

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

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Z. Naturforsch. **53c**, 1055–1060 (1998); received June 3/June 17, 1998

L-Phenylalanine, Rat Diaphragm, Acetylcholinesterase, Na⁺,K⁺-ATPase

The effect of different L-phenylalanine (Phe) concentrations (0.24–12.1 mM), on acetylcholinesterase (AChE) and Na⁺,K⁺-ATPase activities of diaphragm homogenates from 21-day old rats and pure enzymes, was investigated at 37 °C. AChE and Na⁺,K⁺-ATPase activities were determined after preincubation with Phe. AChE activity in diaphragm homogenate or in pure eel *E. electricus* enzyme showed a decrease, which reached a maximum of 18% with Phe concentrations of 0.9–12.1 mM. However lower Phe concentrations (0.24 mM) increased the enzyme activity (by approximately 22%), only in the diaphragm homogenate. Diaphragm-associated Na⁺,K⁺-ATPase activity showed a progressive and concentration-dependent decrease, by about 30–35% in the presence of high Phe concentrations. Pure enzyme activity (from porcine cerebral cortex) was not affected by high Phe concentrations (>0.48 mM), while it was increased by low concentrations. The above results suggest: a) A direct inactivating effect of high Phe concentrations on AChE and an indirect activating effect induced by low concentrations. b) A direct activating effect of low Phe concentrations and an indirect inactivating effect of high ones on Na⁺,K⁺-ATPase. c) The combination of high Phe concentrations effects on AChE and Na⁺,K⁺-ATPase could influence the levels of the diaphragm synaptic ACh.

Introduction

Phenylketonuria (PKU) is caused by mutation in the phenylalanine hydroxylase gene, resulting in impaired conversion of phenylalanine (Phe) to tyrosine and consequently in abnormally high levels of Phe in body fluids (Kupfermann, 1991). An excessive increase in blood Phe results in mental retardation (IQ<60) (Missiou-Tsagaraki *et al.*, 1988) seizures and other neurophysiological and psychological dysfunctions (Behbenhani and Langenbeck, 1982).

Phe was found to influence, by several mechanisms: a) the neural excitability (Iarosh *et al.*, 1987) b) the axonal conduction velocity (Oldendorf, 1973; Antonas and Coulson, 1975; Taub and Johnson, 1975; Huges and Johnson, 1977; Burri *et al.*, 1990) and c) the synaptic transmission (Blau, 1979; Aragon *et al.*, 1982; Herrero *et al.*, 1983; Krause *et al.*, 1986; Fernstrom, 1994), implicated in brain dysfunction in PKU. Regarding cholinergic

systems, experimental results showed their possible involvement in Phe action (Ohue *et al.*, 1991; Hommes, 1993 and 1994).

Since neural excitability and synaptic transmission are important signaling mechanisms and since cholinergic systems appear to be affected by Phe action, a study of changes in the activity of implicated enzymes, during Phe action, seemed worthwhile. 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 acetylcholine (ACh) cycle, including ACh release (Kouniniotou-Krontiri, 1985; Kouniniotou-Krontiri and Tsakiris, 1989) and b) Na⁺,K⁺-ATPase, an enzyme implicated in neural excitability (Sastri and Phillis, 1977), activity-dependent metabolism of energy (Mata *et al.*, 1980) and Na⁺-dependent choline uptake system (Vaca *et al.*, 1982; Tuček, 1984). In order to study the Phe action on the above mentioned enzymes exclusively in cholinergic systems and to avoid involvement of the possible action of other neurotransmitters (Tsakiris *et al.*, 1998), this study was performed on

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homogenised diaphragm, the innervation of which is purely cholinergic.

Materials and Methods

The enzymes activities measurements were carried out on rat homogenised diaphragm and pure enzymes (*E. electricus* AChE and porcine cerebral cortex Na⁺,K⁺-ATPase).

For the experiments conducted on rat homogenised diaphragm, 21-day old Albino Wistar rats of both sexes (Saint Sabbas Hospital, Athens, Greece) were used. The suckling rats with their mother were housed in a cage at a 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.

The diaphragms were placed in a bath, perfused with Krebs solution, and trimmed of connective tissue after washing out the blood from the freshly dissected muscles. The Krebs solution (pH 7.4) had the following composition (in mM): NaCl, 115.2; KCl, 5.9; CaCl₂, 2.6; MgCl₂, 1.2; NaHCO₃, 26.2; NaH₂PO₄, 1.2; Na₂SO₄, 1.2; glucose, 5.5. Considerable mincing was necessary before homogenising. The homogenisation medium contained 70 mM Tris (hydroxymethyl) aminomethane-HCl buffer (Tris-HCl), pH 7.4 and 300 mM sucrose. 20 mg of tissue/ml medium were disrupted for 8 min with an Omni mixer and then were homogenised in a Potter-Elvehjem at 4 °C. The protein content of the homogenate was determined according to Lowry *et al.* (1951).

The enzymes activities determinations were carried out at 37 °C. In the experiments, in which enzyme preincubation with Phe was needed, the activity was determined after preincubation with 0.24, 0.48, 0.9, 1.8 or 12.1 mM of Phe at 37 °C.

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-HCl (pH 8.0), 240 mM sucrose and 120 mM NaCl. The protein concentration of the incubation mixture was 300 µg/ml for the homogenised diaphragm 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 by the 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 (ΔOD) at 412 nm.

Na⁺,K⁺-ATPase was calculated as the difference between total ATPase activity (Na⁺,K⁺,Mg²⁺-dependent ATPase) and Mg²⁺-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 MgCl₂, 240 mM sucrose, 1 mM ethylenediamine tetraacetic acid K₂-salt (K⁺-EDTA), 3 mM disodium ATP and 100–130 µg protein for the homogenised diaphragm and 40 µg 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 Mg²⁺-ATPase. The values of Mg²⁺-dependent ATPase were similar in the presence of ouabain in the reaction mixture as well as in the absence of ouabain and without NaCl and KCl. The reaction was started by adding ATP and stopped after an incubation period of 30 min by the addition of 2 ml mixture of 1% lubrol and 1% ammonium molybdate in 0.9 M H₂SO₄ (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

The time-course of AChE activity during incubation of the diaphragm homogenate with 0 (control), 0.24, 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 0.24 mM of Phe, AChE activity increased progressively to a higher level (22% increase). The above increase, (*p*<0.01), attained a steady level after 40 min of Phe action. On the contrary, when Phe concentration was 0.9 or 1.8 mM, AChE activity showed a

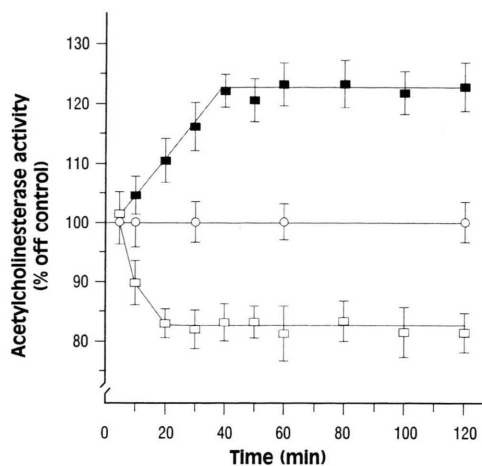


Fig. 1. Time-dependent effect of Phe on AChE activity determined in 21-day old rats homogenized diaphragm. Points and vertical bars represent mean values \pm SD for Phe concentrations 0 mM (○), 0.24 mM (■) as also 0.9 and 1.8 mM (□). Values represent means \pm SD of three experiments. The average value of each experiment arise from two determinations. Control value of AChE activity was 0.18 ± 0.01 Δ OD/min \times mg protein.

progressive decline during the first 20 min of Phe action. Then the activity attained a steady level which was approximately 18% lower than the control activity level ($p < 0.01$).

Since the above blood Phe concentrations, observed in PKU, were accompanied by 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 homogenised diaphragm AChE as well as on the activity of pure eel *E. electricus* AChE. The results of this study, are illustrated in Fig. 2. As shown, 1 hour of Phe action on the pure eel *E. electricus* AChE resulted in a statistically significant decline of AChE activity, which reached an inhibition of about 18% for Phe concentration, of 0.9, 1.8 and 12.1 mM ($p < 0.01$). As shown in the same Figure, the decrease of AChE activity induced by high Phe concentrations (0.9, 1.8 and 12.1 mM) was similar in homogenised diaphragm AChE and in the eel *E. electricus* non membrane-bound enzyme. Therefore, in the above concentrations, Phe has a direct effect on AChE. On the contrary, in lower Phe concentrations (0.24 and 0.48 mM) differences were observed between

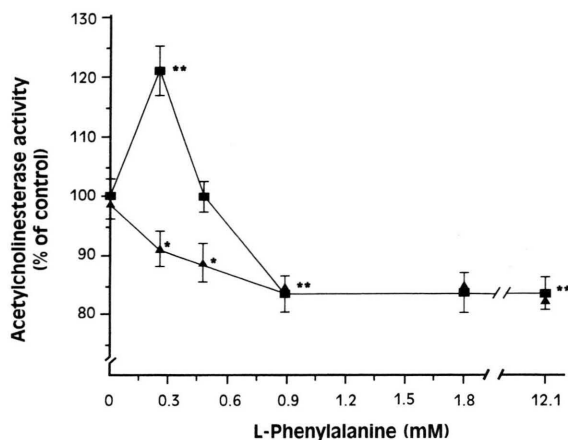


Fig. 2. Effect of different Phe concentrations on AChE activities determined in homogenized diaphragm (■) and in eel *E. Electricus* pure enzyme (▲). The activity control values were 0.18 ± 0.01 Δ OD/min \times mg protein for diaphragm homogenate and 1.23 ± 0.04 Δ OD/min \times mg protein for the pure enzyme. Values represent means \pm SD of four experiments. The average value of each experiment arise from three determinations. In all cases a preincubation for 1 h of the enzyme incubation medium with different Phe concentrations preceded the substrate addition. *: $p < 0.05$, **: $p < 0.01$.

the curve of diaphragm-associated AChE activity and the curve of the activity of pure eel *E. electricus* AChE. Thus, 0.24 and 0.48 mM of Phe induced a decline in pure enzyme activity. On the contrary, 0.24 mM of Phe caused a rise of the activity of diaphragm-associated AChE, of about 22% ($p < 0.01$); in the presence 0.48 mM of Phe the homogenised diaphragm AChE activity displayed a descending phase, reaching the same value with that of the control.

The effects of different Phe concentrations on homogenised diaphragm Na⁺,K⁺-ATPase and on pure Na⁺,K⁺-ATPase from porcine cerebral cortex are illustrated in Fig. 3. Regarding homogenised diaphragm Na⁺,K⁺-ATPase, preincubation of the diaphragm homogenate with different concentrations of Phe for 1 hour induced a concentration-dependent progressive decrease in the enzyme activity (up to a Phe concentration of 1.8 mM), while higher concentrations (up to 12.1 mM) did not change the activity level, which was attained by 1.8 mM of Phe. In this case the Phe induced decrease in the enzyme activity reached a value of 30–35% ($p < 0.01$).

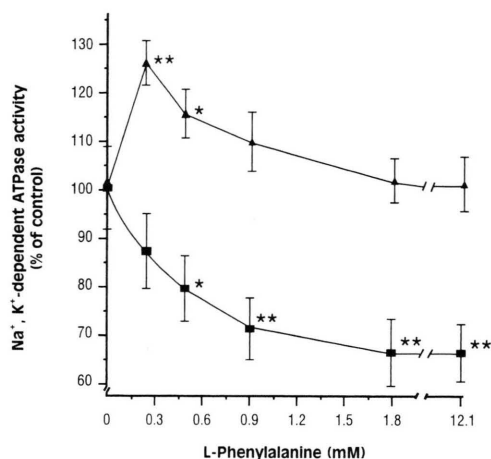


Fig. 3. Effect of different Phe concentrations on Na⁺,K⁺-ATPase activities determined in homogenised diaphragm (■) and in pure enzyme from porcine cerebral cortex (▲). The activity control values were 0.85 ± 0.07 $\mu\text{mol Pi/h} \times \text{mg protein}$ for homogenised diaphragm Na⁺,K⁺-ATPase and 14.80 ± 1.60 $\text{Pi/h} \times \text{mg protein}$ for the pure enzyme. Values represent means \pm SD of four experiments. The average value of each experiment arise from three determinations. In all cases preincubation of the enzyme incubation medium with different Phe concentrations was realised for 1 h before the substrate addition. *: $p < 0.05$; **: $p < 0.01$.

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 shown in Fig. 3, the activity of 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 ($p > 0.05$).

Mg²⁺-ATPase activity was found to be 5.9 ± 0.3 $\mu\text{mol Pi/h} \times \text{mg protein}$ in homogenised diaphragm of 21-day old rats. High Phe concentrations (0.9–12.1 mM) decreased the enzyme activity by about 14% ($p < 0.05$), while, lower concentrations appeared unable to affect the enzyme activity ($p > 0.05$).

Discussion

The AChE, whose role is very important in cholinergic transmission, is primarily associated with nerve and muscle, localised typically at synaptic

contacts (Taylor, 1991). Most of the AChE at the neuromuscular junction is associated with the basal lamina in the synaptic cleft. The catalytic subunits of the enzyme are bound by disulfide linkage to a long, collagen-like tail that attaches the enzyme to heparan sulfate proteoglycan, a component of basal lamina. The synaptic enzyme appears to be made by the muscle and deposited in the extracellular matrix (Hall, 1992). Diaphragm-associated AChE has been affected by Phe. The time-course of diaphragm homogenised AChE activity during Phe action showed that low Phe concentrations induce an increase in the enzyme activity while high concentrations lead to an activity decrease. The above results are consistent with observations concerning brain homogenate (Tsakiris *et al.*, 1998). A decreased AChE activity can lead to synaptic ACh increase, while a long term AChE inhibition could induce a progressive neuronal ACh depletion (Kouniniotou-Krontiri, 1985; Kouniniotou-Krontiri and Tsakiris, 1989). Thus, high Phe concentrations, which appear in PKU, could cause an AChE activity decline, resulting in transmitter cycle perturbation. The decline of AChE activity, induced by high Phe concentrations, was also observed in the eel *E. electricus* pure AChE, suggesting that the above activity decrease is a direct effect of Phe on the enzyme, possibly due to an interaction with AChE positively charged sites. On the contrary, the activity rise induced by low Phe concentrations (0.24 mM) was observed only in diaphragm homogenate, indicating that this Phe effect is indirect. This indirect effect could be due to an interaction with cellular factor(s) and/or with the positively charged membrane lipids. Moreover the activity of the membrane-bound AChE can be modulated by the physical state of its lipid environment, despite the fact that the enzyme as such is not lipid-dependent (Frenkel *et al.*, 1980).

Regarding Na⁺,K⁺-ATPase, Phe induced a progressive concentration-dependent decline in the diaphragm homogenate activity, while its action on the pure Na⁺,K⁺-ATPase from porcine cerebral cortex appeared to be an activation of the enzyme by low Phe concentrations and a stabilisation of the activity by 0.9, 1.8 and 12.1 mM. These results suggest that Phe has a direct effect on the Na⁺,K⁺-ATPase only at low concentrations while high Phe concentrations have no effect on the enzyme. Thus,

it seems that PKU can not influence Na⁺,K⁺-ATPase directly. On the contrary Phe produced an indirect inhibition of the enzyme and this inhibition was more pronounced with high Phe concentrations. Therefore, PKU can influence the enzyme activity by an indirect way. Since an inhibition of Na⁺,K⁺-ATPase is implicated in neural excitability (Sastri and Phillis, 1977) and in activity-dependent metabolism of energy (Mata *et al.*, 1980) an inhibition of the enzyme by high (toxic) Phe concentrations could influence neural excitability and diaphragm metabolism of energy. Moreover, since an inhibition of Na⁺,K⁺-ATPase is correlated with increased release of ACh (Meyer and Cooper, 1981), we can conclude that PKU could increase the ACh release at the diaphragm end-plate. Such an enhancement of ACh release in correlation with the AChE activity decline, induced, as we have seen, by high Phe concentrations, could lead to an increase of the synaptic ACh at the diaphragm. On the other hand, a

long-term increased release of ACh related to an AChE inhibition could result in ACh depletion (Kouniniotou-Krontiri, 1985; Kouniniotou-Krontiri and Tsakiris, 1989). It is noteworthy that the recently observed indirect effect of Phe on brain homogenised Na⁺,K⁺-ATPase (Tsakiris *et al.* 1998) and the Phe effect on diaphragm Na⁺,K⁺-ATPase are quite different. However, taking into account the pure cholinergic innervation of the diaphragm, this difference is consistent with the point of view that the indirect Phe effect on brain homogenate Na⁺,K⁺-ATPase could be, at least in part, the result of a norepinephrine enhancement by high Phe concentrations (Tsakiris *et al.*, 1998).

Acknowledgements

This work was funded by the University of Athens.

Many thanks are extended to C. Dinos for his significant assistance in this work.

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