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Aluminum Activation and Inactivation of Bovine Caudate Acetylcholinesterase

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Aluminum has been implicated as a toxic agent in a number of neurological disorders (Alfrey et al., 1976; Perl and Brody, 1980) and may be a significant environmental health hazard (Johnson, 1979; Cronan and Schofield, 1979). Elevated Al^{3+} concentrations have been related to impaired motor function (Bowdler et al., 1979) and to a number of cognitive deficits, including loss of memory and a form of dementia often compared to senile dementia of the Alzheimer type (Crapper and Dalton, 1973; Hetnarski et al., 1980). Neurofibrillary degeneration similar, but not identical to, the Alzheimer type of degeneration has been observed in brain regions with elevated Al^{3+} levels (Perl and Brody, 1980). In addition, a form of dementia can be correlated to elevated brain Al^{3+} levels in patients undergoing chronic dialysis therapy and ingesting significant amounts of aluminum hydroxide to balance phosphate metabolism (Alfrey et al., 1976; McDermott et al., 1978).

We have found that Al^{3+} is a noncompetitive inhibitor of electric organ acetylcholinesterase (AChE; EC 3.1.1.7), and that Al^{3+} binds to a peripheral anionic site distinct from that which binds Ca^{2+} or decamethonium (Marquis and Lerrick, 1982). However, studies with human serum cholinesterase (ChE; EC 3.1.1.8) suggest that Al^{3+} and Ca^{2+} interact with the same anionic site and that aluminum toxicity may, in fact, be provoked by a hypocalcemic condition, since the enzyme is protected from Al^{3+} inhibition in high Ca^{2+} media (Marquis, 1983). Furthermore, Wenk and Stemmer (1981) have shown that the toxic effects of ingested aluminum on central adrenergic transmitters are enhanced in animals fed diets deficient in Cu^{2+}, Zn^{2+}, Fe^{2+}, or Mg^{2+}.

Al^{3+} has previously been reported to inhibit enzyme systems involving both ATP and Mg^{2+} (Harrison et al., 1972) and the brains of rabbits injected intraventricularly with $AlCl_3$ salts exhibit significant alterations in cholinergic enzyme activity. The present studies are designed to examine in vitro the toxicity of Al^{3+} with AChE partially purified from bovine caudate nucleus and enzyme chemically modified to mask at least one class of anionic sites.

MATERIALS AND METHODS

Whole calf brains were obtained fresh from a nearby slaughterhouse and transported on ice to the laboratory where the caudate nucleii were dissected and stored at -70°C until used for extraction and purification of AChE. Membrane-bound AChE was partially purified by the procedures of Rakonczay et al. (1981). Briefly, the caudate nucleii were homogenized in 10 ml per caudate of saline buffer (12.5 mM sodium phosphate, pH 7.2, 0.4M NaCl) and the suspension was centrifuged at 53,000 x g for 2 hours. The supernatant was discarded and the pellet rehomogenized in an equivalent volume of distilled water. The suspension was added slowly to a 9-fold excess of buffer containing 0.55% Triton X-100, stirred for 30 minutes, and centrifuged as above. The resulting pellet was discarded, and the supernatant was purified by affinity chromatography. The solubilized enzyme was absorbed on a Concanavalin A-Sepharose 4B column and eluted with 0.5 M α-methyl-D-mannoside. This partially purified enzyme preparation, eluted from the Concanavalin A column with a specific activity of 600-1200 μmoles/hr/mg protein, was utilized for the present studies (Table 1).

Table 1: Summary of purification of calf brain AChE

	PROTEIN		AChE ACTIVITY		
FRACTION	mg	%	Specific Activity μmoles/hr/mg prot.	Total Units	% Recovered
Homogenate	977	100	26.5	25,890	100
Supernatant 2 (Triton X-100)	738	76	30.1	22,214	86
Con-A Sepharose	11.9	1.2	1018.7	12,122	48.8

This preparation was obtained from 3 pairs of calf caudate nuclei, wet wgt.=13.7g. Solubilization was carried out in 13.75mM PO4/0.4M NaCl/0.5% Triton X-100, pH 7.2. AChE activity is defined as the amount of AChE required to hydrolzye 1μmol of acetylthiocholine (ASCh) per hour. Specific activity is expressed as μmol of ASCh hydrolyzed/hour/mg protein.

Protein content was measured by the procedure of Wang and Smith (1975) which excludes interference by Triton X-100. Enzyme activity was assayed by the Ellman procedure (1961) in 2 mM TRIS buffer, pH 7.2, chosen to optimize solubility. High ionic strength experiments were run in 2mM buffer with 0.1M NaCl added. Controls demonstrated optimum enzyme activity in the high ionic strength media. K_{Mapp} and V_{Max} were determined from double-reciprocal plots of reaction rate as a function of substrate concentration for acetylthiocholine (ASCh) concentrations of 0.05mM to 1.2mM.

For carbodiimide modification of AChE, the water-soluble carboxyl group affinity reagent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) was prepared in 2mM Pipes buffer, pH 6.8. The slightly lower pH should favor the reaction of EDAC with carboxyl groups (Means and Feeney, 1971). Reversible and irreversible effects of EDAC on enzyme activity were assayed as follows: 1) to measure reversible effects, the reaction was carried out in 1mM EDAC, 2mM Pipes buffer, 0.05-1.2mM ASCh and 0.33mM DTNB; 2) to measure irreversible effects of the carbodiimide, the enzyme was pretreated with 1mM EDAC and ASCh hydrolysis was measured in the same reaction buffer as above but without EDAC.

Aluminum chlorohydrate was purchased from Pfaltz & Bauer, Stamford, CT. All other compounds were purchased from Sigma Chemical Co., St. Louis, MO.

RESULTS AND DISCUSSION

Table 2 exhibits V_{Max} and K_{Mapp} values, determined from double-reciprocal plots as described above, for varying concentrations of Al^{3+}. In low ionic strength, low concentrations (1-10μM) increase the activity of AChE with no change in the affinity of the catalytic site for the substrate ASCh. Higher concentrations (50-200μM) produce a predominantly noncompetitive inhibition of enzyme activity; and 500μM exerts a mixed competitive-noncompetitive inhibition. Reversal of inhibition by 100μM and 500μM Al chlorohydrate was measured by adding a 10-fold excess of EDTA (ethylene diamine tetraacetic acid), a cation chelating agent, to the incubating media. Assays were run at both low and high ionic strength and demonstrated that enzyme inhibition can be completely reversed by chelation. Dilution experiments, similarly, demonstrated complete reversal of the multivalent cation effects in the concentration range studied.

At low ionic strength, rapid enzyme activation is seen with Al chlorohydrate concentrations three orders of magnitude below the total ionic strength of the buffer. The activation is an apparent allosteric effect and involves no change in the affinity of the catalytic site of AChE for ASCh. At higher concentrations, still well below the ionic strength of the buffer, a competitive effect becomes evident. In high ionic strength buffer, on the other hand, Al^{3+} appears to be exclusively an inhibitor of bovine brain AChE, exhibiting largely noncompetitive inhibition at lower concentrations and mixed competitive-noncompetitive inhibition at higher concentrations. The enzyme preparation used in this study is about 100-200-fold less pure than homogeneous bovine caudate AChE.

Thus, the possibility of nonenzymic binding sites for Al^{3+} cannot be ruled out. The observation, however, that noncompetitive inactivation persists at high ionic strength indicates that peripheral ligands may bind to the enzyme in physiological milieu and may influence enzyme reactivity *in situ*.

Table 2. Effects of aluminum on the V_{Max} and K_{Mapp} of bovine caudate AChE in low and high ionic strength (μ) media*

CATION conc. (μM)	K_{Mapp}		V_{Max}	
	‡Low μ	High μ	Low μ	High μ
0	.07 ± .02	.09 ± .02	627 ± 24	718 ± 28
1	.06 ± .01	.08 ± .01	695 ± 10	751 ± 23
2	.05 ± .02	.09 ± .02	no data	no data
5	.06 ± .02	.09 ± .02	no data	no data
10	.07 ± .01	.08 ± .02	527 ± 22	680 ± 51
50	.06 ± .03	.11 ± .02	496 ± 24	543 ± 30
100	.07 ± .01	.13 ± .06	434 ± 13	629 ± 47
200	.14 ± .02	.20 ± .07	420 ± 22	525 ± 21
500	.63 ± .33	.41 ± .20	266 ± 53	301 ± 48

*Values for maximum enzyme activity and substrate affinity were determined by Lineweaver-Burk plots, with 0.05mM-1.2mM ASCh, calculated on an Apple II computer interfaced to an LKB Ultrospec 4050. Experimental data are expressed as the mean ± standard error of between 3 and 5 kinetic assays.

‡Experiments were run in buffers of low μ, i.e. low ionic strength (2mM TRIS, pH 7.2) and high ionic strength (with 0.1M NaCl added).

As shown in Table 3, the water-soluble carboxyl group affinity reagent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), exerts a small but significant effect on the catalytic activity of AChE. When assayed with increasing substrate concentrations (0.05mM-1.2mM), in the presence of a fixed EDAC concentration, reversible competitive inhibition of enzyme activity is evident. If the enzyme is incubated 4-7 hr with 1mM EDAC (5°C), and aliquots are removed for assay to a buffer-substrate reagent without EDAC, irreversible enzyme inhibition is evident as a 17 ± 8% decrease in V_{Max} with no change in the K_{Mapp} for ASCh. In addition, the carbodiimide-treated enzyme is not activated by 1mM Ca^{2+} or by 10 μM Al^{3+} A minimum of 4 hr treatment of enzyme with EDAC is required to reduce the activation by cations and, as reported by Roufogalis and Wickson (1973), this effect of EDAC increases progressively with time. It is furthermore interesting to note that inhibition of carbodiimide-modified enzyme by higher concentrations of Al^{3+} is exclusively competitive inhibition, with no involvement of peripheral anionic sites, presumably evident as noncompetitive inhibition of ASCh hydrolysis (see Table 3).

Table 3. Carbodiimide* modification of anionic site reactivity of bovine caudate AChE‡

A. Reversible Effects: The enzyme reactions were carried out in the presence of 1mM EDAC*, 2mM Pipes buffer, pH 6.8, with 0.05-1.2mM ASCh and 0.3mM DTNB

SAMPLE	K_{Mapp} (mM)	V_{Max} (μmoles/hr/mg prot.)
Control	.06 ± .01	213.5 ± 13.3
.02mM EDAC	.12 ± .01	221.2 ± 6.3
.05mM EDAC	.31 ± .04	188.6 ± 9.7

B. Irreversible Effects: Enzyme was pretreated with 1mM EDAC* and ASCh hydrolysis was measured in the same reaction buffer as above but without EDAC. The effects of calcium and trivalent cations were assayed on pretreated enzyme by adding the cations to the reaction buffer.

Control (no EDAC)	.06 ± .02	636.6 ± 65.8
+1mM EDAC	.05 ± .02	484.5 ± 45.9
+1mM Ca^{2+}	.05 ± .01	520.2 ± 34.0
+10μM Al^{3+}	.05 ± .02	466.9 ± 49.3
Control'+ 1mm EDAC	.11 ± .01	252.6 ± 8.0
+100μM Al^{3+}	.44 ± .13	249.6 ± 9.4
+200μM Al^{3+}	.24 ± .04	237.2 ± 14.0

*EDAC = 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
‡Values of each parameter are expressed as the 5 replicate mean± the standard error of the mean.

Several investigators have shown that activation of AChE by Ca^{2+} is entirely allosteric and that Ca^{2+} binds to peripheral anionic sites on the enzyme (Roufogalis and Quist, 1972; Marquis and Webb, 1974; Gordon et al., 1977). The Ca^{2+}-binding activator sites, labelled β- by Roufogalis and Quist (1972) and P_1 by Rosenberry (1975), quite likely involve a carboxyl group. In erythrocyte enzyme, the allosteric activation by Ca^{2+} was found to be blocked by the water-soluble carbodiimide EDAC (Roufogalis and Wickson, 1973, 1975). The data shown with bovine brain enzyme demonstrate that this carboxyl group modifying reagent does indeed block the activating effects observed with both Ca^{2+} and Al^{3+} under conditions in which the reagent itself exerts only a minimal inhibition

of enzyme activity. Thus, in the 1-10 μM range, Al^{3+}, like Ca^{2+} may bind to β- or "activator" peripheral anionic sites on bovine brain AChE, and binding involves a carboxyl group that can be blocked by carbodiimides.

Overall, these data support the suggestion that high tissue Al^{3+} levels modify cholinergic neurotransmission, and that aluminum neurotoxicity may be modulated by the levels of tissue and cytoplasmic Ca^{2+}.

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