Vol. 119, No. 2, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1984 Pages 516-523

EFFECTS OF AMMONIA ON SYNAPTOSOMAL MEMBRANES

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Received December 7, 1983

Acute and sustained hyperammonemia in mice resulted in a decrease of the transition temperature of Arrhenium plots of synaptosomal (Na^+-K^+)ATPase. The activation energies in both phases of the plots were increased. "In vitro" addition of ammonia produced similar changes. This seems to indicate that ammonia alters the physical properties of synaptosomal membranes. The "in vitro" interaction of ammonia and ethanol at the membrane level was also investigated. Both agents together produced a further shift in the transition temperature and affected the activation energies. The relevance of these findings regarding the mechanism of ammonia toxicity and the protective effect of ethanol thereon is discussed.

High levels of ammonia in blood and brain due to metabolic disorders or to experimentally induced ammonia intoxication are associated with neurological abnormalities in humans and in animals (1,2). The mechanism of ammonia toxicity at the CNS level is still unknown; alterations in brain energy metabolism have been reported (3,4) and direct effects of ammonia on the transmission of the nervous impulse have also been proposed (5-8).

Recently, we have found that ethanol had a protective effect on acute (9,10) and chronic (unpublished) ammonia intoxication in mice. Other alcohols of different chain length, including tert-butanol (11) also had an excellent protective effect. The hypnotic action of alcohols has been related to their solubility in the lipid fraction of the neural cell membrane (12). Since these agents clearly improved some of the neurological manifestations of acute ammonia intoxication, their effect on the membrane might be important. To clarify this we have studied

0006-291X/84 $1.50

the possible changes in the physical properties of synaptosomal membranes induced by "in vivo" and "in vitro" exposure to ammonia and ethanol. The implication of this finding relative to the mechanism of ammonia toxicity and its prevention, as well as the effect of ethanol are discussed.

MATERIALS AND METHODS

All the chemicals were of the highest purity available. Urease was from Sigma Chemical Co. Glutamate dehydrogenase was from Boehringer Mannheim.

Male Swiss albino mice weighing 25 to 30 grams were used. Acute ammonia intoxication was established in one group by a single i.p. injection of 12 mmoles of ammonium acetate/kg body weight (as a 0.6% w/v solution). Since death occurs in about 15 min, they were sacrificed 10 min after the injection. For chronic toxicity, urease (5 units/ml of saline) was given to another group of mice in an i.p. injection of 33 units/kg body weight daily up to five days. The animals were sacrificed 6 h after the last dose.

For ammonia determination, blood samples were drawn from the tail vein at designated times, precipitated with 10% trichloroacetic acid and centrifuged. Ammonia was measured in the supernatants by a glutamate dehydrogenase assay (13). At the time of sacrifice, brains of mice were homogenized in cold 0.25 M sucrose, precipitated with 10% trichloroacetic acid and centrifuged. Ammonia was measured in the supernatants as described above.

For synaptosome preparation, normal and ammonia intoxicated mice were sacrificed as indicated and their brains quickly removed. The cerebellum and brain stem were discarded and a pool of the remaining areas of ten brains was weighed and homogenized in five volumes of ice-cold 0.32 M sucrose. Synaptosomes were prepared according to the method of Cotman (14). The synaptosome fraction, recovered from the interphase of the 6 and 13% Ficoll solutions, was diluted four-fold with 0.05 M Tris-HCl pH 7.0. Synaptosomes were sedimented by centrifugation at 33000 g for 35 min and resuspended in the same buffer. This suspension was frozen (-40°C) overnight, thawed and used for the (Na^+K^+) ATPase activity assay (15).

Arrhenius plots were calculated from (Na^+K^+) ATPase activities at different temperatures, as previously described (15). Specific activity values were plotted against 1/T. Protein was determined by a biuret method (16) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Fig. 1 shows the levels of ammonia in the blood and brain of mice acutely intoxicated with ammonium acetate. Before death all the animals exhibited clear symptoms of ammonia toxicity, progressing from hyperexcitability and drowsiness to deep coma, associated with clonic and tonic convulsions. The levels of am-

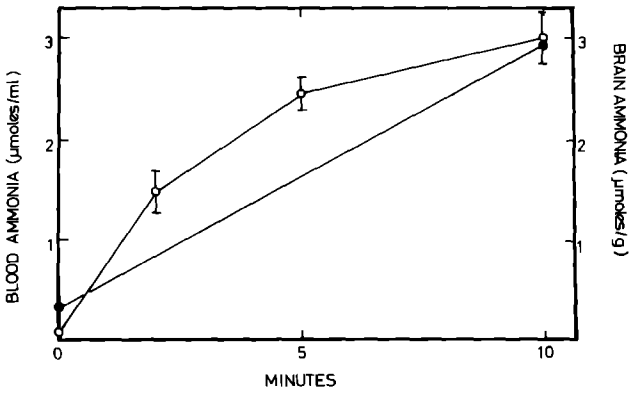

Fig. 1. Levels of ammonia in the blood and brain of mice injected with ammonium acetate. The animals received 12 mmoles ammonium acetate/kg body weight by intraperitoneal injection. Blood (o) and brain (●) ammonia were determined as indicated in Methods. Results are the mean ± S.D. of four animals.

monia in mice injected with urease are illustrated in Fig. 2. As can be seen, blood ammonia rose sharply after the injection, decreasing gradually until the levels were normal twenty-four hours later. The levels of brain ammonia were lower than those in blood, but its disappearance was slower and levels remained significantly increased after twenty-four hours. When a second injection of urease was administered to the mice, ammonia again

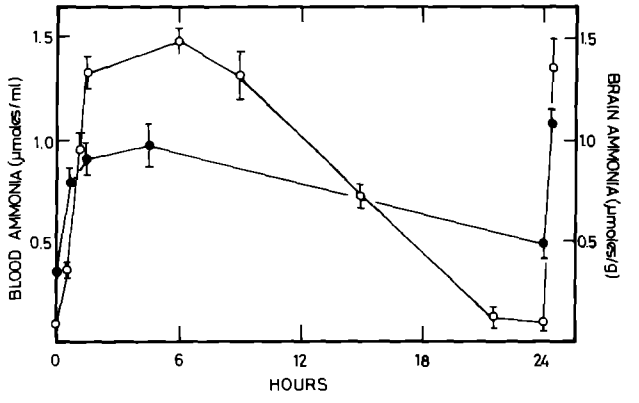

Fig. 2. Levels of ammonia in the blood and brain of mice injected with urease. The animals received 33 units of urease/kg body weight in a daily intraperitoneal injection, as indicated in Methods. Blood (o) and brain (●) ammonia were determined as indicated. Since blood ammonia variations were essentially the same after every injection of urease, the figure only reflects the time course in the early hours of the experimental period. Results are the mean ± S.D. of six animals.

rose rapidly to the same levels as on the first day. Although not shown in the figure, this occurred after each injection of urease. Thus, this treatment produced a sustained hyperammonemia with concomitantly increased brain ammonia. In spite of this, the animals showed no appreciable symptoms of ammonia toxicity and appeared normal during the experimental period.

Many exogenous agents induce changes in lipid fluidity in membranes which are evidenced by shifts in the discontinuities of Arrhenius plots (18,19). Discontinuities in the Arrhenius plots of membrane-bound enzymes could be related to phase changes in membrane lipids, which are specific for each enzyme at given conditions (17). (Na^+-K^+)ATPase is a membrane-bound enzyme whose activity has been shown to be altered by changes in membrane characteristics (17,19). In order to investigate the effect of ammonia on neural membranes, we tested the (Na^+-K^+) ATPase activity of synaptosomes prepared from animals submitted to acute and sustained hyperammonemia.

Fig. 3 shows the effect of ammonia on the Arrhenius plots of (Na^+-K^+)ATPase activity. In synaptosomes from normal mice, a biphasic plot was seen, with a discontinuity appearing at 23.7° C. A shift of that discontinuity towards lower temperatures was seen in both acute and chronic exposure to ammonia, being greater in the synaptosomes from urease-treated mice, but the specific activity remained similar in all cases. "In vitro" addition of ammonia induced similar changes. When (Na^+-K^+)-ATPase of control synaptosomes was assayed in the presence of 50 mM ammonium chloride, the break in the diagram was shifted to 17.3°C. Also, the specific enzyme activity was markedly reduced.

From these results it seems clear that ammonia, both "in vivo" and "in vitro", interferes with synaptosomal membranes,

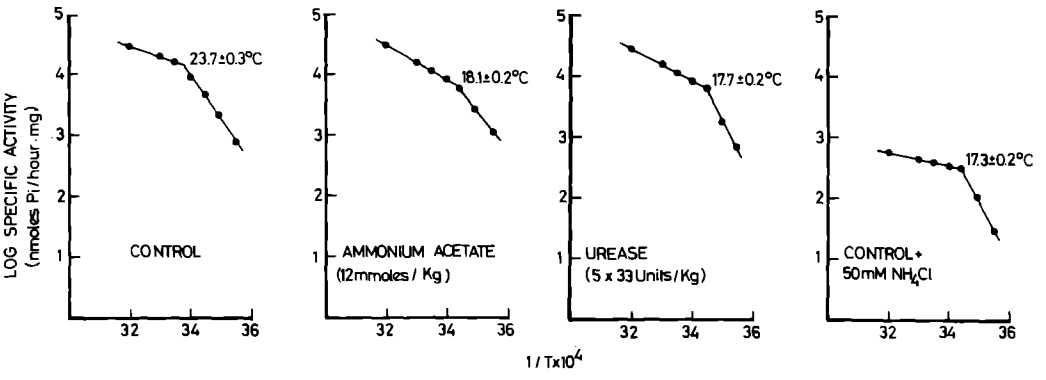

Fig. 3. "In vivo" and "in vitro" effects of ammonia on the Arrhenius plots of (Na^+-K^+)ATPase activity in mouse synaptosomes. The mice received ammonium acetate or urease as described in Methods. Synaptosomal (Na^+-K^+)ATPase activity was assayed as indicated. "In vitro" effect of ammonia was determined by asaying control brain synaptosomal (Na^+-K^+)ATPase in the presence of NH_4Cl. Arrhenius plots were drawn from the values of enzyme V max at temperatures from 10-40°C. The transition temperatures for each plot are indicated. The values are the mean ± S.D. of 4-6 different values from 2-3 preparations, i.e., at least twenty mice.

altering their physical properties. Since the break is shifted towards lower temperatures, the change could be interpreted as an increase in lipid fluidity. This increase in disorder can be translated as conformational changes in membrane-bound functional proteins (17).

Some authors have related the convulsant properties of ammonia to a direct effect on the mechanisms regulating neuronal excitability. In 1970, Lux et al. found that ammonia inhibited the postsynaptic hyperpolarizing inhibition in cat spinal motoneurones (6), making it ineffective for the suppression of neuronal excitation. This phenomenon has since been observed in a number of vertebrates and invertebrates (7,20-22), including, more recently, the motor cortex of the cat "in vivo" (23). It has been shown to depend on an ammonia-induced inhibition of the mechanism of chloride ion extrusion (24). This might result from the disordering effect of ammonia on membranes since conformational changes in the chloride pump, due to variations in

the state of the lipid phase of the neurone membrane, might cause a loss in its efficiency.

Some effects of long-term exposure to ammonia could be related to its disordering action on brain membranes. There is increasing evidence pointing to alterations of blood-brain barrier permeability following portacaval shunting, a condition leading to hyperammonemia; increased permeability to different dyes and ferric oxysaccharides (25), horseradish peroxidase (26) and neutral amino acids (27) has been reported. Hepatectomized rats dying in coma showed an increased permeability of the blood-brain barrier to insulin, sucrose and trypan blue (28). Changes in the glial compartment have also been described in the brain of animals with urease-induced hyperammonemia which consist in an alteration of glial cells nuclei accompanied by an inhibition of astrocyte mitosis (29). Since there are compounds which affect both cell membranes and microtubuli (30), it is possible that ammonia could play a role in these processes.

Regarding the relationship between the protective effect of ethanol and other alcohols on ammonia intoxication (9-11) and their action on membranes (12), an opposing action of these compounds at this level is not clearly suggestd by our results. In fact, since ammonia increases membrane fluidity in the same way as alcohols do (17), an additive effect might be expected with both. Fig. 4 shows the "in vitro" effect of ammonia plus ethanol on the Arrhenius plots of synaptosomal (Na^+-K^+)ATPase. As can be seen, ethanol at low doses had no effect on the transition temperature, but at high doses induced a further shift to lower temperatures. Therefore, it may be assumed from these data that the acute cytotoxicity of ammonia is not due to a primary effect on membrane fluidity.

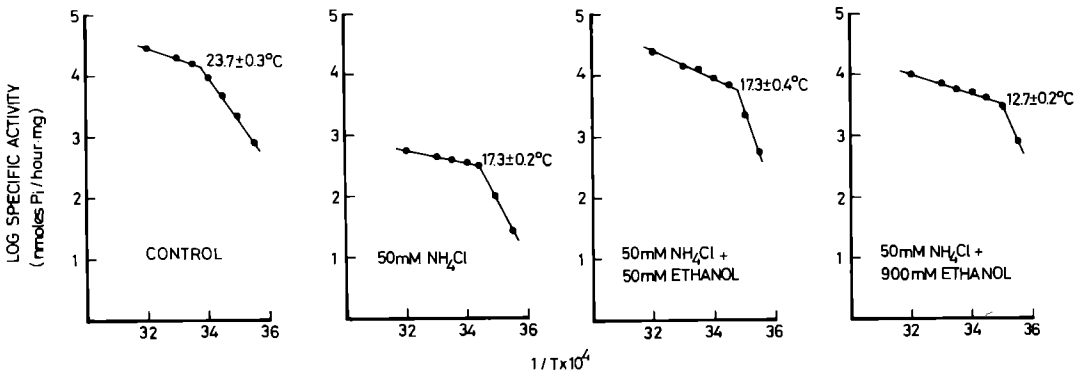

Fig. 4. "In vitro" effects of ammonia and ethanol on the Arrhenius plots of (Na^+-K^+)ATPase activity in mouse synaptosomes. Synaptosomal (Na^+-K^+)ATPase from control brains was assayed in the presence of the stated additions. Experimental conditions are as described in legend to Fig. 3. The results are the mean ± S.D. of 4-6 different values of 2-3 preparations.

At this stage, very little can be said about the nature of the interaction of ammonia with synaptosomal membranes. Ammonia may exist in two equilibrium forms, anionic (NH_3) and ionic (NH^+_4). The first of them is a lipophilic gas, readily diffusible through biological membranes. If it is responsible for the action on membranes, it is likely that it is a gaseous anesthetic-like effect. Ammonium ion might interact with the hydrophilic moiety of the membrane, disrupting the molecular forces that rule lipid-lipid or lipid-protein interactions at this level, thereby increasing disorder and mobility. Also, ammonium ion is highly basic in nature. As the plasmic membrane is quite rich in acidic functions (sialo glycoproteins, phospholipids, etc.) the neutralization of them by $NH4^+$ may therefore induce *per se* notable membrane alterations.

ACKNOWLEDGEMENTS

This work has been supported in part by grants from the Fondo de Investigaciones Sanitarias de la Seguridad Social, Comisión Asesora de Investigación Científica y Técnica of the Spanish Ministry of Education and by the International

Molecular Program of the IIC-KUMC. We thank Dr. F. Thompson for critically reading the manuscript.

REFERENCES

1. Walser, M. (1982) The Metabolic Basis of Inherited Disease, pp. 403-438, McGraw-Hill Book Co., New York.
2. Duffy, T.E. and Plum, F. (1982) The Liver: Biology and Pathobiology, pp. 693-715, Raven Press, New York.
3. Hindfelt, B., Plum, F. and Duffy, T.E. (1977) J. Clin. Invest. 59, 386-396.
4. McCandless, D.W. and Schenker, S. (1981) Exp. Brain Res. 44, 325-330.
5. Lorente de Nó, E., Vidal, F. and Larramendi, L.M.H. (1957) Nature 179, 737-738.
6. Lux, D.H., Loracher, C. and Neher, E. (1970) Exp. Brain Res. 11, 431-447.
7. Llinás, R., Baker, R. and Precht, W. (1974) J. Neurophysiol. 37, 522-532.
8. Deisz, R.A. and Lux, D.H. (1982) J. Physiol. 326, 123-138.
9. O'Connor, J.E., Guerri, C. and Grisolía, S. (1982) Biochem. Biophys. Res. Commun. 104, 410-415.
10. O'Connor, J.E., Guerri, C., Jordá, A. and Grisolía, S. (1982) Biochem. Biophys. Res. Commun. 107, 1508-1516.
11. O'Connor, J.E., Guerri, C. and Grisolía, S. (1982) New Eng. J. Med. 347, 254.
12. Sun, A.Y. (1979) Biochemistry and Pharmacology of Ethanol (vol. 2), pp. 81-100, Plenum Press, New York.
13. Grisolía, S., Quijada, C.L. and Fernández, M. (1964) Biochim. Biophys. Acta 81, 61-70.
14. Cotman, E.W. (1974) Methods in Enzymology (vol. 31), pp. 445-452, Academic Press, New York.
15. Guerri, C., Ribelles, M. and Grisolía, S. (1981) Biochem. Pharmacol. 30, 25-30.
16. Richterich, E. (1969) Clinical Chemistry, pp. 241-249, Academic Press, New York.
17. Lenaz, G., Curatola, G. and Masotti, L. (1975) J. Bioenerg. 7, 223-299.
18. Guerri, C. (1982) Biochem. Pharmacol. 31, 449-453.
19. Raabe, W. and Gumnit, R.J. (1975) J. Neurophysiol. 38, 347-355.
20. Nicoll, R.A. (1978) J. Physiol. 283, 132.
21. Iles, J.F. and Jack, J.J.B. (1980) Brain 103, 555-578.
22. Raabe, W. (1982) Physiology and Pharmacology of Epileptogenic Phenomena, pp. 73-80, Raven Press, New York.
23. Aickin, C.C., Deisz, R.A. and Lux, D.H. (1982) J. Physiol. 329, 319-339.
24. Lewantovsky M.I. (1930) Z. Gesante Neurol. Psychiatr. 126, 484-495.
25. Laursen, H. and Westergaard, E. (1977) Neuropathol. Appl. Neurobiol. 3, 29-43.
26. James, J.H., Escourron, J. and Fischer, J.E. (1978) Science 200, 1395-1397.
27. Livingstone, A.S., Potvin, M., Gorsky, C.A., Finlayson, M.H. and Hinchey, E.J. (1977) Gastroenterology 73, 697-704.
28. Diemer, N.H. (1977) Acta Neurol. Scand. 55, 16-32.
29. Diemer, N.H. and Laursen, H. (1977) Acta Neurol. Scand. 55, 425-442.
30. Marx, J.L. (1976) Science 192, 455-457.