

Alanine Reverses the Inhibitory Effect of Phenylalanine on Acetylcholinesterase Activity

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The aim of this work was to evaluate, *in vitro*, the effect of L-alanine (Ala) on suckling rat brain acetylcholinesterase (AChE) and on eel *Electrophorus electricus* pure AChE inhibited by L-phenylalanine (Phe) as well as to investigate whether Phe or Ala is a competitive inhibitor or an effector of the enzyme. AChE activity was determined in brain homogenates and in the pure enzyme after 1 h preincubation with 1.2 mM of Phe or Ala as well as with Phe plus Ala. The activity of the pure AChE was also determined using as a substrate different amounts of acetylthiocholine. Ala reversed completely the inhibited AChE by Phe (18–20% in 500–600 μ M substrate, $p < 0.01$). Lineweaver-Burk plots showed that V_{\max} remained unchanged. However, K_M was found increased with Phe (150%, $p < 0.001$), decreased with Ala alone (50%, $p < 0.001$) and unaltered with Phe plus Ala. It is suggested that: a) Phe presents a competitive inhibitory action with the substrate whereas Ala a competitive activation; b) Ala competition with Phe might unbind the latter from AChE molecule inducing the enzyme stimulation; c) Ala might reverse the inhibitory effect of Phe on brain AChE in phenylketonuric patients, if these results are extended into the *in vivo* reality.

Introduction

Phenylketonuria (PKU) is a group of metabolic disorders in which the amino acid phenylalanine (Phe) is highly elevated in blood (higher than 20 mg/dl or 1.2 mM) (Missiou-Tsagaraki *et al.*, 1988) resulting in mental retardation, seizures, which are the main clinical manifestation in the untreated patients (Ludolph *et al.*, 1996). According to our previous *in vitro* studies (Tsakiris *et al.*, 1998a; Tsakiris *et al.*, 1998b; Schulpis *et al.*, 1998), Phe was found to inhibit acetylcholinesterase (AChE, EC 3.1.1.7) activity up to 20% at concentrations 1.0 to 12 mM in rat brain, in eel *Electrophorus electricus* AChE as well as in diaphragm. Furthermore, Wyse *et al.* (1995) have showed that L-alanine (Ala) reverses the inhibitory effect of Phe on Na^+, K^+ -ATPase (EC 3.6.1.3) activity in synaptic plasma membranes from the cerebral cortex of rat. Additionally, it has been reported that Ala reverses the inhibitory effect of Phe on other enzyme such as pyruvate kinase from liver and brain of rats (Weber, 1969).

Additionally, the ability of AChE to undergo ligand-induced conformational changes was first suggested by Changeux (1966), who, by double inhibition studies, deduced the presence of “peripheral” (allosteric) anionic sites, distinct from the catalytic anionic site. The binding of ligands on peripheral sites could influence the catalytic properties of AChE. This hypothesis has been also supported by the studies of several authors (Kitz *et al.*, 1970; Rosenbery and Bernhard, 1971). Also, the kinetic properties of AChE have been reported to be markedly affected by the presence of both organic (Wermuth and Brodbeck, 1973; Pattison and Bernhard, 1978) and inorganic ions (Tomlinson *et al.*, 1982; Kouniniotou-Krontiri and Tsakiris, 1984; Hughes and Bennett, 1985; Perez-Guillermo *et al.*, 1987; Tsakiris and Kouniniotou-Krontiri, 1988).

Therefore, the aim of this study was to evaluate, *in vitro*, the effect of Ala on rat brain AChE and on eel *E. electricus* AChE inhibited by Phe as well as to investigate whether Phe or Ala is a competitive inhibitor or an effector using acetylthiocholine as an enzyme substrate.

Materials and Methods

Animals

For the experiments conducted on homogenized rat brain, 21 days old Albino Wistar rats of both sexes (Saint Savvas Hospital, Athens, Greece) were used. The suckling rats with their mother were housed in a cage at constant room temperature (22 ± 1 °C) under a 12 hL:12 hD (light 08.00–20.00 h) cycle. Animals were cared for in accordance with the principles of the “*Guide to the Care and Use of Experimental Animals*” (Committee on Care and Use of Laboratory Animals, 1985).

Tissue preparation

Animals were sacrificed by decapitation. Whole brains from six rats were rapidly removed, weighed and thoroughly washed with isotonic saline. Tissues were homogenized in 10 vol. ice-cold (0–4 °C) medium containing 50 mM Tris (hydroxymethyl) aminomethane-HCl (Tris-HCl), pH 7.4 and 300 mM sucrose using an ice-chilled glass homogenizing vessel at 900 rpm (4–5 strokes). The homogenate was centrifuged at $1000 \times g$ for 10 min to remove nuclei and debris. In the resulting supernatant, the protein content was determined according to the method of Lowry *et al.* (1951) and then the enzyme activities were measured. The enzyme incubation mixture was kept at 37 °C.

Amino acids (Phe, Ala, Phe plus Ala) preincubation

As it is known (Missiou-Tsagaraki *et al.*, 1988), the common intracellular concentration of 1.2 mM Phe in brain is 6 times lower than that found in plasma (7 mM) of phenylketonuric patients. The mean Phe levels in normal brain and plasma are 0.04 ± 0.01 and 0.09 ± 0.02 mM, respectively.

AChE activities were measured after preincubation of 1.2 mM Phe, or Ala or Phe plus Ala concentrations with 100 µg protein of whole brain homogenates or with 0.13 µg protein of eel *E. electricus* pure AChE (Sigma) for 1 h. The preincubation medium (about 1 ml) contained 50 mM Tris-HCl, pH 8.0 and 240 mM sucrose in the presence of 120 mM NaCl.

Determination of AChE activity

AChE activity measurements were determined on rat brain homogenates and pure enzyme (eel *E. electricus*) (Sigma) according to the method of Ellman *et al.* (1961). The reaction mixture (1 ml) contained 50 mM Tris-HCl, pH 8.0 and 240 mM sucrose in the presence of 120 mM NaCl. Protein concentration was 80–100 µg/ml incubation mixture for the homogenized brain and 0.13 µg/ml for the eel *E. electricus* pure AChE. Then, 0.030 ml of 5,5'-dithionitrobenzoic acid (DTNB) and 0.050 ml of acetylthiocholine iodide, used as a substrate, were added and then the reaction started. The final concentration of DTNB and substrate were 0.125 and 0.5 mM, respectively. Moreover, AChE activities in eel *E. electricus* soluble enzyme were measured by using different concentrations of the substrate. K_M values and n_H coefficients were determined from Lineweaver-Burk and Hill plots, respectively. The enzymatic reaction was followed spectrophotometrically by the increase in absorbance ($\Delta\overline{OD}$) at 412 nm.

Statistical analysis

The data are expressed as mean \pm SD of five separate experiments performed in triplicate. Differences between means were calculated by one way ANOVA using SPSS 7.5 statistical program on an IBM computer. P values < 0.05 were considered statistically significant.

Results

As it is shown in Table I, AChE activity in rat whole brain homogenate as well as in that of eel *E. electricus* pure enzyme was equally inhibited (18–20%, $p < 0.01$) when incubated with 1.2 mM of Phe. Lower concentrations of the amino acid induced a statistically significant minor inhibition of the enzyme activity, whereas higher concentrations (> 1.2 mM) did not add further inhibitory effect on AChE (Tsakiris *et al.*, 1998a). On the contrary, the amino acid Ala had no effect on the enzyme activity when incubated with the previously mentioned concentration. Moreover, the presence of Ala together with Phe in the mixture of the enzymatic reaction reversed completely ($p > 0.05$) the inhibitory effect of Phe on AChE activity. Since the obtained results of the effect of

Table I. AChE activity in rat brain and in eel *E. electricus* when incubated with Phe, Ala and Phe+Ala vs control.

	Concentration [mM]	Activity ($\Delta\text{OD}/\text{min}$)	
		Brain AChE	eel <i>E. electricus</i> AChE
Control	0	0.087±0.003	0.106±0.004
Phe	1.2	0.071±0.001**	0.085±0.002**
Ala	1.2	0.089±0.003	0.112±0.005
Phe+Ala	1.2	0.087±0.002	0.108±0.003

** $p < 0.01$.

Values are expressed: mean±SD.

Protein concentration was 80–100 $\mu\text{g}/\text{ml}$ incubation mixture for the homogenised brain and 0.13 $\mu\text{g}/\text{ml}$ for the eel *E. electricus* pure AChE. The final concentration of acetylthiocholine iodide (substrate) was 500 μM . For other details see Materials and Methods.

the amino acids on rat brain membrane-bound AChE as well as on that of soluble AChE were similar, we assume that Phe and Ala act directly on AChE molecule in the same way. So, we proceeded with our experiments on eel-AChE pure enzyme only.

Figure 1 presents the effect of various concentrations of Ala on the inhibited AChE by Phe (1.2 mM). Ala induced a statistically significant reversion of the inhibited enzyme by Phe at concentration 0.3 mM (50% protection, $p < 0.05$) with a maximum reversion at concentrations 1.0–1.2 mM of the amino acid (100% protection, $p < 0.01$).

Furthermore, in order to investigate Ala action on the inhibited AChE by Phe, pure AChE activity was determined using different amounts of acetylthiocholine as a substrate in the presence or absence of 1.2 mM Phe, Ala, or Phe plus Ala (Fig. 2). As it is shown in this figure, Phe was an enzyme inhibitor (50%, at 50 μM of substrate) whereas Ala an effector (50%, at the same substrate concentration). The degree of Phe inhibition and Ala stimulation on the enzyme was decreased by increasing the substrate concentration. For example at concentrations 500–600 μM of the substrate, Phe inhibited the enzyme activity about 20% ($p < 0.01$), whereas Ala alone or together with Phe had no effect ($p > 0.05$).

Moreover, the reduced AChE activity was reversed to control values in the presence of Phe plus Ala at concentrations of the substrate lower than 600 μM , while it was inhibited at higher concentrations (>600 μM) and not reversed to control

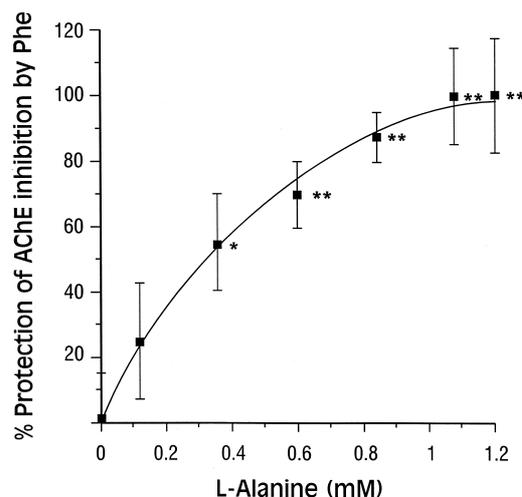


Fig. 1. Protection of different concentrations of Ala on the inhibited eel *E. electricus* pure AChE by Phe (1.2 mM). Values represent means±SD of five experiments performed in triplicate. The control value of pure AChE activity was $0.106 \pm 0.04 \Delta\text{OD}/\text{min}$ and that induced by Phe $0.086 \pm 0.002 \Delta\text{OD}/\text{min}$ for protein enzyme concentration 0.13 $\mu\text{g}/\text{ml}$ incubation mixture. One hour preincubation of the enzyme with Phe and Ala different concentrations preceded the addition of substrate at concentration of 500 μM . * $p < 0.05$; ** $p < 0.01$; as compared to control.

values. Also, enzyme inhibition was obtained with Phe or Ala alone at high concentrations of acetylthiocholine (>600 μM).

Figure 3 shows Lineweaver-Burk plots of the results presented in Fig. 2. These plots were obtained with substrate concentrations 20–250 μM , in which we also observed in Fig. 2 a 100% maximum reversal effect on the enzyme activity with Phe plus Ala compared to that with Phe alone (inhibition about 50–30%, $p < 0.001$). V_{max} remained unchanged in all cases. On the contrary, the K_M for the substrate was increased (about 150%, $p < 0.001$) with Phe, decreased (about 50%, $p < 0.001$) with Ala alone but it remained unaltered ($p > 0.05$) when Phe plus Ala were added (see Table II). Furthermore, the Hill plots of the results of Fig. 2 are illustrated in Fig. 4. The Hill coefficient (n_H) remained unchanged ($p > 0.05$) at value about 2 with Phe, Ala, or Phe plus Ala (see also Table II).

Discussion

Our previous *in vitro* studies (Tsakiris *et al.*, 1998a; Tsakiris *et al.*, 1998b; Schulpis *et al.*, 1998)

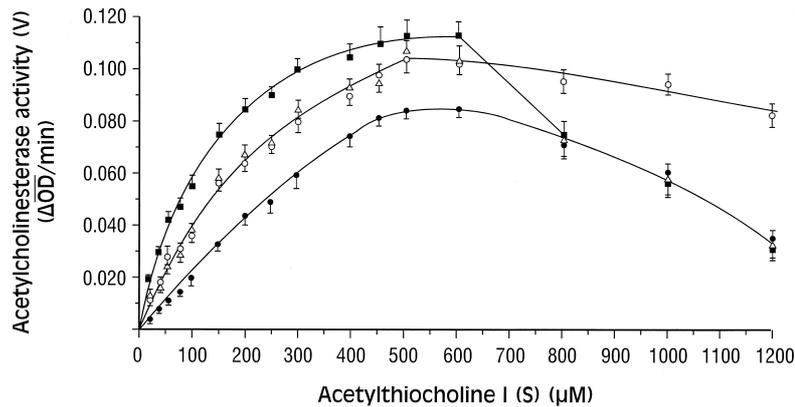


Fig. 2. AChE activity of eel *E. electricus* pure enzyme as a function of acetylthiocholine concentration, in the absence (○) and in the presence of 1.2 mM Phe (●), or Ala (■) or Phe plus Ala (△). Each point represents the average value of five experiments performed in triplicate ($\Delta\overline{OD}$, change in average optical density).

Table II. Effects of Phe, Ala and Phe+Ala on K_M values derived from Lineweaver-Burk plots and on n_H coefficients from Hill plots of eel *E. electricus* AChE activity.

	Concentration [mM]	K_M^a [μM]	Hill coefficients n_H^b
Control	0	154 ± 14	1.93 ± 0.17
Phe	1.2	$400 \pm 39^{***}$	1.99 ± 0.10
Ala	1.2	$74 \pm 10^{***}$	1.91 ± 0.15
Phe+Ala	1.2	154 ± 16	1.95 ± 0.16

*** $p < 0.001$.

Values are expressed: mean+SD.

^a The K_M values were calculated at substrate concentrations of 20–250 μM .

^b The Hill coefficients were calculated at substrate concentrations of 80–400 μM .

Protein concentration was 0.13 $\mu\text{g/ml}$ incubation mixture. For details see Materials and Methods.

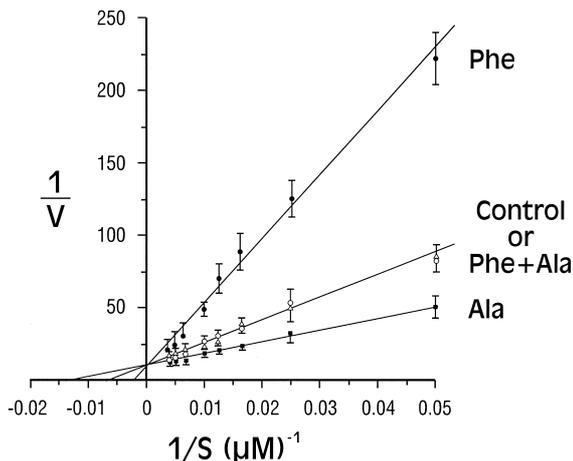


Fig. 3. Lineweaver-Burk plots of the results of Fig. 2 in substrate concentrations of 20–250 μM .

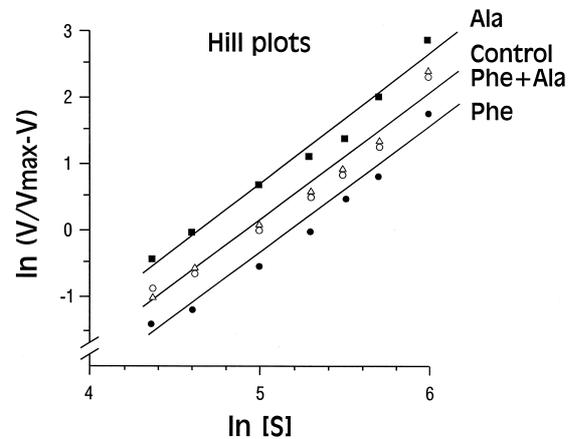


Fig. 4. Hill plots of the results of Fig. 2 in substrate concentrations of 80–400 μM , at which the rate (V) versus (S) curve was sigmoid. The correlation coefficients (r^2) for the straight lines were > 0.97 .

have shown that concentration of 1.2 mM Phe, which is the common concentration in the brain of phenylketonuric patients (Missiou-Tsagaraki *et al.*, 1988), inhibits (20%) AChE in rat brain, in rat diaphragm and in eel *E. electricus* soluble enzyme in a similar way, having a direct action on AChE molecule. Our unpublished results have recently shown *in vitro* that high Phe concentrations (1.2 mM) preincubated with human normal erythrocyte membranes caused a higher degree (up to 40%, $p < 0.001$) AChE inhibition. Moreover, Ala (1.2 mM) was able to reverse this enzyme inhibition by Phe to control values. Therefore, the degree of AChE inhibition by Phe is dependent on the kind of tissue. Wyse *et al.* (1995) have shown that Ala also reverses the inhibitory effect of Phe

on Na⁺,K⁺-ATPase activity in synaptic plasma membranes from cerebral cortex of rat. Such an action of Ala on the enzyme was assumed, when the amino acid completely reversed the inhibitory effect of Phe on AChE activity in brain and soluble enzyme (see Table I). Therefore, Ala might reverse the inhibitory effects of Phe on brain AChE and Na⁺,K⁺-ATPase in phenylketonuric untreated patients.

Additionally, the Lineweaver-Burk plots of the results of Fig. 2 showed in Fig. 3 that V_{\max} remained unaltered in all cases. On the contrary, the K_M for the substrate was increased with Phe, decreased with Ala whereas it remained unchanged with Phe plus Ala. Therefore, Phe showed a competitive inhibitory action with the substrate whereas Ala a competitive activation. Phe and/or Ala may combine with the enzyme at a position other than the active (substrate) site. The combination of the inhibitor or the effector with the en-

zyme could cause a change in the conformation (tertiary or quaternary structure) of the enzyme that may modulate the substrate site and thereby prevent (as Phe did) or promote (as Ala did) the substrate from binding. This reversal effect of Phe plus Ala on the K_M value, as compared to that of Phe (Table II), might indicate that Phe and Ala bind on the same site of the enzyme. So, Ala competition with Phe might unbind the latter from AChE molecule inducing the enzyme stimulation. If these *in vitro* results extend to the *in vivo* reality, Ala supplementation in the special therapeutic diet of the phenylketonuric patients might ameliorate the outcome of their disease.

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