Biochemical Pharmacology, Vol. 17, pp. 129–141. Pergamon Press. 1968. Printed in Great Britain

EFFECTS OF TETRODOTOXIN AND OUABAIN ON ELECTRICALLY STIMULATED CEREBRAL CORTEX SLICES

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(*Received* 15 *May* 1967; *accepted* 19 *July* 1967)

Abstract—Isolated slices of guinea pig cerebral cortex were subjected to alternating electrical pulses and found to gain non-inulin Na^+ and to lose non-inulin K^+. Creatine phosphate levels dropped 80 per cent during electrical stimulation. These changes were largely reversed after cessation of pulses. Tetrodotoxin at 1×10^{-6} M largely prevented the effects of electrical stimulation by its action at the cell membrane. Ouabain prevented the post-stimulation gain in K^+ and extrusion of Na^+, suggesting interference with active cation transport. In the presence of ouabain, creatine phosphate levels dropped after stimulation had ceased, suggesting secondary inhibition of energy-producing mechanisms.

THE TECHNIQUE of stimulating cerebral cortex slices with alternating pulses has made it possible to study, *in vitro*, effects of drugs on the electrical excitability of brain.[1] In such preparations, by measuring changes in electrolytes and in high energy phosphates before, during, and after electrical stimulation, differentiation can be made between a drug's action on passive cation movements, on energy-utilizing processes such as active transport, or on energy-producing processes.

Effects of the cardiac glycoside ouabain have been previously examined with unstimulated cerebral slices. This agent brings about the inward shift of Na^+ ions and the loss of K^+ ions, effects which have been suggested to result from inhibition of the Na^+, K^+-stimulated adenosine triphosphatase, the enzyme system thought to be important in active cation transport. Ouabain also brings about a fall in high energy phosphates and has effects on respiration which are rather difficult to explain by the above mechanism alone.[2, 3] In the present experiments this agent was added to slices which were electrically stimulated and additional information was obtained about its mode of action in cerebral tissues.

Tetrodotoxin is a potent neurotoxin which is thought by neurophysiologists to interfere specifically with the membrane changes that result in increased permeability toward Na^+ ions during depolarization.[4] This agent has been most thoroughly studied in peripheral nerve preparations, though it has toxic effects on the central nervous system as well. It seemed of interest to explore its effects on isolated cerebral tissues in order to examine whether in this system interference with passive Na^+ movements would occur, whether the K^+ shifts which normally take place with electrical stimulation would also be blocked, and whether this agent would interfere with the toxic effects of ouabain.

METHODS

Preparation and incubation of tissue slices. Guinea pigs were used in all experiments. A young animal, weighing about 200 g, was stunned by a blow on the back of the head. The brain was removed and slices of 39 ± 6 mg (S.D. for 21 slices) were prepared with moistened blade and guide.[5] Three slices were prepared from each hemisphere. In most experiments, after weighing on a torsion balance, the slice was floated into a quick-transfer electrode holder[5] and placed in a beaker containing 7 ml of medium with the following composition: NaCl, 124 mM; KCl, 5 mM; KH_2PO_4, 1·24 mM; $MgSO_4$, 1·3 mM; $NaHCO_3$, 26 mM; glucose, 10 mM; and usually inulin, 1%; equilibrated with 5% CO_2–95% O_2 at 37°. The pH of the equilibrated medium was 7·45. The medium contained 0·75 mM $CaCl_2$ unless stated otherwise in the text. In 13 experiments, the mean time elapsing between killing the animal and incubation was 7½ min. Incubation was carried out in a water bath equipped with a gantry for application of electrical pulses. The electrical stimulator was obtained from Specialized Laboratory Equipment, Thornton Heath, Surrey, England. The stimulator supplied condenser pulses at 100/sec, with an amplitude of 10 V, and a time constant of 0·2 msec. Usually, the slice was incubated for 30 min before application of electrical pulses. In some experiments (Table 1), the slices were held in grid electrodes in Warburg electrode vessels.[5] In the Warburg experiments the medium was buffered at pH 7·4 with 30 mM Tris-HCl.

At the end of an experiment, the slice was floated into incubation medium at room temperature, removed with a platinum wire, briefly drained against the side of a beaker, and placed in a glass homogenizer tube which contained 2 ml of 6% trichloroacetic acid (TCA). The suspension was handled as stated by Swanson[3] and the supernatant fluid was analyzed for Na^+, K^+, inulin, and acid-soluble phosphates.

Na^+ and K^+ analyses. These were determined on a Perkin–Elmer model 290 atomic absorption spectrophotometer. Glassware was soaked in dilute nitric acid prior to rinsing and drying.

Analyses for inulin, acid-soluble phosphates, and medium lactate. These were carried out as described earlier.[3] Inulin analysis was by a resorcinol method of Varon and McIlwain.[6] Creatine phosphate, "10-min phosphate," and inorganic phosphate were separated and analyzed by a barium precipitation method. "Ten-min phosphate" includes the β and γ phosphates from ATP as well as the β phosphate from ADP.

Calculation of non-inulin Na^+ and K^+ contents. All calculations were referred to the initial wet weight of the slice. Non-inulin Na^+ or K^+ content was:

$$\text{Total } Na^+ \text{ or } K^+ \text{ content in slice } (\mu\text{Equiv./g initial wet wt.}) - \left(\begin{array}{l} Na^+ \text{ or } K^+ \text{ concentration} \\ \text{in the medium } (\mu\text{Equiv./ml}) \end{array} \times \frac{\mu\text{g inulin in the slice} \,/\, \text{initial wet wt. of the slice (g)}}{\text{inulin concentration in the medium } (\mu\text{g/ml})} \right)$$

Materials. Tetrodotoxin (mol.wt. 319) was kindly supplied by Dr. F. A. Fuhrman, Fleischmann Laboratories, Palo Alto, Calif. Ouabain was from Sigma Chemical Co. and inulin from Pfanstiehl Laboratories, Inc. Deionized water was used in all experiments.

RESULTS

Effects of tetrodotoxin

Slices incubated in Tris-buffered media containing 10^{-8}M tetrodotoxin respired at essentially normal rates and responded with a markedly increased respiration rate to the application of electrical pulses (Table 1). In contrast, the respiration of unstimulated slices was depressed 26 per cent by 10^{-7} to 10^{-6} M tetrodotoxin. Moreover, the respiration response to electrical stimulation was decreased by 37–46 per cent in the presence of tetrodotoxin. Stimulation of lactate formation by electrical pulses was also markedly diminished by 10^{-6} M tetrodotoxin.

Experiments were carried out to determine if the above effects on respiration and lactate formation were accompanied by alterations in levels of creatine phosphate, other acid-labile phosphates, or inorganic phosphate. Slices were held in quick-transfer holders and incubated in bicarbonate-buffered media. In control slices which were subjected to 10-min electrical stimulation, levels of creatine phosphate dropped by about 80 per cent (Table 2). Ten min after cessation of stimulation, creatine phosphate levels had recovered to within 25 per cent of those of unstimulated slices. These differences were highly significant ($P < 0{\cdot}001$). Changes in "10-min phosphate" were in the same direction but were less marked and were accompanied by reciprocal changes in levels of inorganic phosphate.

Tetrodotoxin to a large extent prevented the shifts in levels of acid-soluble phosphates. At 10^{-6} M tetrodotoxin, electrical stimulation was accompanied by a drop in creatine phosphate of only 23 per cent, and at 5×10^{-6} M, by a drop of 15 per cent. Tetrodotoxin also prevented the changes in "10-min phosphate" and inorganic phosphate that were observed in control slices. Since slice levels of high energy phosphate were maintained even in electrically stimulated slices, it seemed unlikely that tetrodotoxin directly interfered with energy-producing processes.

Effects on slice Na^+ and K^+ contents. Interference with energy-consuming processes seemed a more likely explanation of the above effects of tetrodotoxin, since electrical stimulation in the presence of this agent was accompanied by much less loss of K^+ or increase of Na^+ cations than in control slices (Fig. 1). After 10 min of electrical stimulation of control slices, tissue content of non-inulin K^+ had fallen by 38 per cent, and that of non-inulin Na^+ had risen by 71 per cent. These differences were highly significant ($P < 0{\cdot}001$). During the 10-min period after electrical stimulation, control slices regained 63 per cent of the lost K^+ and extruded 80 per cent of the Na^+ which had been gained during stimulation. These changes also were statistically significant ($P < 0{\cdot}01$ for the change in Na^+; $P < 0{\cdot}001$ for the change in K^+). In the presence of 1×10^{-6} M tetrodotoxin, stimulation resulted in a loss of only 12 per cent of the tissue K^+, and a gain of Na^+ of 42 per cent. K^+ fell to 49 μEquiv./g compared with the control level of 32·5 μEquiv./g, and Na^+ rose to 37 μEquiv./g, compared with the level of 60 μEquiv./g reached in control slices, differences which did not achieve statistical significance ($0{\cdot}1 < P < 0{\cdot}2$ for the change in Na^+). At 5×10^{-6} M, tetrodotoxin completely obliterated the Na^+ and K^+ responses to electrical stimulation.

When tetrodotoxin was added to the incubation medium during a period of electrical stimulation, the effects of electrical stimulation were reversed even when stimulation continued (Fig. 2). Recovery of prestimulation levels of Na^+ and K^+ began to take place during continued electrical stimulation, and was accompanied by a significant ($P < 0{\cdot}001$) rise in the slice content of creatine phosphate (Fig. 3). Thus,

TABLE 1. EFFECTS OF TETRODOTOXIN ON RESPIRATION AND LACTATE FORMATION OF ELECTRICALLY STIMULATED CEREBRAL SLICES*

Tetrodotoxin concn. (M)	Respiration rate (μmole/hr/g wet wt.) (a) Unstimulated (30 min)	(b) Stimulated (60 min)	Stimulation by electrical pulses (%)	Lactate formation (μmole/g wet wt.) (c) Unstimulated (30 min)	(d) Stimulated (10 min)	(e) Recovered (10 min)
No addition	62 ± 12 (6)	97 ± 6	56	43 ± 8 (10)	63 ± 18 (8)	77 ± 17 (8)
10^{-8}	61 (4)	98	61			
10^{-7}	46 (4)	62	35			
10^{-6}	46 ± 8 (7)	60 ± 11	30	33·4 (4)	38·2	46·3

* Slices were incubated in Tris-buffered media in Warburg vessels equipped with electrodes (a, b), or in bicarbonate-buffered media in quick-transfer holders (c, d, e), and subjected to electrical stimulation for the periods shown. Tetrodotoxin was present at indicated concentrations from the onset of incubation. Values are means ± S.D. of number of slices in parentheses.

TABLE 2. EFFECTS OF TETRODOTOXIN ON ACID-SOLUBLE PHOSPHATE CONTENTS OF ELECTRICALLY STIMULATED CEREBRAL SLICES*

Tetrodotoxin concn. (M)	Creatine phosphate (μmole/g wet wt.) (a) Unstimulated (30 min)	(b) Stimulated (10 min)	(c) Recovered (10 min)	10-Min phosphate (μmole/g wet wt.) (a) Unstimulated (30 min)	(b) Stimulated (10 min)	(c) Recovered (10 min)	Inorganic phosphate (μmole/g wet wt.) (a) Unstimulated (30 min)	(b) Stimulated (10 min)	(c) Recovered (10 min)
No addition	1·47±0·40 (10)	0·32±0·09 (10)	1·11 ± 0·35 (9)	4·3 ± 0·4	3·2 ± 0·7	3·6 ± 0·9	3·0 ± 0·4	4·3 ± 0·4	2·7 ± 0·6
1×10^{-6}	1·59±0·54 (6)	1·23±0·37 (6)	1·16±0·30 (6)	4·1 ± 0·9	4·7 ± 0·7	4·2 (4)	4·1 ± 1·4	4·2 ± 1·3	4·5 ± 1·2
5×10^{-6}	1·30 (2)	1·11 (2)	1·36 (2)	3·8	3·5	3·8	2·8	3·4	2·4

* Slices were held in quick-transfer holders and incubated at 37° in bicarbonate-buffered media (pH 7·4), containing 0·75 mM $CaCl_2$ and indicated concentrations of tetrodotoxin. All slices were incubated for 30 min. Unstimulated slices (a) were removed at that time; other slices were stimulated for 10 min. Of these, some (b) were removed at the end of stimulation and the others (c) were incubated for an additional 10 min without stimulation. Values are means ±S.D. of number of slices in parentheses.

active movements of Na^+ and K^+ were not inhibited by tetrodotoxin in concentrations which blocked passive movements.

Effects of ouabain

Ouabain has marked effects on cation and creatine phosphate levels of unstimulated cerebral cortex slices. The concentration of ouabain which is effective in lowering the

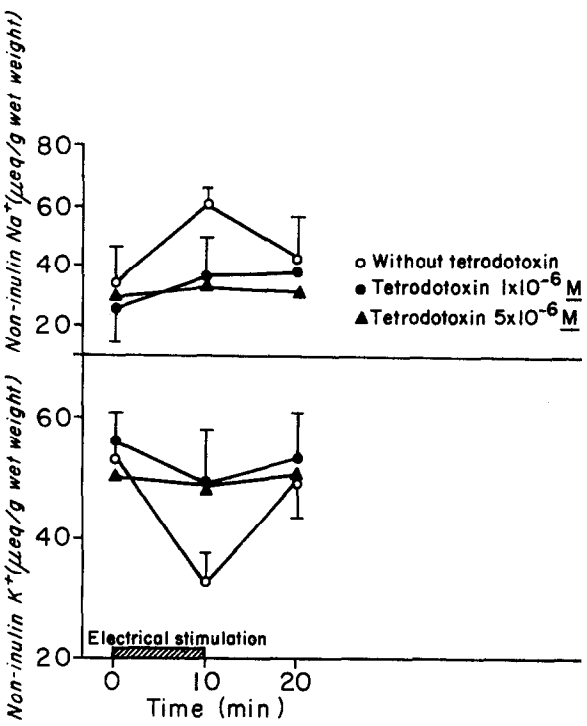

FIG. 1. Effects of tetrodotoxin on slice contents of non-inulin K^+ and Na^+ before, during, and after electrical stimulation. Cerebral cortex slices were incubated in bicarbonate-buffered medium which contained 0·75 mM $CaCl_2$ and which were equilibrated with 5% CO_2–95% O_2. After 30 min of preincubation in the presence of indicated concentrations of tetrodotoxin, slices were either removed for analysis (0 time) or electrically stimulated for 10 min, after which some slices were removed and others incubated for a further 10 min without electrical stimulation. Vertical bars represent standard deviations of mean values from 6–10 slices; other points represent mean values from 2–4 slices. ○ = No addition; ● = tetrodotoxin, 1×10^{-6} M; ▲ = tetrodotoxin, 5×10^{-6} M.

slice content of K^+ and raising Na^+ is about 10^{-5} M in the presence of Ca^{2+}, and 1 to 5×10^{-6} M in the absence of Ca^{2+}.[7] When slices were electrically stimulated, lower concentrations of ouabain were found to bring about changes in slice contents of these cations. In the presence of 0·75 mM $CaCl_2$, recovery of Na^+ and K^+ levels after stimulation did not appear to be significantly affected by 1×10^{-6} M ouabain (Fig. 4). At $2{\cdot}5 \times 10^{-6}$ M, ouabain prevented the post-stimulation rise in K^+ and extrusion of Na^+ while having less effect on the prestimulation concentrations or on

the movements of these cations with electrical stimulation. Higher ouabain concentrations brought about marked cation shifts in unstimulated slices. The shifts were augmented by electrical stimulation, and recovery processes appeared completely ineffective. At a medium Ca^{2+} concentration of 2·8 mM, electrical stimulation for 10 min in the presence of 5×10^{-6} M ouabain brought about a drop of non-inulin K^+ from 35·8 to 17·5 μEquiv./g wet wt. and a rise in Na^+ from 47 to 72 μEquiv./g

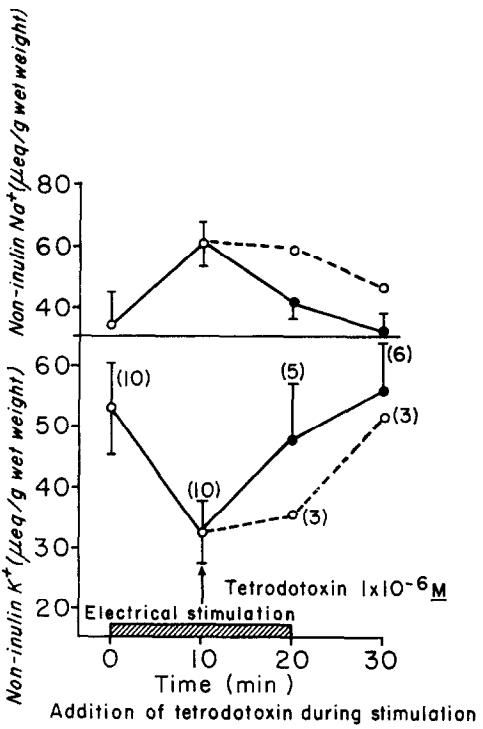

FIG. 2. Effects of 1×10^{-6} M tetrodotoxin on non-inulin Na^+ and K^+ contents of electrically stimulated cerebral slices. Conditions as in Fig. 1, except that electrical stimulation was continued for 20 min and tetrodotoxin was added after stimulation had proceeded for 10 min (arrow). Number of slices in parentheses. ○ = No tetrodotoxin; ● = 1×10^{-6} M tetrodotoxin.

wet wt., with little reversal of these changes in the post-stimulation period. When Ca^{2+} was omitted from the medium, the cation changes with electrical stimulation were similar, though they occurred at lower concentrations of ouabain, so that at 1×10^{-6} M ouabain, there was no extrusion of Na^+ and only 1/3 of the K^+ was recovered after electrical stimulation (Fig. 5).

Ouabain also altered the way in which slice creatine phosphate content changed during electrical stimulation, and the effect obtained was closely dependent upon the ouabain concentration used and on the medium Ca^{2+} concentration (Fig. 6). In medium containing 0·75 mM Ca^{2+}, $2{\cdot}5 \times 10^{-6}$ M ouabain lowered prestimulation levels of phosphocreatine by half. Electrical stimulation further lowered phosphocreatine levels to 0·4 μmole/g, but the phosphocreatine levels rose only slightly during

the 10-min post-stimulation recovery period (Fig. 6B). At the higher ouabain concentration of 5×10^{-6} M, creatine phosphate levels no longer fell on electrical stimulation. However, after stimulation at a time when the non-inulin K^+ level was 13 μEquiv./g (Fig. 4), the creatine phosphate level dropped to 0·5 μmole/g. A similar effect was found with the higher medium Ca^{2+} of 2·8 mM. In two experiments, the

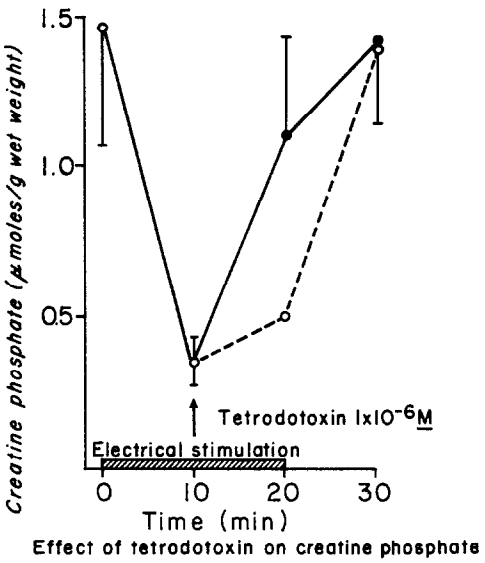

FIG. 3. Effects of 1×10^{-6} M tetrodotoxin on creatine phosphate content of electrically stimulated cerebral slices. Tetrodotoxin was added after electrical stimulation had proceeded for 10 min (arrow). ○ = No tetrodotoxin; ● = 1×10^{-6} M tetrodotoxin.

drop in creatine phosphate during electrical stimulation was only 33 per cent compared with a 73 per cent fall in control slices, and in the post-stimulation period, ouabain-treated slices continued to lose creatine phosphate, which fell to 0·77 μEquiv./g.

In Ca^{2+}-deficient media, prestimulation levels of phosphocreatine were only 55 per cent of the levels in Ca^{2+}-containing media (Fig. 6A). At ouabain concentrations between 5×10^{-7} M and 10^{-6} M, the patterns of creatine phosphate change on electrical stimulation were more variable than in media which contained Ca^{2+}. In some experiments, 5×10^{-7} M ouabain prevented the creatine phosphate loss on electrical stimulation, and was followed by a further drop during the post-stimulation period. In other experiments the creatine phosphate content did drop with electrical stimulation, though recovery was incomplete, and at the higher concentration of 2×10^{-6} M ouabain, the prestimulation level of creatine phosphate had already fallen to 0·3 μmole/g and rose slightly during electrical stimulation.

Combined effects of tetrodotoxin and ouabain

Since tetrodotoxin largely prevented the effects of electrical stimulation on slices, the effects of this agent were examined on the cation shifts brought about by an excess of ouabain. Inclusion of tetrodotoxin in concentrations of 1 to 5×10^{-6} did

temper by 10–26 per cent the cation shifts brought about by ouabain (Table 3A), but the shifts were still profound. The ouabain-induced drop in creatine phosphate content was lessened to a greater degree by inclusion of tetrodotoxin in the medium. When 10^{-6} M tetrodotoxin was added 15 min after addition of 10^{-5} M ouabain, there was no reversal of the ouabain-induced cation shifts or creatine phosphate loss (Table 3B).

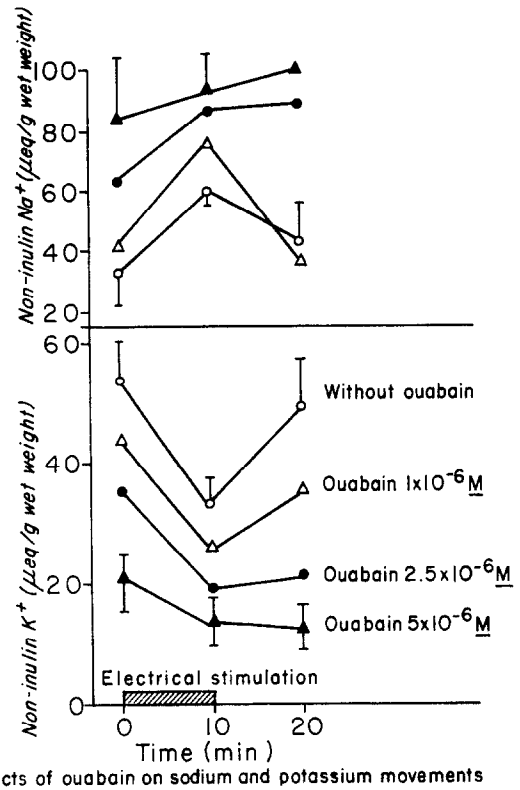

FIG. 4. Effects of ouabain on slice contents of non-inulin K^+ and Na^+ before, during, and after electrical stimulation in bicarbonate-buffered medium containing 0·75 mM Ca^{2+}. Vertical bars represent S.D's of mean values from 6–10 slices; other points are average values from 2–4 slices. ○ = No addition; △ = ouabain, 10^{-6} M; ● = ouabain, 2·5 × 10^{-6} M; ▲ = ouabain 5 × 10^{-6} M.

DISCUSSION

The two agents examined in the present study have exhibited interesting differences in their effects on electrically stimulated cerebral slices. The normal response of a thin slice of cerebral cortex to stimulation by alternating pulses of the type used in this study is a rise in non-inulin Na^+ and a fall in K^+ content. Upon cessation of stimulation, reversal of these changes occurs and under optimal circumstances, prestimulation levels of these cations may be reached.[8] The net cation shifts measured in such experiments reflect the balances between cation effluxes and influxes which are found during and after electrical stimulation. In the present experiments, electrolyte and labile

phosphate contents were measured after 10 min of stimulation or recovery. Maximum changes in electrolyte content have taken place by these times,[8] though the actual rates of cation flux increase much more rapidly than this. Thus the rate of ^{42}K efflux increased from 400 μEquiv./g tissue/hr to 600–750 μEquiv./g tissue/hr within 10 sec of applying pulses of the type used in this study.[9] An increase of ^{24}Na influx took

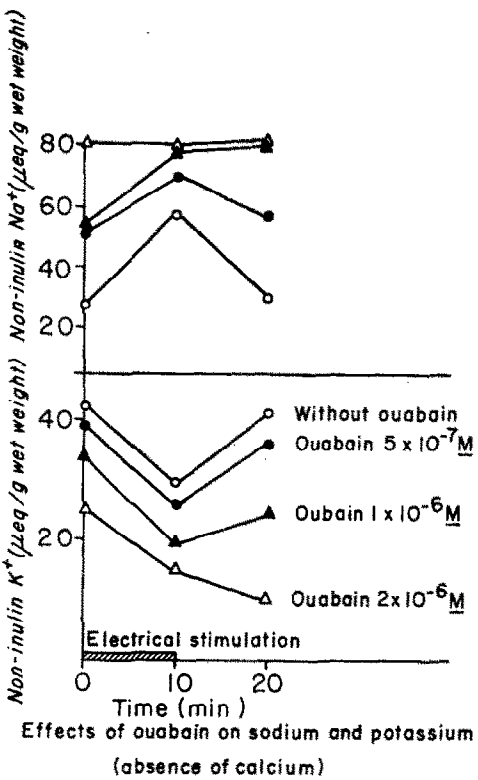

FIG. 5. Effects of ouabain on slice contents of non-inulin K^+ and Na^+ before, during, and after electrical stimulation in the absence of Ca^{2+}. Conditions were similar to those in Fig. 1, except that the medium contained no added $CaCl_2$. ○ = No addition; ● = ouabain, 5×10^{-7} M; ▲ = ouabain, 10^{-6} M; △ = ouabain, 2×10^{-6} M.

place during the first minute or two of stimulation, the rate rising from a resting value of 175 μEquiv./g/hr to about 1060 μEquiv./g/hr.[10] After significant shifts of Na^+ and K^+ ions have occurred, the above flux rates are balanced by active transport processes which prevent further net cation shifts. Upon cessation of stimulation, the active transport mechanisms are able to restore prestimulation levels of these cations under optimal circumstances. The "downhill" cation shifts that occur during stimulation probably result from an increased membrane permeability brought about by the electrical pulses, and are a property of excitable tissue. The changes, however, are slower than spike discharges, and may be analogous to the permeability changes that take place in axons in voltage clamp experiments.[11]

It is well established that cerebral tissues utilize increased amounts of energy during periods of electrical stimulation, since levels of creatine phosphate drop while rates of lactate formation and respiration increase.[12] Levels of creatine phosphate are also restored in the period after electrical stimulation. If the period of stimulation is brief, restoration may be complete within 4–7 sec.[13]

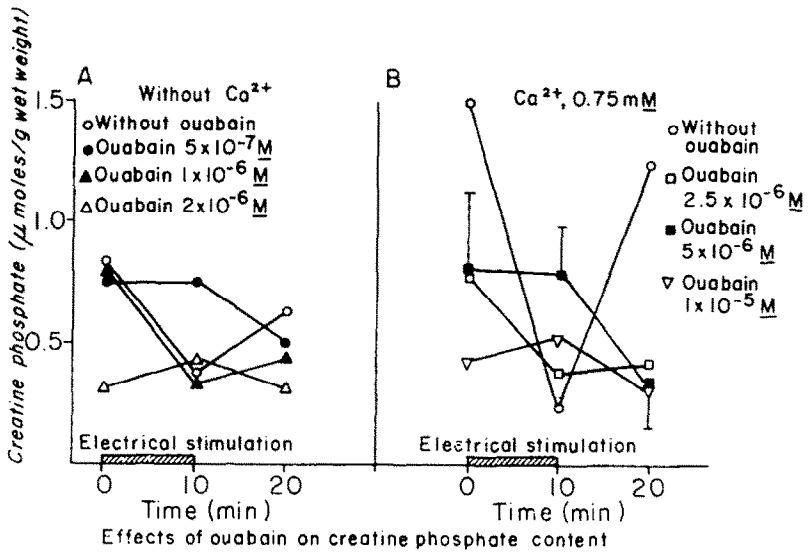

FIG. 6. Effects of ouabain on slice contents of creatine phosphate as affected by electrical stimulation: (A) in absence of Ca^{2+}; or (B) with 0·75 mM Ca^{2+}. Conditions as in Figs. 4 and 5. ○ = No addition; ● = ouabain, 5×10^{-7} M; ▲ = ouabain, 10^{-6} M; △ = ouabain, 2×10^{-6} M; □ = ouabain, $2{\cdot}5 \times 10^{-6}$ M; ■ = ouabain, 5×10^{-6} M; ▽ = ouabain, 1×10^{-5} M.

The tetrodotoxin experiments strongly suggested that in cerebral tissues this agent, in concentrations of 1 to 5×10^{-6} M directly interferes with the passive downhill movements of both Na^+ and K^+ which normally occur upon electrical stimulation. The absence of direct interference with energy-producing mechanisms was reflected in the maintenance of creatine phosphate and other high energy phosphate levels during stimulation. Of further interest were the experiments in which slices which had been stimulated for 10 min were then exposed to tetrodotoxin (Fig. 2). Here, the slice K^+ rose to prestimulation levels even during continued stimulation, while Na^+ was extruded. This suggests that in cerebral tissues, the movements of Na^+ and K^+ that take place during the active processes of recovery are not inhibited by tetrodotoxin. Thus, presumably the membrane groups with which tetrodotoxin combines are different from those groups which are important in cation selection during the active transport process. Substances which may combine with the latter category of membrane group include protamine[14] and chlorambucil,[15] both of which appear to block the active transport of cations without inhibiting the Na^+, K^+-ATPase and while having no effect on the passive movements which are affected by tetrodotoxin. In squid giant axon and other peripheral nerve preparations, electrophysiological measurements have suggested that tetrodotoxin selectively prevents the increased permeability to Na^+ which takes place during depolarization.[4] If in cerebral tissues the

TABLE 3. EFFECT OF TETRODOTOXIN ON OUABAIN-INDUCED CHANGES IN ELECTROLYTE AND CREATINE PHOSPHATE CONTENTS OF UNSTIMULATED SLICES

	Tetrodotoxin concn.	Non-inulin K^+ (μEquiv./g wet wt.)		Non-inulin Na^+ (μEquiv./g wet wt.)		Creatine phosphate (μmole/g wet wt.)	
		Without ouabain	Ouabain, 10^{-5} M	Without ouabain	Ouabain, 10^{-5} M	Without ouabain	Ouabain, 10^{-5} M
A*	None	60·3 (3)	8·5 ± 2·0 (6)	28	106 ± 13	1·31	0·50 ± 0·08
	10^{-6} M	60·6 (3)	22·1 (3)	22	85	1·44	1·11
	5×10^{-6} M		15·3 (3)		72		0·89
B†	None		13·1 (2)		92		0·61
	10^{-6} M		9·6 ± 1·3 (4)		93 ± 9		0·62 ± 0·09

* (A) Slices were incubated for 30 min in presence of indicated concentration of tetrodotoxin. Ouabain was then added to 1×10^{-5} M and slices were removed after a further 30-min incubation.

† (B) After preincubation for 30 min without tetrodotoxin, ouabain was added to 1×10^{-5} M. After 15 min, control slices were removed for analysis and tetrodotoxin was added to the remaining slices, which were incubated for a further 30 min.
Number of slices in parentheses. Ca^{2+}, 0·75 mM.

locus of action of this agent is also only at sites of importance for Na^+ movement, then it may be inferred that the K^+ efflux which results from electrical pulses cannot take place without concurrent or preceding Na^+ influx. Thus, in cerebral tissues, as well as in peripheral nerve, K^+ efflux may be regarded as a secondary phenomenon which follows the gain of Na^+ and which is not itself a direct result of electrical stimulation.

In this study, ouabain brought about changes of a different type, which were more complex than those of tetrodotoxin. The effects of ouabain in electrically stimulated slices lend support to the idea that this agent acts primarily to prevent active extrusion of Na^+ and uptake of K^+, since the downhill shifts of these cations with electrical stimulation were of the same magnitude as in control slices, although recovery in the post-stimulation period was severely inhibited. Moreover, the failure of creatine phosphate levels to fall during electrical stimulation of some of the ouabain-treated slices (Fig. 6) may then be attributed to a decrease in energy consumption due to inhibition of the energy-requiring active cation transport system. However, in these experiments creatine phosphate levels fell appreciably in the period which followed stimulation, while in other ouabain experiments creatine phosphate levels fell during stimulation and did not recover in the post-stimulation period. It appears then that the creatine phosphate level in ouabain-treated slices depends upon the relative balance between at least two processes: the first, inhibition of the Na^+, K^+-ATPase, blocks active transport of Na^+ and K^+ and tends to prevent utilization of creatine phosphate during electrical stimulation; the second process brings about a fall of creatine phosphate levels.

Yoshida *et al.*[16] have suggested that the ouabain-induced creatine phosphate drop was due to an increase in membrane permeability, since the effects of 5×10^{-6} M ouabain were prevented by 10^{-3} M cocaine. However, cocaine might prevent access of ouabain to its site of action on the active transport system. In the present study, at a concentration which completely blocked downhill cation movements with electrical stimulation, tetrodotoxin modified by only 12–26 per cent the loss of K^+ and gain in Na^+ brought about by 10^{-5} M ouabain (Table 3). Moreover, after ouabain had been added, addition of tetrodotoxin did not reverse the changes either of cation or creatine phosphate content. Since stimulation-induced passive cation movements did not appear to be appreciably accelerated by ouabain, it does not seem likely that this compound has a major direct effect on membrane permeability. It has been suggested that the ouabain-induced creatine phosphate loss may represent a secondary effect on energy-producing mechanisms.[3] Failure of creatine phosphate levels to rise in the period following electrical stimulation supports this suggestion, and the fall of respiration in ouabain-treated slices which were incubated in Ca^{2+}-deficient medium may reflect this effect. It is still true, however, that in Ca^{2+}-containing media, ouabain brings about a respiration increase.[2,7,17] This effect was suggested to result from an incomplete inhibition of the Na^+, K^+-ATPase, which allowed the uninhibited part of this enzyme to still respond to cation shifts by consuming high energy phosphate and thereby stimulating respiration.[2] If this proposal is correct, it is difficult to understand why the stimulation of respiration is maximal at 10^{-4} M ouabain, a concentration which is 10–20 times greater than that shown in the present experiments to markedly inhibit active cation transport. Since recovery of creatine phosphate levels was also inhibited in Ca^{2+}-containing media, it would seem that here too, production of high-energy

phosphate is inadequate. It seems possible that the respiration increase reflects an uncoupling of oxidative phosphorylation, which only occurs when ouabain-induced changes take place in Ca^{2+}-containing media, a suggestion that has also been made by Bourke and Tower.[18] When ouabain was added directly to isolated cerebral mitochondria, uncoupling of oxidative phosphorylation did not take place.[3] Uncoupling has, however, been observed in liver mitochondria which were incubated in media containing Ca^{2+} ions at a concentration of 1 mM.[19] The respiration increase was accompanied by mitochondrial swelling and uptake of Ca^{2+} by the mitochondria. Moreover, mitochondrial Ca^{2+} uptake was maximal in media containing high concentrations of Na^{+}.[20] It is reasonable then to suggest that in Ca^{2+}-containing media ouabain may bring about an increase in intracellular Ca^{2+} of cerebral slices in addition to the shifts of Na^{+} and K^{+}. When guinea pig cerebral cortex slices were incubated in the presence of 5×10^{-5} M ouabain and 1·4 mM Ca^{2+}, a 67 per cent increase of $^{45}Ca^{2+}$ influx was found.[21] Experiments with isolated guinea pig cardiac muscle preparations have shown that toxic concentrations of ouabain increase the exchangeable Ca^{2+} fraction.[22] In cerebral tissues, then, a high internal Ca^{2+} in conjunction with high intracellular Na^{+} concentration may uncouple oxidative phosphorylation and in this way bring about an increased rate of respiration in the slice.

Acknowledgements—I am grateful to Mrs. D. Kenner and Mrs. L. Anderson for skilful technical assistance. This work was supported by Grant No. NB 05424-03 from the United States Public Health Service.

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