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THE EFFECT OF DIPHENYLHYDANTOIN ON SODIUM, POTASSIUM, MAGNESIUM-ACTIVATED ADENOSINE TRIPHOSPHATASE IN MICROSOMAL FRACTIONS OF RAT AND GUINEA PIG BRAIN AND ON WHOLE HOMOGENATES OF HUMAN BRAIN*

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Abstract—Microsomal fractions were prepared from rat and guinea pig cerebral cortex. The Na, K, Mg-activated adenosine triphosphatase (Na, K, Mg-activated ATPase) activity of these fractions was consistently inhibited by diphenylhydantoin in concentration of 10^{-4} M during experiments *in vitro*. Ouabain-induced inhibition was also increased by the addition of diphenylhydantoin. Neither diphenylhydantoin nor ouabain in concentrations up to 10^{-3} M had any effect on Mg-activated ATPase activity. Whole homogenates of human cerebral cortex showed similar reactions.

THE MEMBRANE-stabilizing properties of diphenylhydantoin, including its anti-epileptic activity,[1, 2] have been attributed to its ability to improve the active extrusion of sodium from brain cells.[3] The drug also has been reported to prevent intracellular increase of sodium caused by maximal electroshock seizures and by hyponatraemia.[3] It seemed reasonable to suppose that diphenylhydantoin might exert its action by stimulating the Na, K, Mg-activated adenosine triphosphatase (ATPase) enzyme system, since there is evidence that this is the carrier mechanism involved in the active transport of sodium.[4] A preliminary study with whole homogenates of rat brain indicated that diphenylhydantoin diminished Na, K, Mg-activated ATPase activity in the presence of ouabain.[5] In the present study, with microsomal fractions of rat and guinea pig brain and whole homogenates of human brain, it is clear that under the conditions of the experiment, which involved a preliminary exposure of cerebral tissue and drugs, diphenylhydantoin inhibits Na, K, Mg-activated ATPase and increases the inhibition produced by ouabain.

METHODS

Preparation of microsomes from cerebral cortex

Albino rats and guinea pigs were decapitated and the brains removed quickly and placed on ice. The white matter was removed as far as possible by suction through a Pasteur pipette. Each brain was then weighed and placed in 10 vol. of ice-cold Tris-buffer at pH 7·6. Homogenization was carried out at 1500 rpm in a glass-Teflon

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homogenizer and the pestle was passed 40 times through the homogenate as rapidly as possible at 0°. The homogenate was centrifuged at 400 *g* for 10 min in a Sorvall RC2-B refrigerated centrifuge. The supernatant was carefully removed and the precipitate was resuspended in an equal volume of Tris-buffer and recentrifuged at the same speed. The precipitate was then discarded and the supernatants were centrifuged at 10,000 *g* for 15 min. The precipitate was discarded and the supernatants centrifuged at 20,000 *g* for 60 min to precipitate a heavy microsomal fraction. The pellet obtained was resuspended in 3 ml of the original Tris-buffer and frozen for 48 hr. Immediately before use it was diluted 1:3 with Tris-buffer.

Preparation of homogenate of human frontal cortex

Approximately 1 g of frontal cortex was obtained from the brain of a 60-yr-old woman who had died 14 hr previously of noncerebral causes. The sample was homogenized and frozen for 48 hr. The concentration of diphenylhydantoin used in this experiment approximates therapeutic levels in the nervous system and is achieved by parenteral administration of 40 mg/kg in the rat.[6]

ATPase assay

Total ATPase activity was estimated in the reaction mixtures containing 3 mM $MgCl_2$, 100 mM NaCl, 30 mM KCl, 3 mM Tris-ATP, and 0·4 ml of enzyme suspension in a total volume of 2 ml Mg-ATPase activity was measured in identical reaction mixtures which omitted Na and K. Then Na, K, Mg-activated ATPase activity was found by subtracting Mg-activated ATPase from total ATPase activity. Appropriate beakers contained diphenylhydantoin (10^{-4} M) or ouabain (10^{-4} M) or both. Incubation was carried out for 10 min in a water bath at 37° with shaking in 25-ml beakers. The mixture containing the enzyme was preincubated at 37° for 5 min before starting the reaction with ATP. All the constituents were made up in 25 mM Tris-buffer at pH 7·6 and the pH change during the reaction was usually in the range pH 7·6 to 7·4. The addition of diphenylhydantoin (10^{-4} M) did not alter the pH. After 10 min, the reaction was stopped by addition of 1 ml of ice-cold 5% trichloracetic acid. The mixtures were transferred to test tubes and the sediment was spun down at 3000 rpm for 10 min. Inorganic phosphate was measured in 1 ml of the supernatant by the method of Fiske and Subbarow.[7] A Beckmann D-6-u.v. spectrophotometer was used for the colorimetric readings at 660 mμ. All the incubations were made in triplicate and an average was taken; no more than a 2 per cent variation was accepted. Tris-ATP was used in all the assays. This was made by treating disodium ATP with an exchange resin.[8]

Method of addition of diphenylhydantoin and ouabain to the reaction mixture

A fresh solution of diphenylhydantoin in water was prepared daily and added to the reaction mixtures to give a final concentration of 10^{-4} M. Consistent inhibition of enzyme activity was obtained only by exposing the enzyme solution to diphenylhydantoin (10^{-4} M) for 10 min before starting the reaction. No further inhibition was obtained when the enzyme was exposed to diphenylhydantoin for 45 min prior to starting the reaction. Correction was made in the control assays for dilution caused by the addition of diphenylhydantoin or ouabain solution or by both solutions combined. A fresh solution of ouabain (10^{-4} M) in water was prepared daily. The

action of ouabain in inhibiting the enzyme was immediate and it was not necessary to pre-expose the enzyme to ouabain before starting the reaction.

Protein determination on the fractions

Protein determinations were made by the method of Lowry *et al.*[9] The amount of protein used in each rat brain assay varied from 0·08 to 0·17 mg. The guinea pig assays contained 0·19 mg/assay. The whole homogenates of human cortex gave 0·4 mg/assay. Increasing the amount of protein in the assay increased the amount of phosphate released in a linear fashion.

Twenty-one assays were made; 11 with rat brain microsomes, 5 with guinea pig brain microsomes, and 5 with whole homogenates prepared from a single human brain. In addition, 5 assays were made with rat brain microsomes with 15 mM sodium instead of 100 mM sodium in the incubation medium.

RESULTS

ATPase activity of the preparations

The characteristics of the microsomal ATPase activities were as described in the literature.[8, 10] In every assay, diphenylhydantoin inhibited ATPase activity and increased ouabain-induced inhibition.

Rat. Table 1 gives the results of the assays on rat microsomes. Mean Na, K, Mg-ATPase activity was 2·8 uMP_i/mg protein/10 min; with diphenylhydantoin in the incubation medium the mean was 2·2. The mean percentage inhibition was 23 per cent ($P = 0{\cdot}0005$). The addition of ouabain to the incubation medium inhibited 73 per cent of the Na, K, Mg-ATPase activity and the combination of diphenylhydantoin and ouabain inhibited 96·8 per cent of the activity, i.e. a further 23 per cent inhibition.

With 15 mM sodium in the incubation medium, the Na, K, Mg-ATPase activity was 1·7 uMP_i/mg protein/10 min and 1·4 with diphenylhydantoin added, which indicated an 18 per cent inhibition. Diphenylhydantoin (10^{-5} M) was also used and was found to produce a similar but less marked inhibition.

By the same technique used for assay *in vitro*, phenobarbital (10^{-4} M) was shown to have no effect on Na, K, Mg-ATPase activity.

Guinea pig. Mean Na, K, Mg-ATPase activity was 5·49 uMP_i/mg protein/10 min. The addition of diphenylhydantoin to the incubation medium reduced the mean activity to 4·49. Mean percentage inhibition caused by diphenylhydantoin was 18·7 per cent ($P = 0{\cdot}03$); with ouabain alone, 66 per cent; with both diphenylhydantoin and ouabain, 79·8 per cent (Table 2).

Human. In assays containing whole homogenates of human cortex, inhibition of Na, K, Mg-ATPase with diphenylhydantoin alone was 14·6 per cent, with ouabain alone, 79·4 per cent; and with both diphenylhydantoin and ouabain, 88·4 per cent (Table 3). Neither diphenylhydantoin nor ouabain had an effect on Mg-ATPase.

These results indicate that diphenylhydantoin inhibits Na, K, Mg-ATPase activity both in the absence and presence of ouabain under conditions *in vitro*. To produce this effect consistently it was necessary to expose the cerebral material to diphenylhydantoin for 10 min before incubating with ATP and electrolytes.

DISCUSSION

These experiments indicate that diphenylhydantoin significantly inhibits Na, K, Mg-ATPase activity in rat and guinea pig cerebral microsomes and in whole

TABLE 1. EFFECT OF DIPHENYLHYDANTOIN AND OUABAIN ON NA, K, MG-ATPASE ACTIVITY ON RAT MICROSOMES*

A Mg^{2+}-ATPase	B Total ATPase	B − A Na-K-Mg-ATPase	B − A +D(10^{-4} M)	Inhibition	B − A +Oub(10^{-4} M)	Inhibition	B − A +D + Oub	Inhibition
4·2	6·7	2·5	1·55	0·95 (38%)	0·45	2·05 (82%)	0·1	2·4 (96%)
4·2	6·25	2·05	1·55	0·5 (24·5%)	0·9	1·15 (57%)	0·65	1·4 (70%)
4·05	6·9	2·85	2·7	0·15 (55%)	0·5	2·35 (83%)	0·15	2·7 (95%)
4·0	7·1	3·1	2·6	0·5 (16%)	0·95	2·15 (70%)	0·65	2·45 (80%)
4·1	7·2	3·1	2·95	0·15 (5%)	0·7	2·4 (78%)	0·2	2·9 (93%)
3·5	6·25	2·75	2·2	0·55 (20%)	0·55	2·2 (80%)	0·2	2·55 (92%)
3·6	5·25	1·65	1·2	0·45 (25%)	0·2	1·55 (93%)	0	1·65 (100%)
3·6	5·8	2·2	1·75	0·45 (20%)	0·95	1·25 (58%)	0·4	1·8 (82%)
5·7	9·1	3·4	2·1	1·3 (38%)	1·2	2·2 (65%)	0·4	3·0 (88%)
4·9	9·8	4·9	3·8	1·1 (22·5%)	1·5	3·4 (70%)	0·3	3·5 (92%)
4·8	7·8	3·0	1·8	1·2 (39%)	1·2	2·8 (60%)	0·6	2·4 (80%)
Mean ± S.E.		2·8 ± 0·25	2·2 ± 0·24	Mean %		Mean %		Mean %
				23 ± 3·6		73 ± 3·2		96·8 ± 3·9

* Values are expressed as μMP_i/mg protein/10 min. D = diphenylhydantoin; Oub = oubain.

TABLE 2. EFFECT OF DIPHENYLHYDANTOIN AND OUABAIN ON NA, K, MG-ATPASE ACTIVITY IN GUINEA PIG MICROSOMES*

A Mg^{2+}-ATPase	B Total ATPase	B − A Na-K-Mg-ATPase	B − A +D(10^{-4} M)	Inhibition	B − A +Oub(10^{-4} M)	Inhibition	B − A +D + Oub	Inhibition
2·68	8·3	5·62	4·72	0·9 (16%)	1·72	3·9 (59%)	1·22	4·4 (78%)
2·68	8·3	5·62	4·82	0·8 (14·5%)	1·72	3·9 (59%)	1·07	4·55 (81%)
2·68	8·0	5·32	4·12	1·2 (22%)	1·72	3·6 (68%)	1·22	4·1 (77%)
2·7	7·65	4·95	3·75	1·2 (24%)	1·6	3·35 (68%)	1·2	3·75 (76%)
2·52	8·45	5·93	4·93	1·0 (17%)	1·48	4·45 (75%)	0·88	5·05 (85%)
Mean ± S.E.		Mean	Mean	Mean %		Mean %		Mean %
		5·49 ± 0·04	4·47 ± 0·17	18·7 ± 1·8		66 ± 2·2		79·8 ± 1·45

* Values are expressed as μMP_i/mg protein/10 min. D = diphenylhydantoin; Oub = oubain.

TABLE 3. EFFECT OF DIPHENYLHYDANTOIN AND OUABAIN ON NA, K, MG-ATPASE IN WHOLE HOMOGENATES OF HUMAN FRONTAL CORTEX*

A Mg^{2+}-ATPase	B Total ATPase	B − A Na-K-Mg-ATPase	B − A +D(10^{-4} M)	Inhibition	B − A +Oub(10^{-4} M)	Inhibition	B − A +D + Oub	Inhibition
0·87	3·1	2·23	1·93	0·3 (13·5%)	0·53	1·7 (77%)	0·33	1·9 (85%)
1·1	3·6	2·5	2·2	0·3 (12%)	0·6	1·9 (76%)	0·4	2·1 (85%)
0·96	3·3	2·34	2·04	0·3 (13%)	0·34	2·0 (85%)	0·19	2·15 (92%)
0·9	3·2	2·3	1·8	0·5 (21·5%)	0·5	1·8 (78%)	0·2	2·1 (91%)
1·0	3·3	2·3	2·0	0·3 (13%)	0·4	1·9 (81%)	0·25)	2·05 (89%)
Mean ± S.E.		Mean	Mean	Mean %		Mean %		Mean %
		2·33 ± 0·04	1·99 ± 0·095	14·6 ± 1·55		79·4 ± 1·6		88·4 ± 0·8

* Values are expressed as μMP_i/mg protein/10 min. D = diphenylhydantoin; Oub = oubain.

homogenates of human cortex. The result is not what one might have predicted on the basis of previous work,[3] which indicated that diphenylhydantoin causes extrusion of sodium from brain cells and prevents cerebral intracellular increase of sodium in hyponatraemic animals and in animals given maximal electroshock seizures. The anticonvulsant action of diphenylhydantoin and its general "membrane-stabilizing" properties are thought to be closely related to the augmentation of this active extrusion of sodium from brain cells.[11] After the demonstration of the Na, K, Mg-activated ATPase enzyme systems by Skou,[12] considerable evidence has accumulated to support the concept that the enzyme is intimately involved in the active transport of electrolytes across biological membranes. It has been suggested that diphenylhydantoin acts on this metabolic sodium pump, although other mechanisms are conceivable.

The inhibition of Na, K, Mg-ATPase produced by diphenylhydantoin seems to be a unique effect among neurologically active drugs. No drug has been shown to stimulate the activity of the enzyme in non-lethal doses, and in fact this enzyme system has been shown to be unaffected by many neurophysiologically active agents, including convulsants, anticonvulsants, sedatives, tranquilizers, and hallucinogens.[5] Ethanol and diuretic agents inhibit the enzyme.[13–16] Experiments in this laboratory with phenobarbital (10^{-4} M) indicated that it has no effect whatever on Na, K, Mg-ATPase activity. Since the use of 100 mM sodium in our earlier assays maximally stimulated the enzyme,[8] it might be unreasonable to expect further stimulation to be achieved by the addition of diphenylhydantoin. Accordingly, the assays were repeated with 15 mM sodium. This concentration of sodium has approximately half the maximal stimulating effect. In this circumstance diphenylhydantoin still produced a similar inhibition of the enzyme.

The inhibiting effects of ouabain and diphenylhydantoin on the enzyme are additive, but rarely reach 100 per cent inhibition. It is not clear how this observation relates to the improvement produced by diphenylhydantoin in digitalis-induced dysrhythmias in dogs and man,[11, 17] although the sodium-extruding properties of diphenylhydantoin have been invoked for this effect.

On the basis of these experiments, the sodium-extruding properties of diphenylhydantoin would appear to be due to some mechanism other than stimulation of the Na, K, Mg-activated ATPase system. This may indicate that Na, K, Mg-ATPase is not the only active sodium pump. An alternative explanation is that diphenylhydantoin interferes with the passive movement of sodium ions along its concentration gradient into the cells, without augmenting the process of active extrusion of sodium from the cell. According to this theory, if diphenylhydantoin were active enough in blocking the entry of sodium ions into the cell, even though it had a mildly inhibiting effect on the sodium pump, the net effect would be to lower the intracellular sodium concentration.

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