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The *in vivo* and *in vitro* effect of diphenylhydantoin and phenobarbitone on K^+-activated phosphohydrolase and (Na^+, K^+)-activated ATPase in particulate membrane fractions from rat brain

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The effect of diphenylhydantoin and phenobarbitone on a K^+-activated phosphohydrolase, hydrolysing the artificial substrates *p*-nitrophenylphosphate and acetylphosphate, and (Na^+,K^+)-activated ATPase in particulate membrane fractions from rat brain, has been studied. Diphenylhydantoin was given orally over 20 weeks without any effect on these enzymic activities, whereas orally phenobarbitone significantly decreased the enzymic activities in the particulate membrane fractions containing synaptosomes, nerve endings and microsomes. *In vitro*, diphenylhydantoin inhibited the enzymic activities in the synaptosomal membrane fraction, but phenobarbitone did not. Several possibilities for the *in vivo* action of diphenylhydantoin are outlined; these are mainly concerned with a blocking of the passive movement of sodium into the cell. The *in vivo* effect of phenobarbitone is possibly a secondary pharmacological effect interfering with the utilization of ATP either directly or indirectly by a depression of some energy dependent processes such as protein synthesis.

5,5′-Diphenylhydantoin exerts anti-epileptic activity (Toman, 1952), stabilizes membrane-potentials (Esplin, 1957) and lowers the intracellular concentration of sodium of neural tissue (Woodbury, 1955; Pincus & Rawson, 1969). Since there is evidence that the Mg^{2+}-dependent (Na^+,K^+)-activated ATPase is the carrier mechanism involved in the active transport of sodium (Skou 1965; 1969), it seemed reasonable to suppose that diphenylhydantoin exerts its action on this multi-enzyme system. A previous study by Rawson & Pincus (1968), clearly showed diphenylhydantoin 10^{-4}M to inhibit (Na^+,K^+)-activated ATPase by approximately 20% and to increase the inhibiting effects of ouabain. Many neuropharmacologically active drugs do not affect the (Na^+,K^+)-activated ATPase (Pincus & Giarman, 1967) and it is not clear how the diphenylhydantoin inhibition can be related to the anticonvulsant action of this drug.

The aim of the present work has been to determine the *in vivo* effect of diphenylhydantoin, given by mouth to rats over 20 weeks, on (Na^+,K^+)-activated ATPase. Experiments were also made to determine the effect of phenobarbitone. The results *in vivo* were compared with *in vitro* experiments, which involved a preliminary exposure of a synaptosomal membrane fraction to the drugs.

Much evidence has been accumulated to support the concept, that the (Na^+,K^+)-activated ATPase reaction proceeds through two main steps: a Mg^{2+}-dependent Na^+-activated transphosphorylation during which the terminal phosphate group of

ATP is bound to the protein moiety of the enzyme, and a Mg^{2+}-dependent K^+-activated dephosphorylation (Fahn, Koval & Albers, 1966). Furthermore, experiments in this laboratory have indicated that step 2) also hydrolyses the artificial substrates acetylphosphate and *p*-nitrophenylphosphate (Formby & Clausen, 1968; 1969) during a Mg^{2+}-dependent and K^+-activated reaction. Since the inhibition of (Na^+,K^+)-activated ATPase by ouabain seems to affect the K^+-activated entity, i.e. step 2), and ouabain and diphenylhydantoin inhibiting effects on the enzyme are supposed to be additive (Rawson & Pincus, 1968), two enzymic activities were determined: (Na^+,K^+)-activated ATPase with ATP as substrate, and K^+-activated phosphohydrolase with *p*-nitrophenylphosphate and acetylphosphate as substrates.

EXPERIMENTAL

Materials and methods

Chemicals of highest commercial purity from Sigma (USA) and Merck (W. Germany) were used. Diphenylhydantoin and phenobarbitone were purchased from DAK (Denmark).

Animals. Male Wistar rats weighing initially 175–200 g, were used. Since diphenylhydantoin was given in the drinking water (pH adjusted to 9), initial experiments were made to determine for each rat the amount of water drunk daily at a constant temperature of 25° and its optimal diet. This was calculated as 91·3 ± 8·4 ml per kg body weight per 24 h and on this basis the drugs were added to the drinking water to give each rat 3–4 mg diphenylhydantoin or 2–3 mg of phenobarbitone every 24 h.

Determination of diphenylhydantoin and phenobarbitone. The method of Huisman (1966) was used for determination of the drugs in serum.

Isolation of subcellular membrane fractions. The method of Whittaker (1966) was used with slight modifications. Whole rat brains removed immediately after decapitation, were homogenized in ice-cold 0·32 M sucrose. The homogenate was diluted to 10% of the original weight (w/v) and centrifuged at 900 *g* (10 min) at 0°. The supernatant was centrifuged 18,000 × *g* (60 min) at 0° and the sediment from this centrifugation was suspended in 0·32 M sucrose and layered on a sucrose gradient containing the layers: 1·2M and 0·8M. The final ratios were 1 : 1 : 1. After centrifugation at 50 000 *g* (145 min) at 0°, the fractions A (myelin), B (synaptosomes) and C (mitochondria) were isolated. The supernatant from the 18 000 *g* centrifugation was recentrifuged at 100 000 *g* (40 min) at 0° yielding the microsomal fraction M.

Determination of protein. The method of Lowry, Rosebrough & others (1951) was used with bovine serum albumin as standard.

Preincubation procedures for in vitro *measurements.* The synaptosomal membrane fraction B from the brains of control rats was exposed to various concentrations of either drug at 37° for 60 min. Aliquots of 100 ml (0·2 mg of protein) were then added to incubation media, to which the same amount of drug had been added, and the enzyme activity assayed.

Assay of enzyme activities. K^+-Activated phosphohydrolase with *p*-nitrophenylphosphate as substrate was assayed as previously described (Formby, 1968). Briefly, the reaction mixture contained 100 μl of aqueous suspension of one of the fractions A, B, C or M (0·2 mg protein) and 300 μl medium (5·0 mM *p*-nitrophenylphosphate as Tris-salt, 5·0 mM $MgCl_2$, 7·5 mM KCl and 50 mM Tris-HCl buffer pH 7·4). After 3 min incubation at 37°, the amount of *p*-nitrophenolate was determined spectrophoto-

metrically at 400 nm. The enzyme activity was expressed as mU (= nmol/min)/mg protein.

K^+-Activated phosphohydrolase with acetylphosphate as substrate was assayed as previously described (Formby, 1968). The reaction mixture contained 100 μl of aqueous suspension of one of the fractions A, B, C or M (0·2 mg protein) and 1000 μl medium (5·0 mM acetylphosphate as Tris-salt, 5·0 mM $MgCl_2$, 7·5 mM KCl and 100 mM Tris-HCl buffer pH 7·4). After 5 min incubation at 37°, the amount of acetylphosphate was determined by the acetohydroxamate method (Israel & Titus, 1967). The enzyme activity was expressed as mU (= nmol/min)/mg protein. (Na^+,K^+) ATPase reaction mixture contained 100 μl aqueous membrane suspension (0·2 mg protein) and 500 μl medium (5·0 mM ATP as Tris-salt, 5·0 mM $MgCl_2$, 100 mM NaCl 20 mM KCl and 50 mM Tris-HCl buffer pH 7·4). After 3 min incubation at 37°, the amount of orthophosphate was determined (Fiske & Subbarow, 1925). The enzyme activity was expressed as mU (= nmol/min)/mg protein.

Besides specific activity, a relative specific activity (RSA) was used and defined as the ratio of the percentage of recovered enzyme activity to the percentage of the recovered protein of each fraction.

Statistical evaluation. Each value of enzyme activity represents the mean of two individual determinations. From the values of each experimental and control group mean and standard error (s.e.) of the mean were calculated (n = 5), as well as the significance $P \leqslant 0{\cdot}025$ was used.

Results

The serum concentrations of diphenylhydantoin and phenobarbitone were respectively 109 ± 29 and 68 + 21 μg/ml of serum. Neither drug was detected in control groups.

Table 1. *The effect of long-term medication of diphenylhydantoin* (DPH) *and phenobarbitone* (PB) *to rats on* K^+*-activated phosphohydrolase and* $(Na,^+K^+)$*-activated ATPase in particulate membrane fractions of the rat brain. Specific activities expressed as* mU (= nmol/min)/mg *protein.*

Enzyme		Particulate subfraction A Specific activity	RSA	B Specific activity	RSA	C Specific activity	RSA	M Specific activity	RSA*
K^+-phosphohydrolase with *p*-nitrophenylphosphate as substrate	DPH	136 ± 14	1·1	152 ± 18	1·3	111 ± 9	1·0	135 ± 15	—
	PB	141 ± 11	1·0	110 ± 9	0·7	101 ±:8	0·7	120 ± 11	—
	Control	141 ± 13	1·0	155 ± 9	0·9	125 ± 11	0·9	123 ± 10	—
K^+ phosphohydrolase with acetylphosphate as substrate	DPH	382 ± 15	1·0	535 ± 28	1·6	456 ± 44	1·2	487 ± 29	—
	PB	359 ± 14	0·9	397 ± 14	1·0	228 ±:32	0·6	378 ± 28	—
	Control	385 ± 21	1·0	521 ± 17	1·3	499 ± 35	1·3	507 ± 24	—
(Na^+,K^+)ATPase	DPH	559 ± 29	1·1	1198 ± 32	2·4	835 ± 23	1·7	1304 ± 46	—
	PB	565 ± 48	1·1	1019 ± 34	2·0	644 ± 32	1·3	1158 ± 32	—
	Control	604 ± 48	1·1	1241 ± 39	2·3	899 ± 22	1·6	1356 ± 48	—

* Relative specific activity: the ratio of % recovered enzyme activity to % recovered protein of each fraction.

In vivo *effect of diphenylhydantoin.* Table 1 shows the results of the enzymic assays with particulate fractions isolated from the experimental and control groups. Concerning the specific activities, only non-significant ($P = 0{\cdot}025$) differences were calculated between the diphenylhydantoin treated rats and the control. Also, the relative specific activities (RSA) are of the same order, except K^+-activated phosphohydrolase with *p*-nitrophenylphosphate as substrate in fraction B, which is increased when compared to the control.

In vivo *effect of phenobarbitone.* Table 1 shows that the specific activities of K^+-activated phosphohydrolase with acetylphosphate as substrate and (Na^+, K^+)-activated ATPase are significant decreased ($P = 0{\cdot}025$) in the particulate fractions B, C and M when compared to the controls; also decreased RSA-values were found.

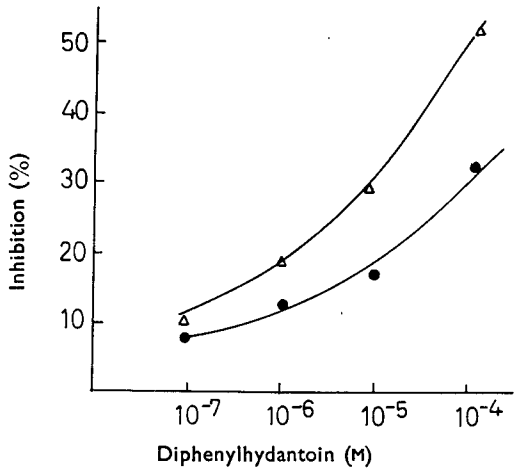

FIG. 1. The effect of pre-incubation with diphenylhydantoin on the specific activities of K^+-activated phosphohydrolase (with acetylphosphate as substrate) (△) and (Na^+,K^+)-activated ATPase (●). Synaptosomal membrane fraction B was preincubated at 37° for 1 h. The activity is expressed as % of the activity of the same enzyme preincubated for the same length of time, but in the absence of diphenylhydantoin.

In vitro *effect of diphenylhydantoin.* Fig. 1 shows the effect of various concentrations of diphenylhydantoin on the activities of K^+-activated phosphohydrolase (assayed with acetylphosphate as substrate) and (Na^+,K^+)-activated ATPase in the synaptosomal membrane fraction B. The synaptosomal membranes were pre-incubated at 37° for 60 min with the drug in various concentrations; membranes in pre-incubated for the same time but without the drug served as control. A half-maximal inhibition of K^+-activated phosphohydrolase was obtained with 10^{-4}M diphenylhydantoin. The same concentration inhibited (Na^+,K^+)-activated ATPase by about 30%. As already demonstrated by Rawson & Pincus (1968), ouabain acts as an additive inhibitor since a concentration of 10^{-5}M it increased the inhibition of K^+-activated phosphohydrolase by about 19% and that of (Na^+,K^+)-activated ATPase by about 26%.

In vitro *effect of phenobarbitone.* Corresponding experiments with phenobarbitone (10^{-4}M) indicated that it has no effect on K^+-activated phosphohydrolase and (Na^+, K^+)-activated ATPase.

DISCUSSION

Diphenylhydantoin, given orally for 20 weeks at 3–4 mg/24 h has no effect on K^+-activated phosphohydrolase or on (Na^+,K^+)-activated ATPase in any of the particulate fractions examined i.e. myelin fraction A, synaptosomal fraction B, mitochondrial fraction C and microsomal fraction M. However, in agreement with other findings (Rawson & Pincus, 1968), *in vitro* studies with this drug showed that pre-incubation of synaptosomal membranes at 37° for 1 h inhibited both K^+-activated phosphohydrolase and (Na^+,K^+)-activated ATPase. This inhibition was further increased by the

presence of ouabain, although the percentage increase was found to be significantly lower than if diphenylhydantoin was absent; this may indicate a competition between the two inhibitors (Conn 1965).

Although determination of diphenylhydantoin in brain tissue was not undertaken and possible effects of metabolites *in vivo* were ignored, it is obvious that the *in vivo* effect of diphenylhydantoin is different from that *in vitro*. One possibility is that the *in vitro* concentrations necessary to give inhibition are unphysiological and much higher than those existing *in vivo*. A second possibility is that there is a cation transport process, other than the (Na^+,K^+)-activated ATPase in neural tissue, upon which diphenylhydantoin acts. Such a "second pump" in nerves has been suggested by Rawson & Pincus (1968) and Pincus & Rawson (1969). A third possibility could be that diphenylhydantoin simply blocks the passive movement of sodium into the cell. Since diphenylhydantoin *in vivo* did not inhibit the (Na^+,K^+)-activated ATPase, the net result thus would be a decrease in intracellular sodium.

The *in vivo* effect of high doses of phenobarbitone is difficult to assess. Several lines of evidence suggest that phenobarbitone decreases the cerebral level of phosphorylated metabolites, leading to a reduced availability of ATP (Gey, Rutishauser & others, 1968; Kroner, Gutenberger & others, 1968). This suggests that phenobarbitone can interfere with the utilization of ATP in the brain, either directly or indirectly by depression of some energy-dependent processes like protein synthesis (Heald, 1960). Also this could explain the synergistic effect of phenobarbitone and diphenylhydantoin in the treatment of epilepsy, the former (for "petit-mal") indirectly inhibiting (Na^+, K^+)-activated ATPase and the latter (for "grand-mal") directly inhibiting the passive movement of sodium into the cell or a "second pump".

Finally, the difference in the *in vivo* and *in vitro* effects of these drugs must influence future design of experiments with anti-epileptic drugs.

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