Alloxan-diabetes alters kinetic properties of the membrane-bound form, but not of the soluble form, of acetylcholinesterase in rat brain

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We examined the effects of alloxan-diabetes on the kinetic properties of the soluble and the membrane-bound forms of acetylcholinesterase (AChE) in rat brain. The K_m (0.15 mM) and V_{max} (1.5 mmol/min per mg of protein) of the soluble form of the enzyme were unchanged in the diabetic animals. The membrane-bound enzyme in the control group displayed a lower K_m (0.09 mM) and a higher V_{max} (7.2 mmol/min per mg of protein)

compared with the soluble form of the enzyme; the diabetic state caused a significant increase (40%) in both K_m and V_{max} . K_i^s values were about 3–4 times higher for the membrane-bound enzyme in both control and diabetic animals. The results suggest that membrane binding and membrane alterations in diabetes can significantly influence the kinetic properties of AChE.

INTRODUCTION

It is well recognized that the disease diabetes mellitus results in altered membrane functions in several tissues [1–3]. Membrane alterations has been recognized as the underlying primary biochemical defect [1]. In the brain, the diabetic state causes a 40% decrease in transport of choline across the blood/brain barrier [4], and decreased synthesis and release of acetylcholine (ACh) in specific brain regions [5]. Additionally, 32 and 41% decreases respectively in the axonal transport of acetylcholinesterase (AChE) and choline acetyltransferase in cholinergic neurons have been demonstrated. The diabetic subjects experience loss of short-term memory and difficulties in concentration [6–8]; the cognitive deficits and memory dysfunctions are associated with cholinergic hypoactivity [9,10]. Besides, decreased/delayed nerve transmission in the peripheral nervous system in the diabetic state has been demonstrated [11,12].

The enzyme AChE indirectly plays an important role in transmission of nerve impulse. It hydrolyses the ACh released at the cholinergic synapse and thus terminates the action of this neurotransmitter. In view of this, it is important to find out whether the diabetic state indeed influences the properties of this enzyme in the brain and thereby impairs the rate of nerve impulse transmission and associated memory and cognitive functions [9–12].

We have therefore carried out experiments to examine the effects of alloxan-diabetes on the kinetic properties of AChE in rat brain. Our results indicate that the kinetic properties of the membrane-bound form, but not of the soluble form, of the enzyme were significantly altered by alloxan-diabetes.

MATERIALS AND METHODS

Chemicals

Alloxan was purchased from Spectrochem, India; 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and acetylthiocholine iodide (ACTI) were from SRL, India. Ethopropazine hydrochloride (ETPZ.HCl) was purchased from Sigma Chemical Co., U.S.A. All other chemicals were of analytical-reagent grade, purchased locally.

Animals

Male albino rats of Charles-Foster strain (8–10 weeks old; 200–250 g body wt.) were fasted overnight and were injected with alloxan intraperitoneally at a dose of 12 mg/100 g body wt. [13]. Alloxan solutions were prepared fresh by dissolving 120 mg of alloxan in 1 ml of 0.9% NaCl. The controls were injected with equivalent volumes of 0.9% NaCl solution. The diabetic state of the animals was ascertained by measuring the urinary and blood sugar levels [13,14]. The animals were killed by decapitation 30 days after alloxan or saline treatment. The brain was quickly removed and placed in a beaker containing chilled (0–4 °C) 38 mM Tris/HCl buffer, pH 8.5. The tissue was freed from adhering blood by repeatedly washing with the buffer, and finally 10% (w/v) homogenates were prepared with a Potter-Elvehjem-type glass-Teflon homogenizer.

Preparation of soluble and membrane-bound AChE

The procedure for isolation of soluble and membrane-bound forms of AChE was essentially as described by Bisso et al. [15]. Briefly, the brain homogenates were centrifuged at 100 000 *g* for 1 h in a Sorvall OTD-Combi ultracentrifuge at 0–4 °C. The supernatant was carefully decanted and was used as a source of soluble enzyme. The pellet was resuspended by homogenization in the same volume of 38 mM Tris/HCl, pH 8.5, containing 0.25% Triton X-100. After a further centrifugation at 100 000 *g* for 1 h, the second supernatant was collected. This was used as the source of membrane-bound enzyme.

Assay of AChE activity

This was measured by the method described by Ellman et al. [16]. The assay system contained, in a final volume of 1.0 ml: 100 mM phosphate buffer, pH 8.0, 0.32 mM DTNB, 0.1 mM ETPZ.HCl and various concentrations of ACTI in the range 0.05–2.0 mM. The enzyme (10–15 µg of protein) was preincubated with the assay medium for 15 min at 37 °C before addition of the substrate. The reaction was started by addition of the substrate, and the increase in A_{412} was recorded in a Shimadzu UV-160A spectrophotometer at 37 °C at intervals of 2–5 s.

Abbreviation used: AChE, acetylcholinesterase.

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Kinetic analyses

For determination of K_m and V_{max} values, the data were subjected to both Lineweaver–Burk and Eadie–Hofstee analyses [17], and K_i for substrate (K_i^s) was calculated from the Murray plot [17]. Protein was determined by the method of Lowry et al. [18].

RESULTS

The diabetic rats lost about 50 % of their body weight; the brain weight decreased by only 15 %. Consequently, the relative brain weight seemed to have increased (+80 %), due to the disproportionately greater decrease in the body weight. The diabetic rats also exhibited polyuria (13-fold increase in urine volume), glucosuria (89 mg of glucose/ml; daily excretion 5.4 g). Their blood sugar levels increased by over 4-fold (Table 1). These

Table 1 Parameters to ascertain diabetic state

The animals were fasted overnight and then injected with alloxan (12 mg/100 g body wt.) intraperitoneally as described in the text. The controls received only the saline vehicle. The animals were killed 30 days after alloxan/saline injection, for further studies. Results are given as means $\pm$ S.E.M. of the numbers of observations indicated in parentheses: * $P < 0.001$ compared with control.

Parameter	Control (10)	Diabetic (6)
Body wt. (g)	339.2 $\pm$ 4.10	163.0 $\pm$ 10.80*
Brain wt. (g)	1.9 $\pm$ 0.01	1.6 $\pm$ 0.03*
Brain wt. as % of body wt.	0.6 $\pm$ 0.01	1.0 $\pm$ 0.06*
Blood sugar (mM)	7.7 $\pm$ 0.22	38.1 $\pm$ 1.56*
Urine volume (ml)	4.7 $\pm$ 0.59	62.3 $\pm$ 8.80*
Urine sugar (mg/ml)	—	88.9 $\pm$ 3.52*
(g/24 h)	—	5.4 $\pm$ 0.62*

Figure 1 Eadie–Hofstee plots for the soluble and the membrane-bound cerebral AChE from control and diabetic animals

AChE activity was determined spectrophotometrically [16] with substrate concentrations in the range 0.05–2.0 mM. Experiments were carried out on enzyme preparations from individual rat brains as detailed in Table 2. The intercept on the ordinate represents V_{max} , and the slope gives the value of $-K_m$ [17]. The plots are typical of 10 or 6 independent observations in the control and diabetic groups respectively. Units: v , mmol/min per mg of protein; $[S]$, mM.

Table 2 Effect of alloxan-diabetes on kinetic properties of soluble and membrane-bound AChE

Soluble and membrane-bound forms of AChE were prepared from individual brains by the procedure of Bisso et al [15]. Kinetic measurements were carried out on the individual preparations of soluble and membrane-bound enzyme. The data were analysed by Lineweaver–Burk and Eadie–Hofstee plots [17] and the values of K_m and V_{max} were averaged. The results are given as means $\pm$ S.E.M. of the numbers of independent observations indicated in parentheses (the ranges of K_m and V_{max} are indicated in brackets): * $P < 0.01$ and ** $P < 0.001$ compared with control. Units: K_m , mM; V_{max} , mmol/min per mg of protein.

Group	Soluble		Membrane-bound	
	K_m	V_{max}	K_m	V_{max}
Control (10)	0.15 $\pm$ 0.01 [0.12–0.18]	1.5 $\pm$ 0.05 [1.1–1.9]	0.09 $\pm$ 0.01 [0.08–0.10]	7.2 $\pm$ 0.74 [6.1–8.1]
Diabetic (6)	0.15 $\pm$ 0.02 [0.12–0.19]	1.5 $\pm$ 0.10 [1.2–1.8]	0.13 $\pm$ 0.01** [0.12–0.15]	10.3 $\pm$ 0.71* [9.8–13.0]

Figure 2 Murray plots for the soluble and the membrane-bound AChE from control and diabetic animals

The experimental details are described in the text. Plots are typical of 3 independent experiments for each group. Units of v as for Figure 1.

parameters are in general agreement with those reported by others [19–21] and noted by us previously [13,14].

The soluble and the membrane-bound enzyme from both control and diabetic rats displayed typical substrate-saturation curves; as expected, higher concentrations of substrate were inhibitory (results not shown). The ascending portions of the substrate-saturation curves were used to obtain the Lineweaver–Burk and Eadie–Hofstee plots. Typical Eadie–Hofstee plots for the soluble and the membrane-bound enzyme are shown in Figure 1. The values of K_m and V_{max} obtained from the two plots were in excellent agreement; these were averaged, and the results are given in Table 2. The soluble AChE in the controls was characterized by a K_m of 0.15 mM and a V_{max} of 1.5 mmol/min per mg of protein; the diabetic state did not significantly influence either of the enzyme parameters. The data in Table 2 also show that the membrane-bound enzyme in the controls had low K_m (0.09 mM) and high V_{max} (7.2 mmol/min per mg of protein) compared with its soluble counterpart. The diabetic state resulted in about 40 % increase in both K_m and V_{max} .

Figures 2(a) and 2(b) show the Murray plots for the soluble and membrane-bound enzymes respectively. It is clear that K_i^s was $(3\text{--}5) \times 10^{-4}$ M for the soluble enzyme, and about $(1.5\text{--}$

1.8×10^{-3} M for the membrane-bound enzyme. Interestingly, the diabetic state did not seem to influence this parameter.

DISCUSSION

The kinetic properties of AChE from electric organs of electric eel and *Torpedo* have been reported in the literature [22,23]. However, it has to be recognized that this enzyme plays a highly specialized role in the electric organ, and hence its properties have no direct bearing on the cerebral enzyme; the cerebral enzyme, on the other hand, plays an indirect but important role in neurotransmission. The results of our present studies have shown that, even in control animals, the kinetic properties of soluble and the membrane-bound forms of cerebral AChE are different, i.e. the membrane-bound enzyme exhibits low K_m and high V_{max} . As far as we are aware, this information for the enzyme from mammalian brain has not been available thus far.

In the brain, AChE is present predominantly in the membrane-bound form; the soluble enzyme makes up only about 15% of the total activity [15]. Obviously, therefore, it is the membrane-bound form of AChE which is physiologically important. Our data on K_m and V_{max} (Table 2) would also substantiate this assumption. In this connection, it is noteworthy that in the brain the membrane-bound enzyme exists in the G4 form, whereas the soluble enzyme comprises a 1:1 mixture of G1 and G4 forms [15]. Inasmuch as the membrane-bound enzyme exhibited strikingly different kinetic properties compared with its soluble counterpart, it may be inferred that it is the membrane binding which plays a crucial role in deciding kinetic characteristics of the enzyme. In other words, G4, when not membrane-bound, displayed properties similar to those of G1.

Our results have shown that the K_m and V_{max} of the membrane-bound AChE, but not the soluble AChE, increased in the diabetic state (Table 2). This may possibly relate to the reported delayed nerve transmission and impaired brain functions [9–12].

AChE is known to be inhibited by greater than saturating concentrations of the substrate, due to the binding of the substrate at a site other than the active site [17]. We found that the K_i s for the membrane-bound enzyme was 3–4 times higher (Figure 2); the diabetic state did not influence this pattern. These observations would thus suggest that substrate inhibition of AChE is also a membrane-dependent phenomenon. To summarize, then, the diabetic state clearly influenced the kinetic properties of the membrane-bound enzyme, but had no effect on substrate binding at higher concentrations.

Since the diabetic state is known to cause membrane alterations [1], it is possible that the changes that we observe here could be attributed to this factor. Noteworthy in this connection is our previous observation that in alloxan-diabetic rats the total membrane-bound Na^+, K^+ -ATPase activity in the brain decreased by 60%, with a 7-fold increase in K_m (ATP) and a 50% decrease in V_{max} . Simultaneously, IC_{50} for ouabain increased by 3 orders of magnitude in the diabetic state. Additionally, we also found

that the membrane glycosylation increased by 2-fold [13]. The last two observations are suggestive of membrane alterations in the diabetic state. Considering these observations together with those of the present studies, it may be inferred that the membrane alterations can significantly affect the kinetic properties of the membrane-bound enzymes. Alterations in the membrane lipid composition could be another possibility. However, Mooradian et al. [24] were not able to demonstrate any significant change in lipid composition of synaptic membranes in experimental diabetes.

Decreased V_{max} without alteration in K_m for erythrocyte AChE from diabetic patients has been reported [25]. The pseudo-cholinesterase activity in the plasma of alloxan-diabetic rats increased significantly, with a concomitant increase in the activities in the liver and adipose tissue [26]. However, the physiological significance of these observations remains unclear, since they are not related to neurotransmission.

REFERENCES

- Alberti, K. G. M. M. and Press, C. M. (1982) in *Complications of Diabetes* (Keen, M. and Jarrett, J., eds.), pp. 231–270, Edward Arnold, London
- Osterby, R. (1988) in *The Kidney and Hypertension in Diabetes Mellitus* (Morgensen, C. E., ed.), pp. 99–105, Martinus Nijhoff Publishing, Boston
- Striker, G. E., Peten, E. P., Carome, M. A., Pesce, C. M., Yang, C.-N., Elliot, S. J. and Striker, J. J. (1993) *Diabetes/Metab. Rev.* **9**, 37–56
- Mooradian, A. D. (1987) *Diabetes* **36**, 1094–1097
- Welsh, B. and Wecker, L. (1991) *Neurochem. Res.* **16**, 453–460
- Perlmutter, L. C., Hakami, M. K., Hodgson, H. C., Ginsberg, J., Katz, J., Singer, D. E. and Nathan, D. M. (1984) *Am. J. Med.* **77**, 1043–1048
- Surridge, D. H. C., Williams, E. D. L., Lawson, J. S., Donald, M. N., Monga, T. N., Bird, C. E. and Letemendia, F. J. J. (1984) *Br. J. Psychiatry* **145**, 269–276
- Ryan, C., Vega, A. and Drash, A. (1985) *Pediatrics* **75**, 921–927
- Bartus, R. T., Dean, R. L., III, Beer, B. and Lippa, A. S. (1982) *Science* **217**, 408–417
- Davis, K. L., Mohs, R. C., Rosen, W. G., Greenwald, B. S., Levy, M. I. and Horvath, J. B. (1983) *N. Engl. J. Med.* **308**, 721–723
- Clements, R. S. (1979) *Diabetes* **28**, 604–611
- Carrington, A. L., Ettlinger, C. B., Calcutt, N. A. and Tomlinson, D. R. (1991) *Diabetologia* **34**, 397–401
- Kumthekar, M. M. and Katyare, S. S. (1992) *Ind. J. Exp. Biol.* **30**, 26–32
- Nerurkar, M. A., Satav, J. G. and Katyare, S. S. (1988) *Diabetologia* **31**, 119–122
- Bisso, Q. M., Briancesco, R. and Michalek, H. (1991) *Neurochem. Res.* **16**, 571–575
- Ellman, G. L., Courtney, K. D., Andres, V., Jr. and Featherstone, R. M. (1961) *Biochem. Pharmacol.* **7**, 88–95
- Dixon, M. and Webb, E. C. (1979) *Enzymes*, pp. 47–206, Longman, London
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Hough, S., Russel, J. E., Teitelbaum, S. L. and Avioli, L. V. (1982) *Am. J. Physiol.* **242**, 451–456
- Bahnak, R. B. and Gold, H. A. (1982) *J. Biol. Chem.* **257**, 8775–8780
- Park, C. and Drake, R. L. (1982) *Biochem. J.* **208**, 333–337
- Lenz, D. E., Maxwell, D. M. and Walden, M. B. (1984) *Life Sci.* **34**, 219–224
- Bakry, N. M., Eldefrawi, A. T., Eldefrawi, M. E. and Riker, W. F., Jr. (1982) *Mol. Pharmacol.* **22**, 63–71
- Mooradian, A. D., Dickerson, F. and Smith, T. L. (1990) *Neurochem. Res.* **15**, 981–985
- Suhail, M. and Rizvi, S. I. (1989) *Biochem. J.* **259**, 897–899
- Oreskovic, K. and Kunec-Vajic, E. (1992) *Res. Commun. Chem. Pathol. Pharmacol.* **78**, 117–120