L-Phenylalanine Effect on Rat Brain Acetylcholinesterase and Na^+,K^+-ATPase

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Z. Naturforsch. **58 c,** 163–167 (1998); received September 22/December 15, 1997

L-Phenylalanine, Rat Brain, Acetylcholinesterase, Na^+,K^+-ATPase, Mg^{2+}-ATPase

The effect of different L-phenylalanine (Phe) concentrations (0.1–12.1 mM), on acetylcholinesterase (AChE) and Na^+,K^+-ATPase activities of brain homogenate and pure enzymes, was investigated at 37 °C. AChE and Na^+,K^+-ATPase activities were determined according to Ellman G. L., Courtney D., Andres V. and Featherstone R. M. (1961), Biochem. Pharmacol. **7**, 88–95 and Bowler K. and Tirri R. (1974), J. Neurochem. **23**, 611–613) respectively, after preincubation with Phe. AChE activity in brain homogenate or in pure eel *E.electricus* enzyme showed a decrease, which reached up to 18% with concentrations of 0.9–12.1 mM. Brain homogenate Na^+,K^+-ATPase activity showed an increase 16–65% with 0.24–0.9 mM of Phe, while an activity increase of 60–65% appeared with 0.9–12.1 mM of Phe. Pure enzyme activity (from porcine cerebral cortex) was not affected by high Phe concentrations, while it was increased by low concentrations. The above results suggest: a) A direct effect of Phe on AChE, b) A direct effect of low Phe concentrations and an indirect effect of high ones on Na^+,K^+-ATPase.

Introduction

Phenylketonuria (PKU) is a group of recessively inherited metabolic disorders, in which the conversion of phenylalanine (Phe) to tyrosine is impaired. This metabolic disorder is due to an autosomal recessive gene that codes for a type of phenylalanine hydroxylase that has reduced enzymatic activity, resulting in abnormally high levels of Phe in body fluids (Kupfermann, 1991). An excessive increase of blood Phe results in mental retardation (IQ<60) (Missiou-Tsagaraki *et al.*, 1988), seizures and other neurophysiological and psychological dysfunctions (Behbenhani and Langenbeck, 1982). High Phe concentrations (0.3–1.8 mM) may occur in sick humans, and are harmful especially during the first year of life, while this "damage" can be prevented with an appropriate dietary control (Missiou-Tsagaraki *et al.*, 1988).

The rapid and precise communication between neurons, necessary for the performance of nervous system functions, is made possible by two signaling mechanisms: excitability and synaptic transmission.

Neural excitability was found to be influenced by Phe in rat brain (Iarosh *et al.*, 1987) while experimental hyperphenylalaninemia in 3–17 day-old rats leads to reduced myelinogenesis (Burri *et al.*, 1990), which could result in a decrease of the axonal conduction velocity. Reduced myelinogenesis is consistent with the observation which showed that high Phe concentrations in the body fluids inhibit brain protein synthesis by inhibition of the transport of amino acids across the blood-brain barrier (Oldendorf, 1973; Antonas and Coulson, 1975) or by direct interference with the protein synthesis apparatus (Taub and Johnson, 1975; Huges and Johnson, 1977).

Alterations in synaptic transmission are also implicated in brain dysfunctions in PKU and several experimental data suggest that the principal cause for the brain dysfunction is the impairment in the neurotransmitter amine synthesis (Blau, 1979). Increased Phe concentrations, by decreasing the availability of the precursors tryptophan and tyrosine (Aragon *et al.*, 1982) might be the primary cause of serotonin and catecholamine depletion in PKU (Herrero *et al.*, 1983). Since the aromatic amino acides (tryptophan, tyrosine, Phe) are the

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0939–5075/98/0300–0163 $ 06.00

biosynthetic precursors for the neurotransmitters serotonin, dopamine and norepinephrine (NE), the aromatic amino acids uptake into the brain can modify their convertion to neurotransmitters and thereby can modify their release from neurons and influence brain functions (Fernstrom, 1994). Moreover high Phe concentrations induce changes of brain electrical function, which may be mediated in part through inhibition of catecholamine synthesis (Krause *et al.*, 1986).

Regarding cholinergic brain systems, experimental results showed their possible involvement during Phe action. The 6R-L-erythro-5, 6, 7, 8,-tetrahydrobiopterin (6R-BH4), a natural cofactor for Phe hydroxylases, has direct acetylcholine (ACh) releasing action *in vivo* in the rat hippocampus (Ohue *et al.*, 1991), while hyperphenylalaninemia leads to a decrease in density of muscarinic receptors of the hippocampus and other brain areas (Hommes, 1993 and 1994). Since the hippocampus has been shown to be involved in the acquisition and long-term storage of information, a decrease in neurotransmitter receptor density may form the basis for a mental retardation in PKU.

In the work reported in this article we studied the effect of different Phe concentrations on the activity of two enzymes: a) Acetylcholinesterase (AChE), the role of which is very important in ACh cycle, ACh release included (Kouniniotou-Krontiri and Tsakiris, 1989) and b) Na^+,K^+-ATPase, enzyme implicated in neural excitability (Sastry and Phillis, 1977), activity-dependent metabolism of energy (Mata *et al.*, 1980) and Na^+-dependent tryptophan uptake system (Herrero *et al.*, 1983).

Materials and Methods

The enzymes activity measurements were carried out on rat homogenised brain and pure enzymes (*E.electricus* AChE and porcine cerebral cortex Na^+,K^+-ATPase) at 37 °C. In the experiments, in which enzyme preincubation with Phe was needed, the determination of the activity was realised after preincubation with 0.1, 0.24, 0.48, 0.9, 1.8 or 12.1 mM of Phe at 37 °C.

For the experiments conducted on rat homogenised brain, 21-day old Albino Wistar rats of both sexes (Saint Sabbas Hospital, Athens, Greece) were used and the preparation of the brain extracts was undertaken as described previously (Tsakiris, 1993). The suckling rats with their mother were housed in a cage at constant room temperature (22±1 °C), under a 12h L: 12h D (light 0800–2000 h) cycle. Animals were cared for in accordance with the principles of the *Guide to the Care and Use of Animals* and were killed by decapitation. Whole brain was homogenised and centrifuged as described previously (Tsakiris, 1993). In the resulting supernatant the protein content was determined according to Lowry *et al.* (1951).

AChE activity was investigated a) as a function of time of Phe action on the enzyme and b) as a function of Phe concentration. The enzyme activity was determined by following the hydrolysis of acetylthiocholine according to the method of Ellman *et al.* (1961). The incubation mixture (1 ml) contained 50 mM Tris (hydroxymethyl) aminomethane-HCl (Tris-HCl), pH 8, 240 mM sucrose and 120 mM NaCl. The protein concentration of the incubation mixture was 80–100 μg/ml for the homogenised brain and 0.1 μg/ml for the eel *E.electricus* pure AChE. The incubation was carried out under continuous magnetic stirring. The reaction was initiated after addition of 0.03 ml of 5,5'-dithionitrobenzoic acid (DTNB) and 0.05 ml of acetylthiocholine iodide, which was used as substrate. The final concentration of DTNB and substrate were 0.125 mM and 0.5 mM respectively. The reaction was followed spectrophotometrically by the increase in absorbance ($\Delta\overline{OD}$) at 412 nm.

Na^+,K^+-ATPase was calculated from the difference between total ATPase activity (Na^+,K^+, Mg^{2+}-dependent ATPase) and Mg^{2+}-dependent ATPase activity. Total ATPase activity was assayed in an incubation medium consisting of 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 240 mM sucrose, 1 mM ethylenediamine tetraacetic acid K_2-salt (K^+-EDTA), 3 mM disodium ATP and 80–100 μg protein for the homogenised brain and 40μg for pure Na^+,K^+-ATPase from porcine cerebral cortex in a final volume of 1 ml. Ouabain (1 mM) was added in order to determine the activity of the Mg^{2+}-ATPase. The values of Mg^{2+}-dependent ATPase were similar in the presence of ouabain in the reaction mixture as also in ouabain absence and without NaCl and KCl. The reaction was started by adding ATP and

stopped after an incubation period of 20 min by the addition of 2 ml mixture of 1% lubrol and 1% ammonium molybdate in 0.9 M H_2SO_4 (Atkinson *et al.*, 1971; Bowler and Tirri, 1974). The yellow colour which developed was read at 390 nm.

All chemicals were analytical grade and purchased from Sigma-Aldrich Vertriebs GmbH (Deisenhofen, Germany). The data were analysed by two-tailed Student's *t*-test.

Results and Discussion

The time-course of AChE activity during incubation of the brain homogenate with 0 (control), 0.48, 0.9 and 1.8 mM of Phe is shown in Fig. 1. In the absence of Phe the enzyme activity remained at a steady level during the 120 min of the experiment. In the presence of Phe, AChE activity declined progressivelly to a lower level (decrease 18%). The above decrease, ($p<0.01$), attained a steady level after 30 min of Phe action when its concentration was 0.48 mM and after 20 min when Phe concentration was 0.9 or 1.8 mM. Since the above Phe concentrations in the blood, observed in PKU, were followed from brain dysfunctions (Missiou-Tsagaraki *et al.*, 1988), and since AChE can influence cholinergic transmission, a more detailed study of Phe action on the enzyme seemed worthwhile. We have therefore investigated the effect of different concentrations of Phe on the activity of brain homogenised AChE as also on the activity of pure eel *E.electricus* AChE. The results

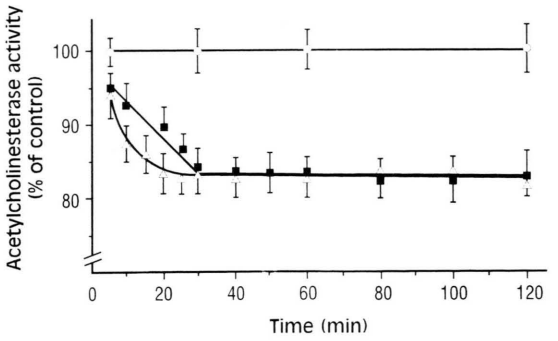

Fig. 1. Time-dependent effect of Phe on AChE activity determined in 21-day old rats homogenised brain. Points and vertical bars represent mean values ± SD for Phe concentrations 0 mM (○), 0.48 mM (■) as also 0.9 and 1.8 mM (△). Each point represents the average value of dublicate determinations from a typical experiment which has been repeated three times. Control value of AChE activity was 0.96±0.04 $\Delta\overline{OD}$/minxmg protein.

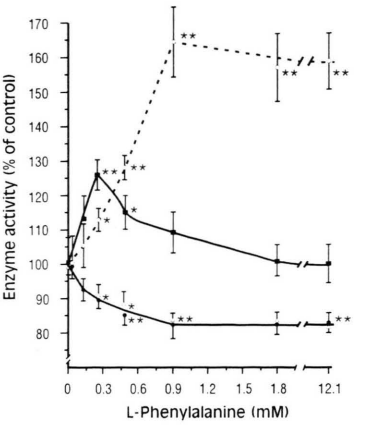

Fig. 2. Effect of different Phe consentrations on AChE and Na^+,K^+-ATPase activities. AChE activity was determined in homogenised brain (●) and in eel *E. Electricus* pure enzyme (△). Na^+,K^+-ATPase activity was determined in homogenised brain (○) and in pure enzyme from porcine cerebral cortex (■). The activity control values were 0.96±0.04 $\Delta\overline{OD}$/minxmg protein for brain homogenate AChE, 1.23±0.04 $\Delta\overline{OD}$/minxmg protein for pure AChE, 1.87±0.17 μmol Pi/hxmg protein for homogenised brain Na^+,K^+-ATPase and 14.80±1.60 Pi/hxmg protein for the pure enzyme. Points and vertical bars represent the average value of three determinations from a typical experiment which has been repeated four times. In all cases preincubation of the enzyme incubation medium with different Phe concentrations was realised for 1 h before the substrate addition. *: $p<0.05$; **: $p<0.01$.

of this study, illustrated in Fig. 2, showed that 1 hour of Phe action on the enzyme resulted in a statistically significant decline of AChE activity about 10% ($p<0.05$) for a Phe concentration of 0.24 mM and 18% ($p<0.01$) for Phe concentrations ranged from 0.9 to 12.1 mM. As seen in Fig. 2, the Phe induced decrease of AChE activity has been similar on brain homogenised AChE and on eel *E.electricus* non-membrane bound enzyme. Therefore, Phe has a direct effect on AChE, possibly interacting with its positively charged sites.

Since the hippocampus has been shown to be involved in long-term storage of information, damage to this structure by hyperphenylalaninemia may provide a clue to the global mental retardation observed in untreated PKU (Hommes, 1993 and 1994). Furthermore, in this structure high Phe concentrations were found to cause loss of muscarinic receptors (Matsuo and Hommes, 1988; Hommes, 1993 and 1994), while 6R-BH4 has a direct ACh releasing action *in vivo*. An AChE activity decrease, induced by Phe, such as the decrease

observed in our experiment, in addition with an increased ACh release, result in enhancement of synaptic ACh and could lead to decrease of muscarinic receptors density, while the AChE activity decrease has been found to be related to decline of spontaneous quantal ACh release on diaphragm (Kouniniotou-Krontiri and Tsakiris, 1989). Since, the decline of AChE activity, observed in our results, could not be excluded from involvement in mental retardation induced in PKU.

The effects of different Phe concentrations on homogenised brain Na^+,K^+-ATPase and on pure Na^+,K^+-ATPase from porcine cerebral cortex are also illustrated in Fig. 2. Regarding homogenised brain Na^+,K^+-ATPase, preincubation of the brain homogenate with different concentrations of Phe for 1 hour induced a concentration-dependent increase of the enzyme activity until the Phe concentration of 0.9 mM, while higher concentations (1.8 and 12.1 mM) did not change the activity level, which has been attained by 0.9 mM of Phe. In this case the Phe induced increase of the enzyme activity reached 60–65% ($p<0.01$). Since Na^+,K^+-ATPase is implicated in several processes, the investigation of a direct or indirect Phe effect on this enzyme seemed interesting. Thus, the activity measurements were repeated on pure enzyme. As we can see in Fig. 2, the activity of the pure Na^+,K^+-ATPase, under the same experimental conditions, showed an enhancement of 28% ($p<0.01$) and 16% ($p<0.05$) for Phe concentrations of 0.24 and 0.48 mM respectively. Higher Phe concentrations had no effect on the enzyme activity ($p>0.05$). The above results show that Phe has mainly a direct effect on Na^+,K^+-ATPase in low concentrations (0.1–0.48 mM), while the marked enzyme activity increase induced by high Phe concentrations are due to an indirect Phe effect on the enzyme.

Regarding the direct effect of Phe, a probable interaction of Phe with positively charged sites of the enzyme could be the basis of its action. The indirect effect of Phe could be the result, at least in part, of a NE enhancement (Swann, 1984), the synthesis of which in non-phenylketonuric rats can be increased by high Phe concentrations. Possible regulation of Na^+,K^+-ATPase by NE includes: 1) An increase of Na^+ influx leading to a compensatory increase in Na^+,K^+-ATPase net synthesis, 2) changes in phospholipids or other membrane components that might unmask additional sites, and 3) modification of otherwise inactive sites, possibly by phosphorylation (Swann, 1984). The increase of Na^+,K^+-ATPase activity is consistent with other cellullar effects as changes in neural excitability (Sastry and Phillis, 1977; Iarosh *et al.*, 1987; Kouniniotou-Krontiri and Tsakiris, 1989) or increase in activity-dependent energy production including stimulation of glycogenolysis (Thomas and Williamson, 1983). Moreover Phe stimulates brain galactose-1-phosphatase (Gulavita *et al.*, 1991) and can convert more galactose to glucose.

Mg^{2+}-ATPase activity was found to be 8.1 ± 0.7 μmol Pi/hxmg protein in homogenised brain of 21-day old rats, while Phe, in the concentration used in our experiments, appeared unable to affect the enzyme activity ($p>0.05$).

Acknowledgements

This work was funded by the University of Athens.

Many thanks are extended to Chr. Plataras for his significant assistance in this work.

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