Effect of Mg^{2+}-ATP on Acetylcholinesterase of *Electrophorus electricus* (L.)

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Z. Naturforsch. **51c**, 65–69 (1996); received July 17/September 8, 1995

Acetylcholinesterase, *Electrophorus electricus* (L.), Mg^{2+}-ATP

The effect of Mg^{2+}-ATP on purified acetylcholinesterase (AChE) from electric tissue of *Electrophorus electricus* (L.) was studied. The enzymatic activities were measured with acetylcholine and acetylthiocholine as substrates. The kinetic parameters V_{max}, K_m and Hill coefficient (n_H), for acetylcholine and acetylthiocholine were modified with Mg^{2+}-ATP. It was shown that acetylcholinesterase presents an apparent activation at high concentration of substrates and an inhibition in the presence of Mg^{2+}-ATP at low concentration of acetylcholine and acetylthiocholine. In addition, the data suggest that Mg^{2+}-ATP induced an allosteric modulation of the acetylcholinesterase obtained from *Electrophorus electricus* (L.), and indicate an active adenosine triphosphate participation during cholinergic activity.

Introduction

Acetylcholinesterase (E.C.3.1.1.7) is a serine hydrolase playing an essential role in cholinergic mechanism,and catalysing the hydrolysis of the natural substrate acetylcholine into acetic acid and choline (Quinn, 1987). The amino acid sequence of AChE showed that serine, histidine and glutamate are important residues for its catalytic activity (Sussman *et al.*, 1991).

Hydrolysis studies of various substrates suggested that the active center is composed of an esteratic subsite containing the active serine, an anionic site which accommodates the choline moiety of ACh and the peripheral anionic site (PAS) (Changeux, 1966; Epstein *et al.*, 1979). The conformation of the active center is affected by the occupation of PAS and its catalytic activity at low ionic strength appears to be altered upon binding of certain ligands (Shafferman *et al.*, 1992). These include d-tubocurarine, decamethonium and gallamine, ligands that bind in exclusive manner with the PAS, suggesting that occupation sites induces different active center conformation (Berman *et al.*, 1981). The control of ionic currents by acetylcholinesterase in excitable membranes is demonstrated by the effects of anticholinesterase agents on electrical activity. Such substances inhibit enzyme catalysis and are generally acylating agents which add carbamate group carbamoylating or phosphorylating of the esteratic serine site. Inhibitors that contain a site of high electronegativity and can interact with the acid group are known to show an apparently non-competitive behavior. Moreover, the inhibitors that interact with the basic group in the active center, such as prostigmine, eserine and carbachol exhibit competitive effects (Rosenberry, 1975). On the other hand, it appears that the adenosine triphosphate exerts its action at different levels during the synaptic activity.

Cholinergic synaptic vesicles containing ACh and a considerable amount of ATP have been iso-

Abbreviations: DTNB, 5-5′-dithiobis(2-nitrobenzoic acid); ATP, adenosine 5′-triphosphate; ACh, acetylcholine chloride; ATC, acetylthiocholine iodide; BSA, bovine serum albumin; PAS, peripheral anionic site.

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lated from the electric organ (Volknandt and Zimmermann, 1986). Also in skeletal muscle, the ATP is released apparently together with ACh, after stimulation of the phrenic nerve of rat (Silinski *et al.*, 1973) and addition of ATP to the incubation medium modifies the evolution of electro-physiological response during the stimulation of electric tissue (Meunier *et al.*, 1975). These obvervations indicate that ATP exerts its action at different levels during the synaptic activity.

In the present report we examine the effect of Mg^{2+}-ATP on the activity of purified AChE obtained from *Electrophorus electricus* (L.).These studies constitute a necessary step towards a more detailed investigation on the alteration of enzymatic activities observed in the presence of different nucleotides.

Material and Methods

Material

Low salt-soluble form of acetylcholinesterase was purified from the electric tissue of *Electrophorus electricus* (L.) by affinity chromatography as described by Hopff *et al.* (1973) and intensively dialysed against 50mM Tris-HCl pH 8.0 buffer. The enzyme was electrophoretically pure and presented a specific activity of 7000–9000 μmoles of acetylthiocholine hydrolysed.min^{-1}.mg $protein^{-1}$ using the Ellman assay (Ellman *et al.*,1961). Protein concentration was determined acording to the method of Lowry (Lowry *et al.*, 1951) using bovine serum albumin as a standard.

BSA, DTNB, ACh, ATC, ATP were obtained from Sigma Chemical Company ST. Louis, MO, USA and other reagents were from Merck, Darmstadt, Germany.

Enzyme assay

Acetylcholinesterase activity was determined by two different assays at 25^0C. In the first case (assay A), the amount of acetic acid produced was measured (Hassón and Liepin, 1963) and in the second (assay B), the prodution of thiocholine (Ellman *et al.*,1961).In the assay A, the standard mixture (in a total volume of 20ml) contained 0.2 M sodium acetate , 0.01 % of BSA and a varied concentration of ACh and $MgCl_2$. The pH was kept constant at 8.0 by addition of 0.1 M NaOH. A Beckman Century (SS) pHmeter was used and 0.1 M NaOH was delivered from an Agla micrometer – syringe. Readings were taken at 1 min intervals over a 4 min period. Specific activity was defined as the μmoles of ACh hydrolyzed. min^{-1} . mg $protein^{-1}$.

For assay B the standard assay mixture [50mM of Tris-HCl pH 8.0, 0.125 mM of 5–5'dithiobis (2-nitrobenzoic acid), 0.01% BSA] was mixed with a variable concentration of $MgCl_2$ and ATP in a total volume of 1ml. The reaction was started by addition of variable amounts of ATC and followed spectrophotometrically at 412 nm in a spectrophotometer (Hitachi model U – 3300).

Data analysis

The kinetic parameters were determined by a non-linear regression computing program (Sigma Plot; Jandel Scientifics, USA). The Hill coefficients were obtained using the formula: $v = V_{max}[s]_n/(K+[S]_n)$, were [S] is the concentration of substrate, n is the Hill coefficient and K is the apparent affinity, $K_{0.5}$.

Results

The effect of Mg^{2+}-ATP on purified acetylcholinesterase of electric organ from *E. electricus* (L.) was determined at varying concentrations of ACh and ATC as substrates. The kinetic data were analysed using the Michaelis-Menten and Lineweaver-Burk plots.

Figure 1 shows the results of enzyme activity (V/Vo) in presence of different concentrations of $MgCl_2$ (0.1–20 mM) and a fixed concentration of ATP (0.5 mM). Inset of the figure shows the AChE activity at increasing concentration of ATP (0.05–5.0 mM), using a fixed $MgCl_2$ (5.0 mM) concentration. The action of Mg^{2+} as an activator of AChE is observed when increasing the Mg^{+2} concentration from 0.1 to 5.0 mM in the presence of 0.5 mM ATP. However, concentrations of 5.0–20 mM of $MgCl_2$ showed an activity decrease, despite the presence of 0.5 mM ATP.

In order to compare the results obtained from the experimental procedure above described, we verified (Fig.2) the enzyme activity at different

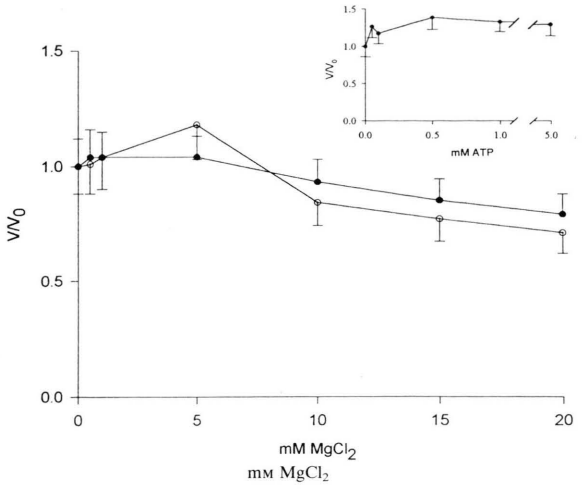

Fig. 1. Effect of $MgCl_2$ on the enzymatic activities of AChE in presence (○) and absence (●) of 0.5 mM ATP. Inset shows the enzymatic activities in function of concentration of ATP in presence of 5.0 mM $MgCl_2$. All assays were performed in presence of 1.0 mM ACh as substrate. The results are the means of three experiments ± SD.

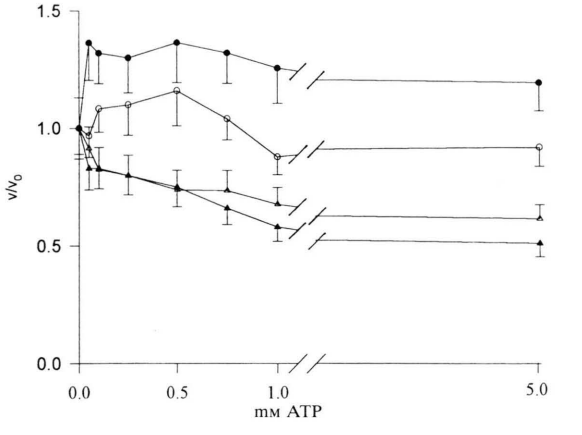

Fig. 2. Effect of ATP on the enzymatic activities of AChE in presence of 5.0 mM $MgCl_2$. The assay was performed with different concentrations of substrate: (▲) 25 μM and 100 μM (●) of ATC, (△) 0.25 mM and 1 mM (○) of ACh. The reaction mixture contained all the components as described under "Material and Methods". The results are the means of three experiments ± SD.

concentrations of ACh (0.25 and 1.0 mM) and ATC (25 and 100 μM) in the presence of ATP (0.05–5.0 mM). The inhibitory effect produced by Mg^{2+}-ATP on AChE activity with these two substrates was more evident at low concentrations of ACh and ATC, moreover, at higher concentrations the activity was increased.When plotting the specific activity versus ACh (0.1–1.5 mM) and ATC (5–150 μM) concentration in presence of Mg^{2+}-ATP (Fig.3), an increase of V_{max} and modifications of kinetic parameters (K_m and n_H) could be observed.

The Hill coefficient (n_H) has a complex physical significance, nevertheless, coefficients greater than 1.0 generally reflect positive cooperativity at

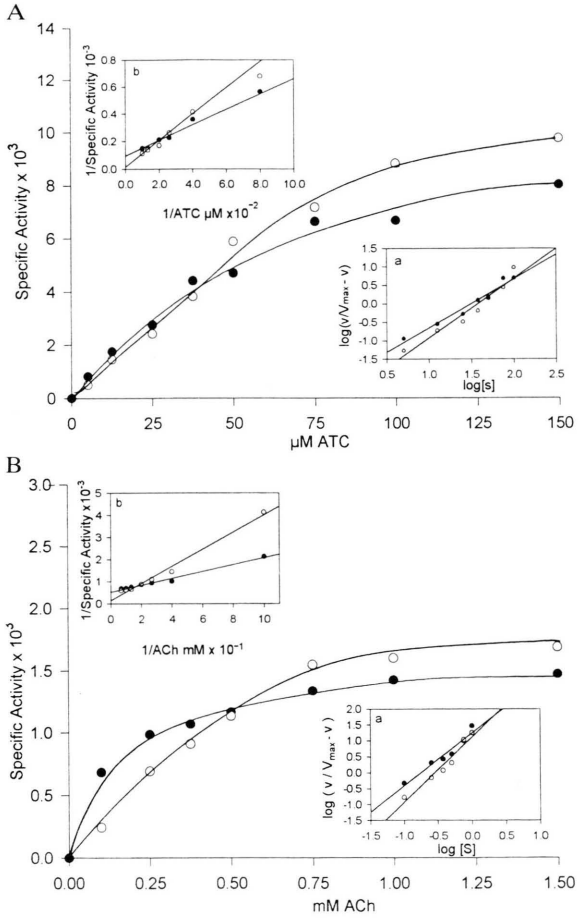

Fig. 3. Influence of ATP on the activities of AChE. All assays were performed in presence of 5.0 mM $MgCl_2$. Inset (a) shows the Hill plots of the two curves and (b) double-reciprocal plots of effect of ATP on hydrolysis of ACh and ATC. (A) ATC and (B) ACh as substrate. (●) Control and (○) in presence of 0.5 mM ATP. The enzymatic activity was expressed in μmoles of substrate hydrolyzed. min^{-1}. mg $protein^{-1}$. The experimental conditions are described under "Material and Methods".

Table I. Effect of Mg^{2+}-ATP on the kinetic parameters of AChE.

Substrate	0 ATP			0.5 mM ATP		
	V_{max}	K_m	n_H	V'_{max}	K'_m	n'_H
ACh	1.72×10^3	0.22	1.66 ± 0.12	2.55×10^3	0.63	2.05 ± 0.12
ATC	11.80×10^3	69.77	1.31 ± 0.19	19.29×10^3	132.01	1.61 ± 0.25

V_{max} and V'_{max} are given as μmoles of substrate hydrol. min^{-1}. mg $protein^{-1}$. K_m and K'_m as mM for ACh and μM for ATC. n_H and n'_H are the Hill coefficient. The activity and other parameters were determined as described in Material and Methods. Assay: A for ACh and B for ATC.

multiple interacting ligand binding sites. The kinetic results of catalytic hydrolysis of ACh and ATC are summarized in Table I.

Discussion

The principal biological role of acetylcholinesterase is termination of the impulse transmission of cholinergic synapses by rapid hydrolysis of the neuro-transmitter acetylcholine. The electrophysiological responses are modified by ATP during the stimulation of electric tissue (Meunier *et al.*, 1975). Some hypothesis of the possible mechanism of action of the adenosine derivatives and ATP is that they depress neuronal excitability indicating that this action is accompanied by hyperpolarization that may result from an increase in membrane K^+ conductance (Tomita and Watanabe, 1973).

Effect of Mg^{2+} and Ca^{2+} on soluble and membrane-bound AChE from *E. electricus* (L.) shows that $MgCl_2$ and $CaCl_2$ cause an increase in the maximum of hydrolysis of ACh by soluble AChE and produce a decrease when the enzyme is in membrane-bound form (Robaire and Kato, 1974). Until the present date was not clear whether the activation of AChE by cations is due to a structure specificity around the active center or at a site distinctly different from the catalytic center. Probably the Mg^{+2} changes the conformation of this active site as postulated by Changeux (1966).

Using acriflavine as an effector, Wermuth and Brodbeck (1973) showed that AChE may exist in two catalytically different states. In state 1, the AChE apparently has a higher affinity for the ACh substrate than in state II. The catalytic constant is also higher in state I than in state II. In state I, AChE effectors such as hexametonium, nicotine, tensilon, atropine and d-tubocurarine compete with acriflavine for the same AChE binding site. While in state II, the effects varied with the nature of the second ligand. The binding of hexametonium, atropine or d-tubocurarine rendered the site less accessible for acriflavine, resulting in an apparent activation of the enzyme and as for tensilon or nicotine they did not decrease the inhibition by acriflavine (Wermuth *et al.*, 1975).

The PAS occupation by different ligands induces active-center conformation as proposed by Berman *et al.* (1981). Recently, Ordentlich *et al.* (1993) suggested the possible involvement of allosteric effect modulation activity a "cross talk" between aromatic residues at the PAS and residues Trp 86 and Tyr 337 at the active center of AChE.

Our observation shows activation of AChE by Mg^{+2}-ATP, using high concentrations of ACh and ATC, but at low substrate concentrations the AChE activity decreases.This suggests that the Mg^{2+}- ATP inhibits or activates the enzyme as a function of substrate concentration probably binding with PAS. Otherwise the Mg^{2+}-ATP may bind tightly to one or more arginine residues of AChE. Until this moment there hasn't been varied endogenous substances, that normally are produced by the organism, that may modify the enzymatic activity of AChE through the PAS binding. Most of the previously described substances normally are not found during neuro-muscular transmission. The possible actions of Mg^{2+}-ATP with PAS explains (in part) the importance of this anionic site, giving evidence for the allosteric property of acetylcholinesterase.

Acknowledgements

Financial support from the CEPEG of the U.F.R.J., CNPq and FINEP is gratefully acknowledged.

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