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SHORT COMMUNICATIONS

Noncompetitive inhibition by aluminum, scandium and yttrium of acetylcholinesterase from *Electrophorus electricus*

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The neurotoxicity of aluminum was first established in 1897 when Dölken [1] injected aluminum tartrate into rabbits and found that they developed neuronal degeneration in different parts of the brain. Changes in the CNS following systemic administration of Al^{3+} salts were described by Seibert and Wells in 1929 [2], and the epileptogenic effect of aluminum hydroxide paste was described by Kopeloff *et al.* in 1942 [3]. To date, however, little is known about the mechanism of the toxic action of aluminum. Al^{3+} toxicity is a serious consideration for a number of medical, industrial and other scientific groups. Recently, aluminum has been implicated as a toxic agent in senile dementia of the Alzheimer type [4–7]; it is a problem of therapeutic importance for dialysis patients receiving aluminum hydroxide gels [8]. The antiperspirant action of aluminum salts remains a subject of several opposing hypotheses to explain the mechanism of the drying action [9]. Furthermore, the neurotoxicity of aluminum is of particular importance to individuals in geographical regions where soluble Al^{3+} salts are believed to be leached out of the soil by acidic rain conditions resulting from acid atmospheric pollutants [10, 11].

There is some experimental evidence for alteration by polyvalent cations in general and aluminum ions in particular of the synthetic and metabolic enzymes of cholinergic neurotransmission. Altered levels of choline acetylase and acetylcholinesterase (AChE) have been reported in brain tissue of rabbits with neurofibrillary degeneration induced by intracisternal injection of Al^{3+} salts [12]. Patočka [13] demonstrated that Al^{3+} activates purified erythrocyte AChE. Mouse neuroblastoma cells showed decreased AChE activity when grown in Al^{3+}-containing culture medium [14]. In our own studies, we have shown that lanthanium (La^{3+}) binds to partially purified AChE from the electroplaques of the electric eel and to the enzyme in intact electroplaques. This trivalent cation exerts a marked, concentration-dependent inhibition of enzyme activity. It was suggested that La^{3+} interacts with regulatory, non-catalytic anionic sites on the enzyme in a manner competitive with Ca^{2+} [15, 16] and partially competitive with decamethonium [16].

The present study examines the influence of aluminum chlorohydrate on the activity of neuronal AChE and the effects of Ca^{2+}, decamethonium (C-10) and eserine on Al^{3+} interaction with the enzyme. The effects on AChE activity of two other trivalent cation salts, scandium chloride ($ScCl_3$) and yttrium chloride (YCl_3), were also studied. These cations have been of interest as possible medicinal agents and cosmetic preparations [17].

Acetylcholinesterase (AChE; acetylcholine hydrolase; EC 3.1.1.7) partially purified from the electric organ of *Electrophorus electricus* was obtained from Worthington Biochemicals, Freehold, NJ. Two different batches of the enzyme preparation were used in this study with optimal specific activities of 42 mmoles ACh hydrolyzed·hr^{-1}·(mg protein)$^{-1}$ and 98 mmoles ACh hydrolyzed·hr^{-1}·(mg protein)$^{-1}$. A pH-stat automatic titrator was used to measure hydrolytic activity as described previously [18]. This technique was chosen above colorimetric assays, as multivalent cations interfere with the latter and the pH-stat permits studies of the natural enzyme substrate. The rate of hydrolysis of 0.1 to 10 mM AChCl was measured. K_M and V_{max} values for AChE were obtained from double-reciprocal plots by the computer-assisted data analysis of Cleland [19] using data points for substrate concentrations below 10 mM. Higher concentrations produced appreciable substrate inhibition and caused the reaction scheme to deviate from Michaelis–Menten kinetics. An apparent inhibitor constant (K_{iapp}) was calculated from the double-reciprocal plot as described by Mahler and Cordes [20] for noncompetitive inhibitors, where $K_{iapp} = i/V/V_i - 1$. The intersection points with the vertical axis give $1/V$ and $1/V_i$, and i is the concentration of inhibitor. A final enzyme concentration of 10 ng protein/ml was used in all the reaction solutions (10 ml total volume). Temperature was maintained at 25° and reaction pH at 7.4. Solutions for all experiments were made up in standard physiological saline for electric eels [21] composed of 188 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 1 mM Tris–HCl (pH 7.4). Al^{3+} was added to the test solutions as aluminum chlorohydrate (Pfaltz & Bauer, Stamford, CT; analysis: Al = 24.4 to 25.4 mol%; Al_2O_3 = 46.0 to 48.0 mol%; and Cl = 15.8 to 16.8 mol%).

The range of concentrations of Al^{3+} tested in this study was restricted by the limited solubility of the Al^{3+} salts. The upper limit of solubility in eel physiological saline was initially observed to be about 0.5 mM. Aluminum chlorohydrate is an internally buffered form of the basic Al salt, $Al_2(OH)_5Cl$ [22]. Measurements of the hydrolysis and precipitation of Al salts in aqueous solutions have indicated that monomeric and polynuclear hydrolyzed ion species are formed in the pH range of the present studies [23]. Clearly, these must be considered in order to estimate the true solubility of the cation. Furthermore, addition of enzyme to the reaction mixture is accompanied by binding of cations to the protein and a subsequent change in the number of free Al^{3+} ions. Because of these considerations and the observed nonlinearity of the dose-related Al^{3+} inhibition of AChE activity, experiments were run to estimate the relative free Al^{3+} in the same reaction mixtures used for kinetic measurements. This was done by measuring the electrical conductivity of 0.01 to 1.5 mM Al chlorohydrate in buffered eel saline (pH 7.25) containing 1 mM AChCl and 10 ng enzyme protein/ml. Conductivity measurements were made in a 4 ml volume using a YSI model 31 conductivity bridge (Yellow Springs Instruments, Yellow Springs, OH). Enzyme activity of these reaction mixtures was also measured.

Decamethonium bromide was obtained from ICN Pharmaceuticals, Plainview, NY, physostigmine ("eserine") sulfate from the Sigma Chemical Co., St. Louis, MO, and scandium chloride and yttrium chloride from K & K Biochemicals, Plainview, NY.

Figure 1A shows that 0.1 and 0.5 mM Al^{3+} chlorohydrate inhibited AChE at all substrate concentrations tested (0.1 to 10 mM). From the Lineweaver–Burk plot in Fig. 1B it can be seen that the inhibition by Al^{3+} was purely noncompetitive, exhibiting an altered V_{max} with no change in the K_M for ACh. This is confirmed in Table 2 showing the values of V_{max} and K_M determined by the Fortran computer program of Cleland [19]. Data are included for 0.01 mM Al^{3+}, in addition to the data from Fig. 1.

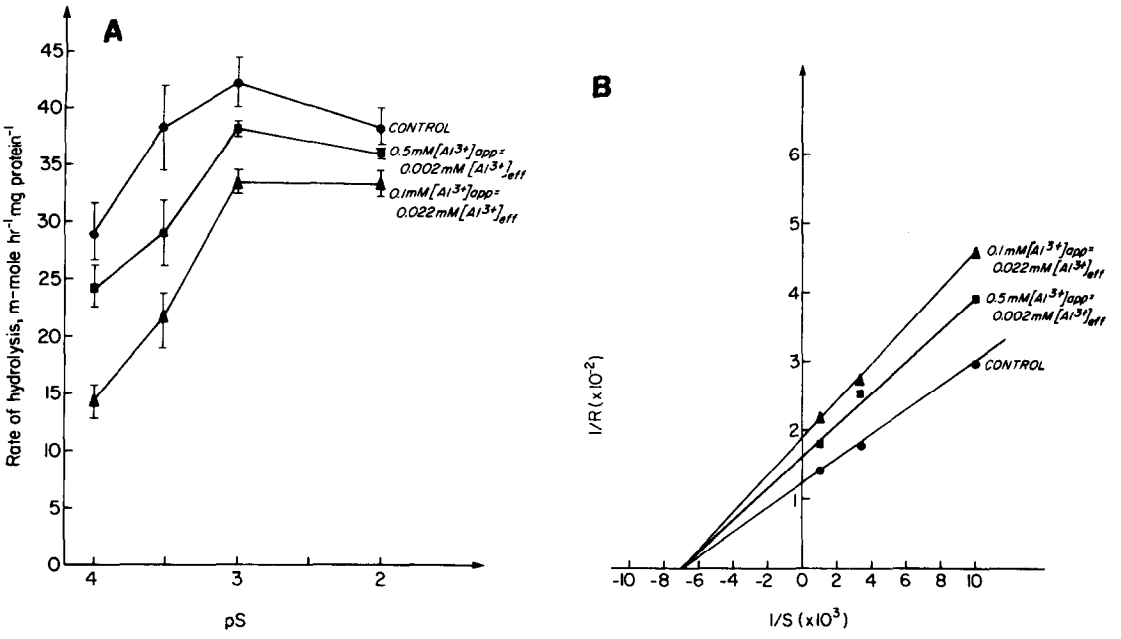

Fig. 1.(A) Effect of 0.1 mM (▲—▲) and 0.5 mM (■—■) "apparent" or "empirical" Al chlorohydrate concentrations and equivalent "effective" concentrations on the activity of purified eel AChE. Substrate is Ach; pS is the negative logarithm of the substrate concentration. Determinations were made at molar concentrations of ACh corresponding to pS = 4 (0.1 mM), pS = 3.52 (0.3 mM), pS = 3 (1 mM) and pS = 2 (10 mM). Rate values (R) were measured for the initial 5 min of the reaction. Controls (●—●) describe the rate of hydrolysis of ACh in normal physiological saline [21]. Daily controls did not demonstrate significant variations in the specific activity of the enzyme over the period of use. Each point is the mean of between five and eight experiments. Vertical bars are used to indicate the range of the mean ± S.E., except where the values lie within the area of the symbol used to denote a point. The standard error is $\sqrt{\Sigma(Xi\text{-}X)^2/n(n\text{-}1)}$. (B) Double-reciprocal plots of the data from panel A for ACh concentrations less than the level producing substrate inhibition.

The observation that 0.5 mM Al^{3+} chlorohydrate was less active than a 5-fold lower concentration of the salt may be due to the solution properties of Al^{3+} salts in water and the formation of so-called "aluminates" or aggregates [24]. The inhibition by 0.5 mM Al^{3+} more closely paralleled the minimal inhibition by 0.01 mM Al^{3+}, a 50-fold lower concentration of the Al^{3+} salt (see Table 2). The results of solution conductivity measurements carried out to resolve this apparent nonlinearity are shown in Table 1. Corresponding "breaks" in both the enzyme inhibition data and the conductivity data suggest that at 0.1 mM Al chlorohydrate a maximum amount of the ionic Al^{3+} species was available in the reaction solution and, at this concentration, the maximum effect of Al^{3+} on AChE may have been observed with these reaction conditions of pH, saline composition and ionic strength. However, it should be noted that in the data shown in Table 2 all three Al^{3+} concentrations lowered the maximum AChE activity to a statistically equivalent level while exhibiting quite different ion conductivity values in solution.

The data in Table 1 indicate that the "effective" Al^{3+} concentrations were substantially different than the molar concentrations which were prepared in the laboratory. By least squares regression analysis, a proportionality curve

Table 1. Electrical conductivity of aluminum chlorohydrate–enzyme–substrate reaction solutions*

Aluminum chlorohydrate (mM) "Empirical"	"Effective"	Conductivity (μmhos × 10^4)	Rate of hydrolysis [mmoles·hr^{-1}·(mg protein)$^{-1}$]
0	0	1.50 ± 0.04 (4)†	17.3
0.01	0.01	1.55 ± 0.01	12.2
0.05	0.05	1.63 ± 0.04	8.9
0.10	0.022	1.59 ± 0.02	9.1
0.50	0.002	1.48 ± 0.02	11.0
1.00‡		1.60 ± 0.01	10.3
1.50‡		1.52 ± 0.01	9.6

* The reaction medium duplicated the reaction conditions of the present studies and contained: 1 mM AChCl, 10 ng enzyme protein/ml, and eel saline. The pH of all solutions was adjusted to 7.25.

† Number of measurements; N = 4 for all solutions.

‡ Precipitation was evident in these solutions.

was derived from the conductivity data and "effective" Al^{3+} concentrations were extrapolated. The assumption was made that the conductivity values are directly proportional to "effective" Al^{3+} concentration between 0 and 0.05 mM "empirical" Al^{3+} concentrations. The following definitions were established: $[Al^{3+}]_{emp}$ = the empirical molar concentrations that the reaction mixtures were prepared to be; $[Al^{3+}]_{eff}$ = the effective molar concentrations that reaction mixtures were determined to be by extrapolation of the ion conductivity data in Table 1. Both concentration values are shown in Table 1. Notably, 0.1 mM $[Al^{3+}]_{emp}$ represented a higher effective ion concentration than 0.5 mM $[Al^{3+}]_{emp}$.

Apparent inhibition constants (K_{iapp}) were calculated from the V_{max} values in Table 2 and the corresponding $[Al^{3+}]_{eff}$ values. K_{iapp} = 0.047 mM at $[Al^{3+}]_{eff}$ = 0.022 mM; and K_{iapp} = 0.018 mM at $[Al^{3+}]_{eff}$ = 0.01 mM. An inhibition constant was not calculated for the data at 0.5 mM $[Al^{3+}]_{emp}$ because of the extremely low conductivity of the reaction solutions at that concentration. It is quite clear that our results qualitatively indicate that Al^{3+} acts as a noncompetitive inhibitor of AChE. Our present data, however, do not permit a better estimate of K_i than the range of 0.02 to 0.05 mM until uncertainties regarding effective $[Al^{3+}]$ are resolved.

Table 2 describes the effects of 0.1 μM eserine sulfate, a competitive carbamylating inhibitor [25], 10 μM decamethonium (C-10), a partly noncompetitive AChE inhibitor [16, 26], and 10 mM Ca^{2+}, an activator cation thought to function primarily via peripheral anionic sites [27, 28], on the enzyme inhibition by 0.1 mM and 0.5 mM Al^{3+} at the optimal ACh concentration of 1 mM. At the concentrations studied, which were selected from earlier studies showing activity in that range [15, 16, 27], Ca^{2+} and eserine had no effect on the noncompetitive enzyme inhibition by Al^{3+}. In the presence of C-10, however, inhibition was increased to the level reported earlier for AChE inhibition by 10 μM C-10 alone, i.e. about 50% [16]. Thus, the C-10 inhibition of AChE, also noncompetitive [16], was fully expressed in the presence of Al^{3+}. These data suggest that Al^{3+} is bound to different anionic sites than those that bind the enzyme modifiers Ca^{2+} and C-10. However, additive effects via "allosterism" cannot be ruled out from simple kinetic experiments on ternary complexes.

The activity of purified AChE was also measured in the presence of 0.1 mM Y^{3+} and 0.1 mM Sc^{3+}, trivalent cations with physical properties suitable for binding to anionic sites in a manner similar to Ca^{2+} and La^{3+} binding [17]. Enzyme activity was inhibited by Y^{3+} and Sc^{3+} at all substrate concentrations tested, and analysis of double-reciprocal plots of the data for suboptimal substrate concentrations (Table 2) shows that inhibition by these trivalent cations also results from noncompetitive interaction with the enzyme. This is similar to the action of Al^{3+} but contrasts with the mixed competitive–noncompetitive inhibition by La^{3+} [15].

Considerable evidence supports the suggestion by Changeux [29] that AChE can undergo ligand-induced conformational changes [30–33]. At least three classes of peripheral anionic sites have been described for bovine erythrocyte AChE [34, 35]. Although these peripheral sites are distinct from the catalytic site, binding of cationic ligands can influence enzyme activity. It has been suggested that the enzyme can exist in at least two distinct functional states dependent on the occupancy of these peripheral sites [36]. Anionic sites on AChE include the α- or catalytic sites, β- or "allosteric" peripheral sites (labeled P_1 by Rosenberry [35], and γ-sites, a second possibly "allosteric" and more hydrophobic group of peripheral anionic sites (labeled P_2-P_4 [35]). Roufogalis and Quist [34] found that Ca^{2+} and C-10 compete for the β-site, a site that also binds tetraethylammonium ions. They attributed the ability of gallamine to antagonize inhibition by C-10 to allosteric perturbations of the α- and β-sites via interaction at the γ-sites. As Al^{3+} inhibition was not altered by Ca^{2+} or C-10 in the experiments presented in Table 1, it is suggested that Al^{3+} may alter ligand binding at the catalytic site by interaction at the γ-peripheral anionic sites. Y^{3+} and Sc^{3+} may also inhibit AChE via the γ-peripheral anionic sites.

The observation that the catalytic activity of AChE can be modified by the binding of multivalent inorganic cations to sites other than the catalytic site suggests a possible mechanism of Al^{3+} neurotoxicity. Many of the symptoms of Al^{3+} toxicity are behavioral modifications, including loss of short-term memory, that have been associated with deficits of central cholinergic transmission [37, 38]. Preliminary *in vitro* studies indicate that, in addition to inhibit-

Table 2. Effects of Al^{3+} in the presence of Ca^{2+}, eserine, and decamethonium (C-10) and the effects of Y^{3+} and Sc^{3+} on the V_{max} and $K_{M_{app}}$ of soluble AChE from *Electrophorus electricus**

	V_{max} [mmoles·hr^{-1}·(mg protein)$^{-1}$]	$K_{M_{app}}$ (mM)
Aluminium experiments		
Control (7)	79.6 ± 5.8	0.09 ± 0.028
0.5 mM Al_{emp} = 0.002 mM Al_{eff} (8)	57.8 ± 4.5	0.121 ± 0.037
+ 10 mM $CaCl_2$ (4)	58.4 ± 6.3	0.083 ± 0.041
+ 0.1 μM eserine SO_4 (3)	66.5 ± 4.5	0.086 ± 0.025
+ 10 μM C-10 (3)	48.6 ± 6.2	0.095 ± 0.054
0.1 mM Al_{emp} = 0.022 mM Al_{eff} (5)	54.1 ± 4.5	0.122 ± 0.037
+ 10 mM $CaCl_2$ (4)	58.4 ± 7.4	0.121 ± 0.056
+ 0.1 μM eserine SO_4 (4)	59.1 ± 6.9	0.137 ± 0.055
+ 10 μM C-10 (3)	44.6 ± 3.3	0.124 ± 0.033
0.01 mM Al_{emp} = 0.01 mM Al_{eff} (5)	42.9 ± 14.6	0.084 ± 0.024
Scandium and yttrium experiments†		
Control (4)	116.4 ± 2.3	0.18 ± 0.02
0.1 mM Y^{3+} (3)	77.1 ± 8.8	0.16 ± 0.05
0.1 mM Sc^{3+} (3)	82.1 ± 12.7	0.37 ± 0.15

* V_{max} and K_M were determined from double-reciprocal plots of reaction rate as a function of substrate concentration using the computer-assisted data analysis techniques of Cleland [19]. The values are given as the mean ± standard deviation and (N), the number of experiments for each data point.

† These experiments were run on a different enzyme batch than the Al^{3+} experiments.

ing AChE, Al^{3+} also inhibits the activity of choline acetyltransferase in frog brain homogenates (N. Ham and J. K. Marquis, unpublished data). Studies are continuing to further define the alterations of cholinergic enzymes by Al^{3+} and other multivalent cations.

In summary, measurements of altered activity of soluble acetylcholinesterase from *E. electricus* electric organ by the inorganic cations aluminum, scandium and yttrium demonstrate that these ions are noncompetitive enzyme inhibitors. Al^{3+} inhibited enzyme activity at all substrate and inhibitor concentrations studied. Inhibition by Al^{3+} did not appear to be sensitive to the active site-specific, competitive ligand physostigmine or to calcium, a peripheral site-binding activator cation. Inhibition by another peripheral site-binding noncompetitive inhibitor, decamethonium, was not altered by Al^{3+}. Al^{3+} appears thus to have interacted with a class of peripheral anionic sites on AChE distinct from the β- or P_1 peripheral anionic sites that bind Ca^{2+} and C-10 and may be a useful probe of a subclass of γ- or P_{2-4} peripheral anionic sites. A possible mechanism for Al^{3+} neurotoxicity, via alterations of the enzymes of cholinergic neurotransmission, is also suggested.

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