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DIPHENYLHYDANTOIN AND MOVEMENT OF RADIOACTIVE SODIUM INTO ELECTRICALLY STIMULATED CEREBRAL SLICES

P. D. SWANSON and P. O. CRANE

Division of Neurology, Department of Medicine, University of Washington School of Medicine, Seattle, Wash. 98105, U.S.A.

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Abstract—Thin slices prepared from guinea pig cerebral cortex responded to electrical stimulation for 1·5–10 min at 1000 c/sec or 100 c/sec with a drop in content of creatine phosphate, ATP and K^+ in the non-inulin space. Non-inulin Na^+ rose. ^{22}Na uptake also rose from an initial rate of about 480 μEq/g initial wt./hr to about 1360 μEq/g initial wt./hr. All of the above changes were diminished by diphenylhydantoin at concentrations of 1–2·7 $\times$ 10^{-4} M. These results suggest that diphenylhydantoin diminishes the ability of neuronal elements to depolarize in response to electrical excitation.

THERE has been a recent resurgence of interest in investigating the biochemical basis for the anticonvulsant action of diphenylhydantoin. A widely accepted view based on studies by Woodbury[1] is that this agent stimulates active Na^+ and K^+ transport in neurons, thereby altering the spread of neuronal depolarization occurring during a seizure. Other investigations have provided evidence both for[2–4] and against[5–7] this hypothesis.

Experiments from this laboratory[6] have shown that diphenylhydantoin prevents and reverses the shifts of Na^+ and K^+ normally occurring in electrically stimulated brain slices, and that levels of ATP and phosphocreatine in stimulated brain slices incubated with diphenylhydantoin are higher than in stimulated controls. These data suggest that diphenylhydantoin causes a decrease in energy utilization in electrically stimulated slices probably brought about by diminishing the downhill movements of monovalent cations.

Intracellular concentrations of monovalent cations are determined largely by the balance between (1) passive movements which depend upon the electrochemical gradient and permeability of the membrane, and (2) active transport in the opposite direction. In the case of sodium, passive movements occur in the inward direction and sodium is actively transported out of the cells. A decrease in intracellular Na^+ could thus be brought about either by increased active transport, or by decreased passive inward movement. Although an accompanying decrease in energy utilization in the presence of diphenylhydantoin strongly strengthens the likelihood that the latter mechanism is operative in cerebral slices, it seemed reasonable to directly examine the effects of diphenylhydantoin on fluxes of radioactive sodium. The results suggest that, during high-frequency electrical stimulation, diphenylhydantoin diminishes the uptake of radioactive sodium into the non-inulin space of cerebral slices, probably due to decreased ability of neuronal elements to depolarize in response to electrical stimulation.

MATERIALS AND METHODS

These experiments were carried out with isolated slices of guinea pig cerebral cortex prepared by standard methods.[8] A thin first slice was discarded to minimize differences between outer slices with onecut surface and slices with twocut surfaces. Three slices weighing from 40–70 mg and approximately 0·3 mm thick were prepared from each hemisphere with a moistened blade and guide, and were then trimmed. The slices, held in quick-transfer holders, were incubated at 37° in medium of 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; $CaCl_2$, 0·75 mM; and inulin, 1%. The medium was equilibrated with 5% CO_2–95% O_2, pH 7·45. Diphenylhydantoin was included at indicated concentrations from the beginning of incubation. Inclusion of diphenylhydantoin did not alter the pH of the medium. Since the stock solution of diphenylhydantoin was prepared in 20% ethanol, a control experiment was carried out in the presence of 0·1% ethanol, the maximum amount added in these experiments. This concentration of ethanol caused no change in the effects of electrical stimulation on cation movements or high energy phosphate content.

^{22}Na uptake. After 30–36 min of preincubation, at "zero time", 10 μl of $^{22}NaCl$ (about 1·18 μc) was added to each beaker. To insure rapid mixing with the medium, the slice in its holder was briefly removed from the beaker while $^{22}NaCl$ was added and the contents were agitated. At time intervals of 1·5–10 min from "zero time", each slice's medium was poured into an evaporating dish, and the slice was released into its medium, and removed within 15 sec. The slices were then drained against the side of the dish, reweighed, homogenized in 2 ml of 6% TCA at room temperature and centrifuged in a Sorvall model RC2-B centrifuge at 3000 *g* for 10 min. Each supernatant was saved. The pellet was resuspended in 2 ml of 6% TCA, and centrifuged at 3000 *g* for another 10 min. This second supernatant was added to the first and samples were taken for subsequent analyses.

Electrical stimulation. From 1 to 6 slices were stimulated with condenser pulses, alternating in polarity at 1000/sec, with an amplitude of 10 V, and a time constant of 0·1 msec, in the manner described by Crane and Swanson.[6] The duration of stimulation lasted from 1·5–10 min. In the experiments reported in Table 3, condenser pulses were applied at 100/sec with an amplitude of 10 V and a time constant of 0·3 msec.

Analysis for Na^+ and K^+. Four hundred μl of supernatant from each slice suspension was diluted to 10 ml. The analysis was carried out with a Perkin–Elmer model 290 atomic absorption spectrophotometer. Glassware was rinsed with demineralized water prior to use.

Analysis for inulin. Inulin was analyzed in the medium and slices by a resorcinol method.[9] Inulin space was calculated according to Keesey *et al.*[10] and was corrected for the additional fluid taken up during incubation.

Measurement of radioactivity. Two hundred μl of sample and 200 μl of its medium in Bray's solution were counted in duplicate for 10 min or less (to give a standard deviation of 2·5 or less for sample and 0·7 or less for media) on a Packard no. 3375 liquid scintillation spectrometer with automatic external standardization. The channel setting was 50–1000 with 3 per cent gain.

Expression of results. The amount of sodium exchanging with the non-inulin space (expressed in microequivalents per gram of initial wet weight of tissue) was determined

by the relationship C/A–I, the justification of which is discussed by Keesey and Wallgren.[11] ^{22}Na exchange is used synonymously with ^{22}Na uptake and should not be confused with Na–Na exchange diffusion, which process may, however, contribute to ^{22}Na uptake (see discussion by Keesey and Wallgren[11]). In this formula, C = counts per min per gram of slice; A = specific activity of sodium in the incubation medium (counts per min per microequivalent of sodium); and I = sodium content of the inulin space (microequivalents per gram of initial wet weight). Thus, C/A is the amount of sodium exchanging with the slice. The inulin sodium content, I, was calculated by: inulin space (microliters per gram) × Na^+ concentration of the incubation medium (microequivalents per milliliter), where inulin space =

$$\frac{\text{Slice inulin content } (\mu g) - [\text{weight gain (g)}] \times [\text{medium inulin concentration } (\mu g/ml)]}{\text{initial wet wt. of slice (g)}}$$

divided by the medium inulin concentration (micrograms per milliliter).

Analyses for acid-soluble phosphates. Creatine phosphate and ATP were analyzed on TCA supernatants from the slice experiments by enzymic methods described elsewhere.[12] All values refer to initial wet weight of slice.

Materials. Diphenylhydantoin was obtained as the sodium salt (mol. wt. 272·25) from Parke Davis & Co., Detroit, Mich. A stock solution of 70 mM was prepared in 20% ethanol. When added to incubation medium at a calculated concentration of $5 \cdot 10^{-4}$ M, a fine precipitate was frequently seen. For this reason, diphenylhydantoin determinations were carried out by Mr. P. Friel on representative incubation media after centrifugation using a gas–liquid chromatographic method.[13] Reported concentrations are those determined on three incubation media.

RESULTS

Electrical stimulation at 1000/sec brought about cation shifts, and a drop in the slice levels of high energy phosphates which were in the same direction as those previously reported[6] with a 10-min stimulation (Fig. 1). The drop in creatine phosphate and ATP, the fall in non-inulin K^+ content and the rise in non-inulin Na^+ content were already evident at 1·5 min, which was the shortest stimulation time examined. In the presence of diphenylhydantoin, all the effects of electrical stimulation were reduced so that at 1·5 min, the stimulation-induced rise in non-inulin Na^+ content was reduced by 95 per cent ($P < 0{\cdot}10$), the fall in non-inulin K^+ content by 45 per cent ($P < 0{\cdot}002$); the fall in ATP by 100 per cent ($P < 0{\cdot}005$) and the fall in creatine phosphate by 50 per cent ($P < 0{\cdot}001$).

^{22}Na uptake. In the absence of electrical stimulation, uptake of ^{22}Na into the non-inulin space occurred to a similar extent as earlier[11] demonstrated with ^{24}Na (Table 1). Inclusion of diphenylhydantoin at a final concentration of $2{\cdot}7 \times 10^{-4}$ M appeared to diminish the amount of exchange noted at 1·5 min, but at later times there was no significant change. Application of electrical pulses at a rate of 1000/sec brought about a marked increase in ^{22}Na exchange (Table 2). Inclusion of diphenylhydantoin in the incubation medium markedly reduced this stimulation-induced increase in ^{22}Na exchange. At the lower concentration of 1×10^{-4} M (data not shown), the effect was similar, with a reduction by 63 per cent of the stimulation-induced increase in ^{22}Na exchange.

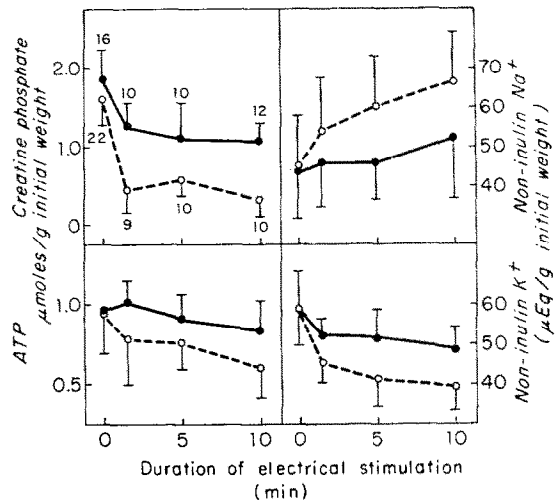

FIG. 1. Effects of diphenylhydantoin on high-energy phosphates and non-inulin cation contents of cerebral slices during electrical stimulation. Slices were preincubated for 30 min without electrical stimulation in the presence or absence of diphenylhydantoin at a final concentration of $2{\cdot}7 \times 10^{-4}$ M. Pulses were applied for indicated times at a frequency of 1000/sec. Bars represent standard deviations of indicated number of slices. ○ = without diphenylhydantoin; ● = with diphenylhydantoin.

TABLE 1. EFFECT OF DIPHENYLHYDANTOIN ON ^{22}Na EXCHANGING WITH NON-INULIN SPACE IN ABSENCE OF ELECTRICAL STIMULATION*

Duration of exposure to ^{22}Na (min)	Sodium exchanged in non-inulin space (µEq/g wet wt) A Without diphenylhydantoin	B With diphenylhydantoin	Significance between A and B
1·5	12·0 ± 3·3 (9)	2·8 ± 8·4 (8)	$P < 0{\cdot}004$
5	16·9 ± 5·6 (8)	20·0 ± 4·5 (7)	NS
10	20·0 ± 2·7 (3)	20·6 ± 9·7 (3)	NS

* Cerebral slices were incubated in standard medium in the presence or absence of $2{\cdot}7 \times 10^{-4}$ M diphenylhydantoin. After a 30-min preincubation, ^{22}Na was added. Slices were removed after indicated times for analysis. Values are means ±S. D. for number of slices in parentheses. NS = Not significant.

TABLE 2. EFFECT OF DIPHENYLHYDANTOIN ON ^{22}Na EXCHANGING WITH NON-INULIN SPACE IN PRESENCE OF ELECTRICAL STIMULATION*

Duration of incubation with ^{22}Na (min)	Sodium exchanged in the non-inulin space (µEq/g wet wt) A without diphenylhydantoin	B with diphenylhydantoin	Significance between A and B
1·5	34·0 ± 17·4 (6)	12·5 ± 11·7 (7)	$P < 0{\cdot}02$
5	36·4 ± 15·0 (12)	22·8 ± 8·2 (11)	$P < 0{\cdot}01$
10	55·0 ± 9·1 (6)	33·5 ± 9·5 (9)	$P < 0{\cdot}001$

* Slices were incubated in presence or absence of $2{\cdot}7 \times 10^{-4}$ M diphenylhydantoin. After a 30-min preincubation, ^{22}Na was added and electrical stimulation at 1000 impulses/sec was begun. Values are means ±S. D. for number of slices in parentheses.

Stimulation at a lower frequency. Diphenylhydantoin was also effective in reducing the changes in cations and ^{22}Na uptake brought about by electrical stimulation at 100 c/sec: a frequency which is more commonly used in stimulation experiments with cerebral slices (Table 3). Diphenylhydantoin reduced by 45 per cent the stimulation-induced increase in ^{22}Na influx. At this frequency, the effectiveness of diphenylhydantoin was somewhat less than demonstrated at the higher stimulation frequency and the difference in creatine phosphate contents did not achieve statistical significance-

TABLE 3. EFFECTS OF DIPHENYLHYDANTOIN ON SLICES ELECTRICALLY STIMULATED AT A FREQUENCY OF 100 c/sec*

		With electrical stimulation			
	Without stimulation	A Without diphenyl-hydantoin	B With diphenyl-hydantoin	Reduction in stimulation induced changes (%)	Significance between A and B
Non-inulin K^+ content (μEq/g initial wt)	59·4 ± 10·8	31·7 ± 1·9	41·3 ± 6·9	35	$P < 0.001$
Non-inulin Na^+ content (μEq/g initial wt)	44·1 ± 13·2	83·8 ± 11·2	66·9 ± 5·4	43	$P < 0.001$
Na^+ exchanged with non-inulin space (μEq/g initial wt)	26·2 ± 5·5	62·2 ± 8·5	45·9 ± 9·1	45	$P < 0.001$
Creatine phosphate (μmoles/g initial wt)	2·09 ± 0·40	0·29 ± 0·25	0·54 ± 0·38	14	NS
ATP (μmoles/g initial wt)	0·93 ± 0·1	0·48 ± 0·1	0·65 ± 0·13	28	$P < 0.01$
No. of slices	3	7	8		

* Slices were incubated as in Fig. 1 and Tables 1 and 2 in presence or absence of 2.7×10^{-4} M diphenylhydantoin except that electrical stimuli were applied to indicated slices at 100 c/sec. Values are means ± S. D. NS = Not Significant.

DISCUSSION

The present experiments demonstrate the ability of diphenylhydantoin to reduce ^{22}Na uptake into the non-inulin space of cerebral slices subjected to electrical stimulation. We have also extended earlier results which showed that diphenylhydantoin diminishes the amount of K^+ lost and Na^+ gained by electrically stimulated slices, and also diminishes the loss of high-energy phosphates. Since stimulation-induced oxygen consumption is also decreased by diphenylhydantoin,[7] it seems unlikely that the agent brings about an increased synthesis of high-energy phosphates.

Reduction by diphenylhydantoin of ^{22}Na exchange with Na of the non-inulin space

strongly suggests a diminution in the ability of neuronal elements to depolarize in response to electrical excitation. Keesey and Wallgren[11] demonstrated an increase in the influx rate of radioactive ^{24}Na from 175 μEq/g wet wt/hr to 1056 μEq/g/hr in response to condenser pulses of alternating polarity applied at a rate of 100/sec. We chose to apply pulses at a more rapid rate in most experiments because of the earlier demonstration that the ability of diphenylhydantoin to diminish the respiration response to electrical stimuli was more easily demonstrable when pulses were applied at a high frequency.[7] The diphenylhydantoin reduction in ^{22}Na uptake was also found at the lower stimulation rate of 100/sec (Table 3).

Could the above findings result from stimulation by diphenylhydantoin of active Na^+ and K^+ transport through an effect on the (Na^+, K^+)-ATPase? If diphenylhydantoin were acting as a stimulator of the Na^+ pump, one might predict that during a state of enhanced electrical activity, as might occur during a seizure, neurones would be able to depolarize with the normal small shifts in Na^+ and K^+ resulting from a transient increase in cation permeability, but that the increased rate of pumping would prevent or lessen any net shifts in these cations from occurring. An increased rate of Na^+ efflux might be seen but the rate of Na^+ influx would not be expected to change. Our experiments have demonstrated a diphenylhydantoin-induced decrease in the ^{22}Na uptake during electrical stimulation. The magnitude of the rate of influx decrease may be compared with the reduction in rate of change of net Na^+ uptake. Because of the time required to come into equilibrium with extracellular Na, it was not possible to calculate the amount of ^{22}Na in the non-inulin space for times shorter than 1·5 min. Thus, the rate calculated for 1·5 min is only an approximation of the initial rate of ^{22}Na influx. Assuming a constant rate during the first 1·5 min after exposure to ^{22}Na, electrical stimulation at 1000 c/sec increased the rate of influx from 480 μEq/g initial wt/hr to 1360 μEq/g initial wt/hr, rates which are close to the ranges found by Keesey and Wallgren[11] under somewhat different conditions. In our stimulation experiments, diphenylhydantoin reduced the influx rate to 500 μEq/g initial wt/hr, a reduction which is more than sufficient to account for the observed reduction in Na^+ content increase in the non-inulin space (Fig. 1).

Recent data with lobster nerve also suggest that diphenylhydantoin acts by limiting the increase in sodium permeability that occurs on electrical stimulation.[14] Diphenylhydantoin had no effect on the rate of efflux of ^{24}Na in resting nerves or in nerves that were stimulated at 50 c/sec. In contrast, ^{24}Na influx was reduced 40 per cent in electrically stimulated nerves. The significance of this study may be altered by other experiments with resting lobster nerve which suggested a stimulating effect of diphenylhydantoin on active ^{42}K uptake.[15] However, physiological studies with crayfish stretch receptor[16] and voltage clamp experiments with squid axon[17] both suggest that diphenylhydantoin does reduce the inward movements of sodium.

Recent studies with nerve ending preparations have been interpreted to show a stimulating effect of diphenylhydantoin on active cation transport.[4] Nerve endings were found to accumulate more K^+ in the presence of diphenylhydantoin when the medium K^+ concentration was below 2 mM and when ouabain was also present. It was suggested that the effect of diphenylhydantoin could not be explained by a block in K^+ efflux because the resultant synaptosomal K^+ was increased above initial values. It would seem possible, however, that a decrease in cation permeability could result in a higher steady state content of intracellular K^+ if the rate of active transport did not

sufficiently decrease in response to the higher K^+ concentration. Further, studies with nerve endings have not included observations of the effects of diphenylhydantoin on energy utilization. It would seem useful to use the techniques of Bradford[18] who has measured oxygen consumption by nerve endings and has shown a response to electrical stimulation. If nerve endings indeed differ from cerebral slices in respect to diphenylhydantoin action, it should be possible to demonstrate that this agent stimulates respiration under appropriate experimental conditions.

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