Neurochemical Research, Vol. 8, No. 11, 1983

INHIBITION OF Na^+, K^+-ATPase ACTIVITY BY δ-AMINOLEVULINIC ACID

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Accepted May 23, 1983

δ-Aminolaevulinic acid (ALA) has been shown to be toxic to cultured neurons and glia at concentrations as low as 10 μM. In an attempt to elucidate the mechanism of toxicity, the effects of ALA on membrane ATPase activity were investigated. Exposure of neuron cultures to 1 mM ALA for 7 days caused a substantial decrease in both Na^+, K^+-ATPase and Mg^{2+}-ATPase activities. At lower concentrations, ALA affected only the Na^+, K^+-component. ALA appeared to act directly, inhibiting Na^+, K^+-ATPase activity in rat brain cortex membrane preparations at 10 μM. Although this effect was slight, it may well represent the mechanism of action of ALA, since ouabain, a potent inhibitor of Na^+, K^+-ATPase activity, proved to be more toxic to cultured neurons than ALA. Furthermore, cardiac glycoside overdosage causes neurological disturbances which are very similar to those observed in the acute attack of porphyria.

INTRODUCTION

Acute phases of the hereditary hepatic porphyrias, lead poisoning and hereditary tyrosinemia are characterized by similar neurological manifestations and are accompanied by an overproduction of δ-aminolevulinic acid (ALA) (1, 2). Increasing evidence suggests that ALA may contribute to the neuropathy of the acute attack. ALA has been shown to cause behavioral changes, as well as decreased motor nerve conduction velocities in mice (3, 4). In addition, at concentrations comparable to those found in the CSF of porphyric patients, ALA has been shown to mimic the neurophysiological effects of γ-aminobutyric acid (GABA), to com-

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pete with GABA for binding to its receptors and to be toxic to neurons and glia in culture, causing a decrease in cell number, protein content and glucose utilization (5–8). The mechanism of toxicity, however, remains unknown. The effects of ALA observed in cultured neurons could not be attributed to its action at GABA receptor sites, since GABA and muscimol did not display any toxicity towards the neurons (5). It was suggested that ALA, in addition to its interaction at GABA receptor sites, could be interfering with energy metabolism in the neuron (5).

The brain is metabolically one of the most active organs of the body and most of its energy consumption is utilized for active transport of ions to sustain and restore the membrane potentials discharged during the process of excitation and conduction (9). Normal nerve function may be expected to be impaired if there is any interference with either the energy supply or the operation of active ion transport systems. ALA has been reported to inhibit rabbit brain microsomal Na^+, K^+-ATPase activity at high concentrations (10). The possibility that ALA may exert its toxic effects on cultured neurons by inhibiting Na^+, K^+-ATPase or Mg^{2+}-ATPase activity was therefore investigated.

EXPERIMENTAL PROCEDURE

Neuron Cultures. Pure neuronal cell cultures were prepared from 7-day-old chick embryo cerebral hemispheres, as previously described (11). Cells were dissociated by passage through a 64 μm nylon sieve and dispersed in Eagle's basal medium supplemented with 20% fetal calf serum. The cell suspension was transferred to plastic Petri dishes (60 mm diameter, 6 ml cell suspension, 5 to 9 $\times$ 10^5 cells/ml) coated with 0.1 M sodium borate buffered polylysine (pH 8.4) to prevent glial proliferation and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The medium was renewed after 48 hr, at which time substances to be tested were added to the cultures in medium at pH 7.4. The medium was renewed after a further 3 days and ATPase activity was determined up to 4 days later. Cell quantitation and protein determination were performed as previously described (11).

Na^+, K^+-ATPase Assay. Cells were washed 3 times with Ca^{2+}-Mg^{2+}-free Tyrode solution, prior to osmotic lysis in large (> 20 ml) volumes of water. Total ATPase activity was assayed at 37°C in a final volume of 1 ml containing 100 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 3 mM Tris-ATP, 30 mM Tris-HCl (pH 7.6) and membrane preparations (30 to 100 μg protein) obtained by osmotic lysis of cells and centrifugation for 30 min at 80,000 *g* (12). Both NaCl and KCl were omitted in the determination of Mg^{2+}-ATPase activity (13). The reaction was initiated by the addition of the membrane preparation and, 10 min later, terminated by the addition of 1.5 ml of ice-cold 5% trichloroacetic acid. Blanks were included in which the membrane preparation was added at the end of the 10 min incubation period. After centrifugation at 1000 *g* for 10 min, the Pi liberated from the substrate in the supernatant fluid was extracted as a complex with molybdate into isobutanol-benzene (1:1, v/v) and quantitated by reduction of the complex with $SnCl_2$ and measurement at 715 nm on a Beckman 5260 spectrophotometer (14, 15).

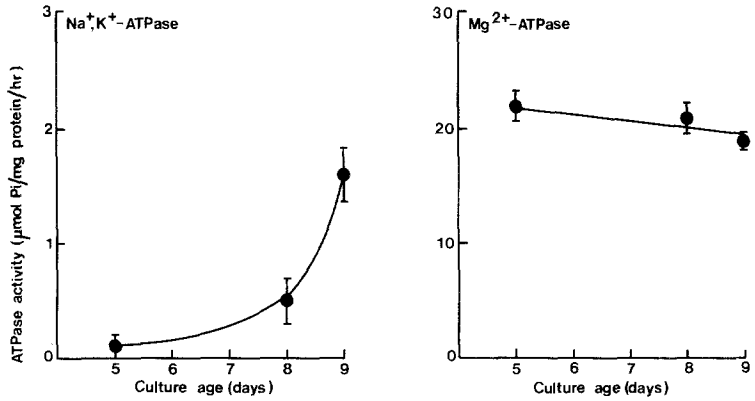

FIG. 1. Na^+, K^+-ATPase activity and Mg^{2+}-ATPase activity in membrane preparations obtained from neurons maintained in culture for different periods of time. Results are the mean ± SEM of 4 to 14 observations.

RESULTS

Na^+, K^+-ATPase activity was found to increase with increasing age of the cultures while Mg^{2+}-ATPase activity remained at a constantly high level (Figure 1). Pure neuronal cultures could not be maintained for periods exceeding 9 days without beginning to show signs of neuronal degeneration. Na^+, K^+-ATPase activity in membrane preparations obtained from the 7-day-old chick embryo cerebral hemisphere neurons, maintained in culture for 9 days was comparable to the activity measured in 16-day-old chick embryo cerebral hemispheres (Table I). Differentiation of the neurons in culture appeared to be comparable to in vivo dif-

TABLE I
A COMPARISON OF ATPASE ACTIVITIES IN CULTURED NEURONS, CHICK EMBRYO CEREBRAL HEMISPHERES, AND RAT BRAIN CORTEX

Membrane preparation	Na^+,K^+-ATPase activity (μmol Pi/mg protein/hr)	Mg^{2+}-ATPase activity (μmol Pi/mg protein/hr)
7-Day-old chick embryo neurons cultured for 9 days	1.6 ± 0.24 (14)	19 ± 0.75 (14)
16-Day-old chick embryo cerebral hemispheres	2.2 ± 0.34 (3)	19 ± 1.1 (3)
Adult rat brain cortex	31 ± 1.3 (6)	24 ± 0.56 (6)

Results are mean ± SEM (*n*)

TABLE II
EFFECT OF ALA ON ATPASE ACTIVITY IN VARIOUS MEMBRANE PREPARATIONS

Membrane preparation and treatment	Na^+,K^+-ATPase activity (% control)	Mg^{2+}-ATPase activity (% control)
Neuron Cultures		
Control	100 ± 3.7 (4)	100 ± 0.12 (4)
100 μM ALA (7 days)	50 ± 11 (5)[a]	108 ± 1.3 (5)
1 mM ALA (7 days)	23 ± 3.3 (5)[b]	36 ± 0.27 (5)[b]
16-Day-old chick embryo cerebral hemispheres		
Control	100 ± 1.7 (5)	100 ± 0.20 (5)
1 mM ALA	35 ± 4.2 (5)[b]	101 ± 0.35 (5)
Rat brain cortex		
Control	100 ± 0.32 (10)	100 ± 0.44 (10)
10 μM ALA	95 ± 0.47 (5)[b]	98 ± 0.24 (5)
100 μM ALA	93 ± 1.2 (5)[b]	97 ± 0.58 (5)
1 mM ALA	85 ± 0.71 (10)[b]	103 ± 0.41 (10)
1 mM GABA	104 ± 0.60 (5)	99 ± 0.36 (5)

Results are mean ± SEM (n); [a] $P < 0.01$; [b] $P < 0.001$

ferentiation. These values were considerably lower than those obtained for membrane preparations derived from adult rat brain cortex (Table I) reflecting the relatively undifferentiated state of the embryonic tissue. In contrast, Mg^{2+}-ATPase activity was found to be comparable in all three tissues (Table I).

Exposure of neuron cultures to 1 mM ALA for 7 days caused a substantial decrease in both Na^+, K^+-ATPase and Mg^{2+}-ATPase activities (Table II). At 100 μM, ALA caused a significant inhibition of Na^+, K^+-ATPase activity without affecting Mg^{2+}-ATPase activity and 10 μM ALA was without effect. The possibility that ALA may exert a direct effect on ATPase activity was investigated by incubating membrane preparations with ALA for 15 min prior to assay of ATPase activity. ALA caused a significant decrease in both chick embryo cerebral hemisphere and rat brain cortex Na^+, K^+-ATPase activity without affecting Mg^{2+}-ATPase activity (Table II). GABA (1 mM) did not cause a similar inhibition of rat cortex Na^+, K^+-ATPase activity (Table II).

Ouabain, at a concentration (0.1 μM) which caused a similar inhibition of Na^+, K^+-ATPase activity in rat brain cortex membrane preparations (Figure 2) as 1 mM ALA, was not as toxic as ALA to cultured neurons (Figure 3, Table III). At higher concentrations (1 μM and 10 μM) however, ouabain caused a considerable loss of both cells and protein from the

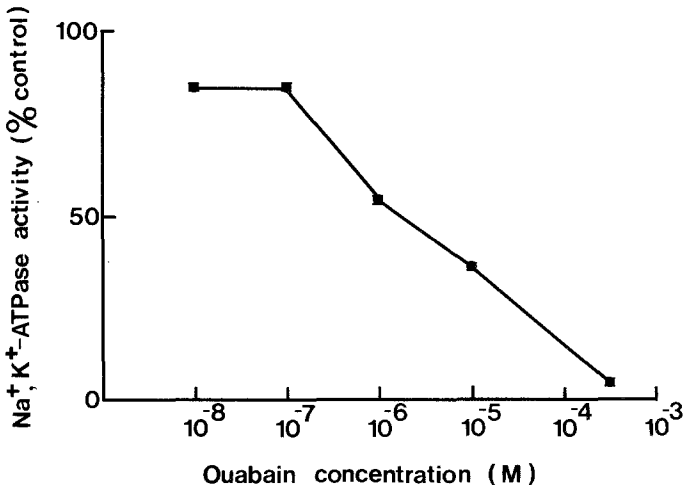

FIG. 2. Na^+, K^+-ATPase activity of rat brain cortex membrane preparations preincubated with various concentrations of ouabain for 15 min prior to assay. Results are the mean ± SEM of 3 to 5 observations.

cultures (Table III). Neuron cultures exposed to 10 μM ouabain for 5 days were similar in appearance to those exposed to 1 mM ALA, the cells were completely rounded up and had lost all connecting processes (Figure 3).

DISCUSSION

Na^+, K^+-ATPase activity was found to increase with increasing differentiation of the neurons in culture, while Mg^{2+}-ATPase activity re-

TABLE III
TOXIC EFFECTS OF OUABAIN ON CULTURED NEURONS

Treatment	Cell number (% control)	Protein recovered (% control)
Control	100 ± 5.4 (5)	100 ± 3.9 (4)
0.1 μM Ouabain	86 ± 6.3 (5)	69 ± 5.8 (5)[a]
1 μM Ouabain	66 ± 7.6 (5)[a]	51 ± 3.0 (5)[b]
10 μM Ouabain	3.4 ± 0.40 (5)[b]	19 ± 2.1 (5)[b]
1 mM ALA	21 ± 3.5 (7)[b]	39 ± 2.5 (7)[b]

Results are mean ± SEM (n); [a] $P < 0.01$; [b] $P < 0.001$

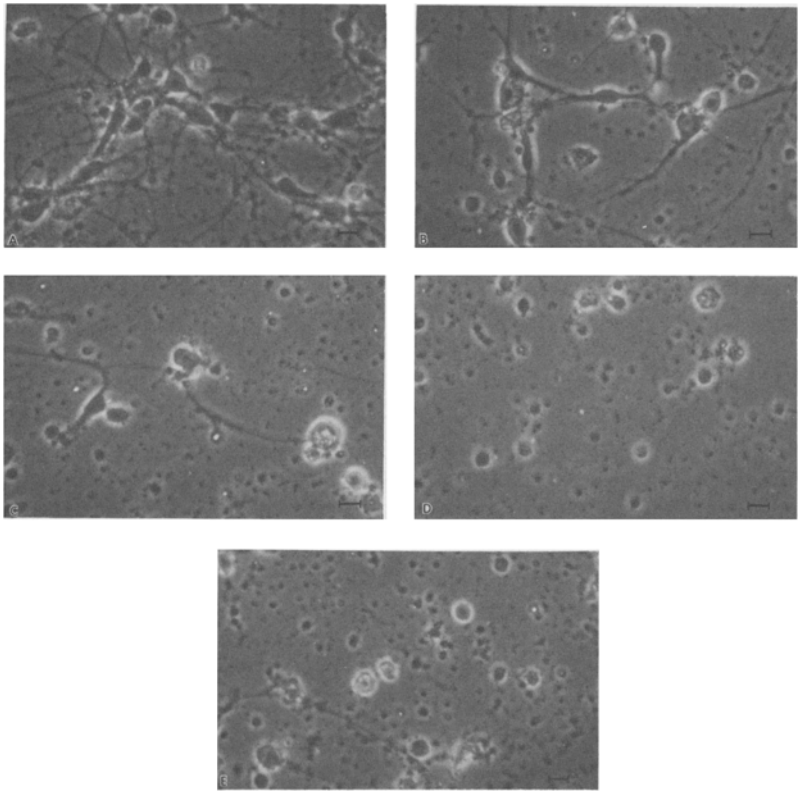

FIG. 3. Morphological changes of cultured neurons after 7 days incubation (a) and after exposure to 0.1 μM ouabain (b), 1 μM ouabain (c), 10 μM ouabain (d) and 1 mM ALA (e) for 5 days. The test compounds were added to the culture medium after 48 hr, as described in Experimental Procedure. Phase contrast. Scale bar 20 μm.

mained constantly high. This is in agreement with the observation that the growth of neuron-like processes in neuroblastoma cell cultures is accompanied by an increase in Na^+, K^+-ATPase activity, whereas Mg^{2+}-ATPase activity increases to a lesser extent (16). The level of Na^+, K^+-ATPase activity observed in primary neuron cultures agrees with previously reported data for neuronal cell lines (16, 17) and is very similar to that obtained for chick embryo cerebral hemispheres of the equivalent age. The chick embryo membrane preparations, however, contained elements from both neurons and glia, which are reported to have similar Na^+, K^+-ATPase activities (17). The low levels of Na^+, K^+-ATPase activity observed in membranes derived from chick embryo cells when

compared to the activity in adult rat brain, reflects their relatively undifferentiated state.

Exposure of neuron cultures to 1 mM ALA for 7 days caused a substantial decrease in both Na^+, K^+-ATPase and Mg^{2+}-ATPase activities. At lower concentrations, ALA appeared to affect only the Na^+, K^+-component. These effects may be attributed to neuronal degeneration. ALA causes a loss of cells (11) and the neurons remaining after long-term exposure to ALA appear to be metabolically less active than controls, having a lower glucose consumption (5). It would appear that these neurons also have less active ion transport systems.

ALA appeared to act directly, inhibiting Na^+, K^+-ATPase activity in rat brain membrane preparations at concentrations as low as 10 μM. Mg^{2+}-ATPase did not appear to be affected. These results agree with and extend an earlier finding that ALA inhibits red cell and rabbit brain microsomal Na^+, K^+-ATPase activity (10). ALA may act directly with the enzyme protein or indirectly with the membrane, perturbing the microenvironment within which the Na^+, K^+-ATPase resides. ALA has been shown to reduce nerve cell membrane resistance, causing an increase in chloride ion conductance (18). This effect may be related to the inhibition of Na^+, K^+-ATPase activity, since ouabain, a potent inhibitor of Na^+, K^+-ATPase activity also increases chloride ion conductance in crayfish giant axons (19). At concentrations causing a slightly greater inhibition of Na^+, K^+-ATPase activity than 1 mM ALA, ouabain was clearly as toxic to neuron cultures as 1 mM ALA. Their mechanisms of action are probably not identical since ALA has been shown to antagonize the contractile effect of ouabain on rabbit duodenum (20). Nevertheless, the inhibitory effect of ALA on Na^+, K^+-ATPase activity may well represent the mechanism of its toxicity towards cultured neurons. The effects on Na^+, K^+-ATPase activity and chloride ion conductance, causing hyperpolarization of nerve cells (18) could explain the inhibition of potassium-stimulated release of GABA (8) and dopamine (21) from synaptosomal preparations, caused by ALA since ouabain also inhibits potassium-stimulated dopamine release from rat striatal slices (22). These effects of ALA cannot be attributed to interaction of ALA at GABA receptors since GABA is not toxic to the cultures (5), does not reduce glucose uptake by cultured neurons (5) and does not inhibit rat brain Na^+, K^+-ATPase activity.

ALA was previously shown to be equally toxic to cultured glial cells (11). Glia have a very active Na^+, K^+-transport system which complements the neuronal pump in restoring and maintaining extracellular potassium concentrations (23) and may also be susceptible to the toxic effects of ALA.

Caution should be exercised in extrapolating these in vitro findings with dispersed chick cells to the in vivo situation where the cell may have a far greater resistance to the effects of toxic agents. Factors which are known to precipitate acute attacks in porphyria are varied and include several drugs, many of which are believed to interact with the GABA receptor-chloride ionophore complex, starvation, stress, infection and steroids. It is possible that these factors perturb neuronal function sufficiently to predispose the nervous system to the toxic effects of ALA.

Additional evidence in support of the hypothesis that inhibition of Na^+, K^+-ATPase activity is the mechanism underlying the neurotoxicity of ALA is the similarity in the neurological effects of digitalis overdosage and the symptoms of acute porphyria. Digitalis, a cardiac glycoside with properties very similar to ouabain, and whose effects are thought to be mediated by inhibition of the Na^+, K^+-ATPase system, causes abdominal pain, anorexia, nausea, vomiting, diarrhea, arrhythmias, tachycardia, headache, fatigue, malaise, generalized muscle weakness, paresthesias, disorientation, confusion and even delirium and hallucinations (24), features which characterize the acute attack of porphyria.

ACKNOWLEDGMENTS

This research was supported by the South African Medical Research Council and the Cape Provincial Administration.

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