster nerve). At the effective concentration (see above) of 1·5 mμmole/g, 9×10^{14} molecules of the toxin are supplied/g tissue, and this carries at a minimum estimate[22] $8{\cdot}2 \times 10^5$ mm^2

of neural surface area. About 10^3 tetrodotoxin molecules are thus supplied per μ^2 of surface, and if these were evenly distributed on the surface they would be at points about 300 A apart. Possibly these are the points of Na entry during excitation; the entry has been evaluated[24] as 6 mμmole Na/g tissue/impulse, with loss of an approximately equivalent quantity of K. Each molecule of tetrodotoxin supplied would then be pictured as blocking a site at which some 4 Na could enter at each stimulating pulse. The effect of tetrodotoxin has been shown above to be reversed by simple washing; thus tetrodotoxin at its sites of action presumably exists in equilibrium with tetrodotoxin elsewhere, especially in the incubation media. For this reason it is probable that each of these calculations underestimates the effectiveness of tetrodotoxin, and further information about the amount attached to the tissue is being sought.

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