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THE INHIBITION OF RED CELL AND BRAIN ATPase BY δ-AMINOLAEVULINIC ACID

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SUMMARY

Red cell membrane and brain tissue ATPase were inhibited by δ-aminolaevulinic acid. The effect was on the $(Na^+ + K^+)$-dependent fraction of ATPase, and δ-aminolaevulinic acid had no effect on the magnesium-dependent fraction of ATPase. The effect of δ-aminolaevulinic acid was reversible. δ-Aminolaevulinic acid had no effect on ATPase enzyme activity on red cell ghost membranes. The difference between red cell membrane and brain tissue ATPase, on the one hand, and red cell ghost membrane ATPase on the other hand may be due to the solubility of the ATPase enzyme in the different experiments. Glycine, cysteine hydrochloride and sodium phenobarbitone had no effect on red cell membrane and brain tissue ATPase enzyme. The findings suggest a possible explanation for the neurological manifestation of acute intermittent porphyria and porphyria variegata, since the inhibition of $(Na^+ + K^+)$-dependent nerve ATPase by δ-aminolaevulinic acid could block nerve conduction and cause paralysis.

INTRODUCTION

The clinical features of the acute phase of both acute intermittent porphyria and porphyria variegata can be explained on the basis of a neurological disturbance[1,2]; yet the pathological descriptions have been variable[1,3–8] and have been described as unimpressive[9]. In view of this it is more likely that the biochemical defect in porphyria gives rise initially to a disturbance in neurophysiology rather than to a neuropathy.

The source of energy for the propagation of a nerve impulse is related to the concentration of sodium on the two sides of the cell membrane[10]. This difference is achieved by the sodium pump, the energy for which is provided by the hydrolysis of ATP by the enzyme ATPase. The enzyme is magnesium-dependent. The rate of hydrolysis is increased by Na^+ and, in the presence of Na^+, is further increased by K^+ (ref. 11). Since δ-aminolaevulinic acid accumulates in the plasma[12] and is excreted in large amounts in the urine[13] in the acute phase of porphyria, it was decided to investigate the effect of this metabolite on ATPase activity. Porphobilinogen is also excreted in increased amounts in the active phase of porphyria. However, there is evidence that cell membranes are more permeable to δ-aminolaevulinic acid than to porphobilinogen[14,15] and thus δ-aminolaevulinic acid was chosen for this study.

MATERIALS AND METHODS

Materials

Red cells were obtained from healthy human donors. Human brain tissue in the form of normal cerebral cortex was obtained at surgery for a deep-seated cerebral tumour. Rabbit brain was removed through a burr-hole after the animals had been anaesthetised with 100–150 mg of sodium phenobarbitone. When the effect of sodium phenobarbitone on brain ATPase was studied, the animal was killed by injection of 40 cm^3 of air into a vein and the skull immediately opened.

Preparation of red cell membrane ATPase

ATPase 'enzyme' was prepared from human red cells as described by POST *et al.*[16]. The final precipitate was washed with $5 \cdot 10^{-4}$ M Tris glycylglycine buffer (pH 8.1) until it was colourless. The enzyme activity was determined as the amount of free phosphate released by the hydrolysis of ATP. The assay system contained 5 μmoles of ATP (Sigma Chemical Co.) (obtained as the sodium salt, adjusted to pH 7.1 with Tris and passed through a Dowex 50 cation-exchange resin in the Tris form to remove Na^+ and any K^+ present), 5 μmoles of $MgCl_2$ and 1 ml of 0.5 M imidazole–histidine buffer, pH 7.1. To this mixture 0.5 ml enzyme preparation was added. Sodium and potassium were added to a final concentration of 0.08 M and 0.03 M, respectively, each in 0.1-ml volumes. When measuring $(Na^+ + K^+)$-independent ATPase activity, sodium and potassium were not added to the assay medium. The final assay volume was made up to 3 ml with distilled water. The mixture was incubated at 40° for 1 h and the reaction stopped with 8% perchloric acid. After centrifugation, 1 ml of supernatant was removed for phosphate determination by a modification of the method of MOZERSKY *et al.*[17]. There was no detectable phosphate in the enzyme preparation and a control without enzyme was included to correct for nonenzymatic hydrolysis of ATP. Where sodium and potassium were added to the medium, the results are referred to as total ATPase activity. The results of assays carried out in the absence of sodium and potassium are referred to as $(Na^+ + K^+)$-independent ATPase activity. $(Na^+ + K^+)$-dependent ATPase is that component of the total ATPase requiring $Na^+ + K^+$, or inhibited by ouabain.

The effect of δ-aminolaevulinic acid (0.1–2.0 mM), glycine (1.0–5.0 mM), cysteine hydrochloride (2.0–10.0 mM) and sodium phenobarbitone (1.0–10.0 mM) on total ATPase and $(Na^+ + K^+)$-independent ATPase activity was studied by adding these substances to the assay system after they had been buffered to pH 7.1 with 0.5 M imidazole–histidine. The amount of sodium present in sodium phenobarbitone was taken into consideration in maintaining the final sodium concentration in the mixture at 0.08 M. δ-Aminolaevulinic acid was obtained from Sigma Chemical Co. as the hydrochloride.

Preparation of red cell ghosts

The red cell 'ghosts' were prepared by a modification of the method of WITTAM[18]. A sample of 50 ml of heparinised blood (heparin, 100 units/ml blood) was centrifuged and the plasma and buffy coat removed. The red cells were then washed three times with a Na-free choline medium containing 147 mM choline chloride, 2 mM $MgCl_2$, 10 mM Tris–HCl buffer, pH 7.6. All the following procedures were then carried out

at 4°. The red cells were lysed by injecting 1 volume of red cells (25 ml) into 5 volumes of deionised distilled water (125 ml). The lysate was immediately centrifuged at 20000 × *g* for 5 min, and the dark red supernatant removed. The stroma was then made up to the original volume of the lysate with choline chloride medium diluted 1:200 with distilled water and containing 2 mM ATP (disodium) and 2 mM $MgCl_2$. At this stage, sufficient 3 M NaCl or 3 M Tris–HCl, pH 7.4 (depending on whether Na^+ was required inside the ghost or not), was added to raise the osmotic pressure of the haemolysate to an osmolality of 0.3 (6.4 ml for 125 ml of lysing fluid). The haemolysate which was now isosmotic with plasma was incubated with occasional shaking, for 30 min in a water bath at 37° to allow the membrane to regain a low permeability to Na^+ and K^+ (ref. 19). The 'ghosts' were then sedimented by centrifugation at 20000 × *g* for 5–10 min. The supernatant was discarded and the 'ghosts' washed once in the medium in which they were to be incubated, and finally made into suspension such that the 'ghosts' from 25 ml of packed erythrocytes provided 11–12 ml of packed 'ghosts' in 20 ml of suspension.

The packed 'ghosts' which had been reconstituted with Tris–HCl were suspended in the Na^+- and K^+-free choline medium. The 'ghosts' reconstituted with sodium were suspended in a buffer containing 10 mM KCl, 2 mM $MgCl_2$ and 10 mM Tris–HCl, pH 7.6. When δ-aminolaevulinic acid was required inside the cell, it was added to the choline medium used to make up the volume after the first centrifugation of the haemolysate, *i.e.* just before reconstitution. When δ-aminolaevulinic acid was required outside the cell, it was added to the potassium chloride medium used to make the final 'ghost' suspension.

In most cases the red cell 'ghosts' were well formed and retained the typical red-cell shape. However, occasionally some of the red cells were slightly crenated, but they were, in any event, whole and not ruptured. The sodium concentration (measured by flame photometry) within the 'ghosts' ranged from 70–150 mM with a mean of 100 mM. δ-Aminolaevulinic acid was measured in 'ghost' red cells and in the medium by a modification of the method of SUN *et al.*[20]. The total volume in each experiment was 2 ml of which approx. 1 ml was packed 'ghosts'. This was incubated at 40° for 1 h and the reaction terminated with 8 % perchloric acid. The precipitated membranes were removed by centrifugation, and the free phosphate measured as for red cell membranes.

ATPase preparation of brain tissue

Brain ATPase was prepared by a modification of the method of SKOU[11]. All visible blood vessels were dissected from the brain tissue which was washed three times in 10 volumes of 0.25 M ice-cold sucrose, made up in 0.5 M imidazole–histidine buffer adjusted to pH 7.4 with 1 M HCl. The brain tissue was then homogenized in a further 10 volumes of fresh buffered sucrose. Stroma and fragments were removed by centrifugation at 2000 × *g* for 15 min at 0°, and mitochondria and some submicroscopic particles by centrifugation at 10000 × *g* for 15 min. The supernatant was then centrifuged at 38000 × *g* for 2 h and the sediment suspended in a volume of 0.25 M ice-cold buffered sucrose, corresponding to one-half the volume of the original homogenate. The suspension was centrifuged at 10000 × *g* for a further 10 min, in order to remove any remaining mitochondria, and the final supernatant was used as 'enzyme' in the experiments. The 'enzyme' obtained from rabbit brain

had a specific activity of approx. 3 units/mg protein (in the absence of sodium and potassium) (1 unit = the release of 1 μmole of inorganic phosphate in 1 h at 40°) and contained 10–15 % of the original activity in the homogenate. As only a limited amount of human tissue was available, the specific activity for human brain could not be estimated. The ATPase activity of the brain preparation was measured as for red cell membrane ATPase.

RESULTS

Red cell membrane ATPase

The results for red cell membrane ATPase are summarized in Table I. The ATPase activity was 50 % greater in the presence of sodium and potassium. Preliminary experiments showed that this fraction could be inhibited by ouabain (1 mg/ml).

TABLE I

THE INHIBITION OF THE $(Na^+ + K^+)$-DEPENDENT FRACTION OF TOTAL ATPase 'ENZYME' ISOLATED FROM RED CELL MEMBRANE BY δ-AMINOLAEVULINIC ACID

δ-Aminolaevulinic acid had no effect on the $(Na^+ + K^+)$-independent fraction of the enzyme.

Mg^{2+} (mM)	Na^+ (mM)	K^+ (mM)	δ-Aminolaevulinic acid (mM/ml wet cells)	Activity (μmoles P_i/ml wet cells per h)
2	0	0	0	2.22
2	80	33	0	3.92
2	0	0	0.1	2.18
2	0	0	0.2	2.30
2	0	0	2.0	2.21
2	80	33	0.1	3.33
2	80	33	0.2	2.64
2	80	33	2.0	2.39
2	80	33	5.0	2.56

There was progressive inhibition of enzyme activity with increasing amounts of δ-aminolaevulinic acid (Table I). The effect was due to inhibition of the $(Na^+ + K^+)$-dependent fraction of the ATPase activity since δ-aminolaevulinic acid had no effect on the $(Na^+ + K^+)$-independent fraction of ATPase (Table I). The effect of δ-aminolaevulinic acid on the $(Na^+ + K^+)$-dependent fraction of total ATPase was reversible (Table II). Enzyme inhibited by δ-aminolaevulinic acid for 1 h was dialysed overnight in cold running tap water which completely removed the δ-aminolaevulinic acid. Enzyme activity could be restored on the addition of ATP and magnesium, and a further 50 % increase occurred when sodium and potassium were added (Table II). Glycine, cysteine hydrochloride and phenobarbitone had no effect on total ATPase activity.

To determine whether δ-aminolaevulinic acid combined with phosphate thereby lowering the amount of measurable free phosphate and giving an apparent inhibitory effect for δ-aminolaevulinic acid on red cell 'ATPase' activity, δ-aminolaevulinic acid (concentration 10 mM) was added to the mixture 1 h after incubation followed by precipitation of the protein. There was no difference in the amount of free phosphate

TABLE II

REVERSIBILITY OF δ-AMINOLAEVULINIC ACID INHIBITION OF $(Na^+ + K^+)$-DEPENDENT RED CELL MEMBRANE ATPase 'ENZYME'

$(Na^+ + K^+)$-dependent and $(Na^+ + K^+)$-independent ATPase 'enzyme' which had been dialysed for 18 h showed less activity than fresh ATPase 'enzyme'. The ATPase 'enzyme' preparation which had been incubated with δ-aminolaevulinic acid for 1 h and then dialysed for 18 h had the same activity as ATPase 'enzyme' which had not been incubated with δ-aminolaevulinic acid but also had been dialysed for 18 h.

	μmoles P_i/h per ml wet cells	
	1 h incubation	*1 h incubation after 18 h dialysis*
$(Na^+ + K^+)$-independent ATPase	1.45	0.52
Total ATPase	2.18	0.97
Total ATPase + δ-aminolaevulinic acid (0.2 mM)	2.06	0.97
Total ATPase + δ-aminolaevulinic acid (2.0 mM)	1.66	1.03

measured as compared with a control to which no δ-aminolaevulinic acid had been added.

ATPase enzyme on red cell 'ghosts'

Red cell 'ghosts' reconstituted with NaCl and incubated in buffer containing potassium had more than 50 % greater ATPase activity than red cell 'ghosts' reconstituted with Tris–HCl and incubated in the sodium- and potassium-free choline chloride medium. This $(Na^+ + K^+)$-stimulated component could be inhibited by ouabain (1 mg/ml).

It was found that the red cell membrane was freely permeable to δ-aminolaevulinic acid. When δ-aminolaevulinic acid was added before reconstitution of the 'ghosts' and thereby placed inside the cell, it soon 'leaked' out into the surrounding medium. One ml of reconstituted 'ghosts', containing 105 μg/ml δ-aminolaevulinic acid internally at zero-time, contained 35 μg/ml internally after a 1-h incubation, and 65 μg in 2 ml of external medium. Ghost red cells incubated with δ-aminolaevulinic acid in the external medium soon reached a concentration internally in equilibrium with the external medium. For example, in one experiment 2 ml of medium, including 1 ml of packed 'ghosts', contained 1000 μg/ml δ-aminolaevulinic acid situated externally at zero time. After a 1-h incubation the external medium contained 500 μg/ml of δ-aminolaevulinic acid and the 'ghosts', 460 μg/ml of δ-aminolaevulinic acid. δ-Aminolaevulinic acid did not have any effect on the $(Na^+ + K^+)$-dependent fraction of total ATPase of red cell 'ghosts' membranes whether the δ-aminolaevulinic acid was initially inside or outside the red cell 'ghost'.

Brain ATPase

The results for brain ATPase are summarized in Tables III and IV. The ATPase activity was increased by more than 50 % in the presence of sodium and potassium. As with red cell membrane, increasing amounts of δ-aminolaevulinic acid resulted in a progressive inhibition of the $(Na^+ + K^+)$-dependent fraction of total ATPase but

TABLE III

THE EFFECT OF δ-AMINOLAEVULINIC ACID ON THE $(Na^+ + K^+)$-DEPENDENT FRACTION OF AN ATPase ISOLATED FROM RABBIT AND HUMAN BRAIN TISSUE

δ-Aminolaevulinic acid had no effect on the $(Na^+ + K^+)$-independent fraction of the enzyme.

Mg^{2+} (mM)	*Na^+ (mM)*	*K^+ (mM)*	*δ-Aminolaevulinic acid (mM)*	*Activity (μmoles P_i/mg protein per h)*
Rabbit				
2	nil	nil	nil	2.88
2	nil	nil	2.0	3.12
2	80	33	nil	5.52
2	80	33	0.1	5.04
2	80	33	0.2	4.32
2	80	33	2.0	2.76
Human				
2	nil	nil	nil	3.16
2	nil	nil	2.0	3.38
2	80	33	nil	5.89
2	80	33	2.0	3.99

TABLE IV

THE EFFECT OF δ-AMINOLAEVULINIC ACID, OUABAIN AND SODIUM PHENOBARBITONE ON $(Na^+ + K^+)$-DEPENDENT ATPase ISOLATED FROM RABBIT BRAIN

δ-Aminolaevulinic acid had no effect on the $(Na^+ + K^+)$-independent fraction of the ATPase 'enzyme'.

Rabbit brain ATPase + various additives	*μmoles P_i/h per mg protein*	
	Expt. 1	*Expt. 2*
$(Na^+ + K^+)$-independent ATPase 'enzyme'	2.99	2.92
$(Na^+ + K^+)$-independent ATPase 'enzyme' + δ-aminolaevulinic acid (2.0 mM)	—	3.15
Total ATPase 'enzyme'	5.14	6.07
Total ATPase 'enzyme' + δ-aminolaevulinic acid (2.0 mM)	3.15	3.09
Total ATPase 'enzyme' + ouabain (1 mg/ml)	2.81	—
Total ATPase 'enzyme' + ouabain (1 mg/ml) + δ-aminolaevulinic acid (2.0 mM)	3.23	—
Total ATPase 'enzyme' + sodium phenobarbitone (2.0 mM)	—	6.07

not the $(Na^+ + K^+)$-independent ATPase. Sodium phenobarbitone had no effect on total brain ATPase (Table IV). Ouabain (1 mg/ml) inhibited the $(Na^+ + K^+)$-dependent fraction of the total ATPase activity (Table IV). There was no summation of effect when both δ-aminolaevulinic acid and ouabain were added, and the effect was the same as if either had been added alone (Table IV).

DISCUSSION

There is a great deal of evidence that in both nerve and red cell cation transport is associated with ATPase activity[21]. Red cell ATPase was studied initially as a mem-

brane ATPase 'model' because of the easy availability of red cells. δ-Aminolaevulinic acid was found to have an inhibitory effect on the $(Na^+ + K^+)$-dependent fraction of ATPase. Glycine and cysteine hydrochloride had no effect. The latter was studied to be sure that the chloride radical of the δ-aminolaevulinic acid hydrochloride, although contributing only 3 parts per 1000 to the total chloride pool in the incubation mixture, was not the cause of the inhibitory effect on the $(Na^+ + K^+)$-dependent fraction of the ATPase. δ-Aminolaevulinic acid was subsequently shown to have a similar effect on brain $(Na^+ + K^+)$-dependent ATPase. An inhibitory effect was detectable with as little as 0.2 mM of δ-aminolaevulinic acid. The finding that δ-aminolaevulinic acid inhibits a red cell membrane $(Na^+ + K^+)$-dependent ATPase and a $(Na^+ + K^+)$-dependent ATPase fraction from brain tissue suggests a possible mechanism for the induction of the neurological disturbance in the acute phase of porphyria. The presence of δ-aminolaevulinic acid at the nerve membrane surface might be expected to inhibit $(Na^+ + K^+)$-dependent ATPase activity thereby interfering with sodium transfer across the membrane resulting in impaired nerve conduction. If such is the case δ-aminolaevulinic acid would have to be located at some site on the nerve membrane to exert an effect. An attempt was made to study this supposition by determining the effect of δ-aminolaevulinic acid on $(Na^+ + K^+)$-dependent ATPase when placed inside and outside red cell 'ghost' membrane. However, δ-aminolaevulinic acid diffused freely across red cell 'ghost' membrane and in each experiment was present on both sides of the membrane. δ-Aminolaevulinic acid had no effect on $(Na^+ + K^+)$-dependent ATPase on 'ghost' red cell membrane. The failure of δ-aminolaevulinic acid to inhibit $(Na^+ + K^+)$-dependent ATPase on 'ghost' red cell membrane in contrast to the inhibition of $(Na^+ + K^+)$-dependent ATPase from red cell and brain tissue is difficult to explain. The δ-aminolaevulinic acid was not metabolised by red cell 'ghosts' since no porphyrins were synthesised, and all the δ-aminolaevulinic acid was recovered after each experiment. The different results between red cell $(Na^+ + K^+)$-dependent ATPase and brain $(Na^+ + K^+)$-dependent ATPase, on the one hand, and 'ghost' red cell membrane $(Na^+ + K^+)$-dependent ATPase on the other hand may be due to the form in which ATPase was present in each experiment. The brain ATPase was isolated in brain particles which could be sedimented by centrifugation at high speed. The results were invariably reproducible. The red cell ATPase was on stromal particles and on occasions no inhibitory effect by δ-aminolaevulinic acid on the $(Na^+ + K^+)$-dependent fraction was detected. The ATPase from 'ghost' red cell membrane was essentially on intact red cell membrane. Here no inhibitory effect by δ-aminolaevulinic acid on the $(Na^+ + K^+)$-dependent fraction was demonstrable. It would appear that the inhibitory effect is related to particle size or solubility. The method of isolation of the enzyme may also explain the variable results. SCHRIER *et al.*[22] noted different proportions of $(Na^+ + K^+)$-activated ATPase and Mg^{2+}-activated ATPase depending on the method of isolation. Another explanation for these variable results may be that brain $(Na^+ + K^+)$-dependent ATPase and red cell $(Na^+ + K^+)$-dependent ATPase are not identical. Recently NEUFELD *et al.*[23] reported a second ouabain-sensitive sodium-dependent ATPase in brain microsomes. The possibility that the ATPase concentration within the 'ghosts' was too low to allow accurate measurement of ATPase activity can be discounted, as a $(Na^+ + K^+)$-dependent component was measurable and could be inhibited by ouabain (1 mg/ml).

While the inhibition of $(Na^+ + K^+)$-dependent brain ATPase by δ-amino-

laevulinic acid is an attractive hypothesis for the development of paralysis in porphyria, there are a number of observations which suggest that δ-aminolaevulinic acid is nontoxic. GOLDBERG *et al.*[24] and JARRETT *et al.*[25] injected haem precursors intravenously into anaesthetised cat and rabbit and recorded nervous impulses or added precursors to preparations of isolated organs which were then stimulated. They found that under these experimental conditions the haem precursors, δ-aminolaevulinic acid and porphobilinogen, were pharmacologically inactive.

δ-Aminolaevulinic acid is excreted in large amounts in rabbits given allyl isopropyl acetamide, yet show none of the features of acute porphyria found in man except constipation and weight loss[26]. δ-Aminolaevulinic acid is excreted in the urine in the quiescent phase of acute intermittent porphyria, although in reduced amount[27]. Finally, while in the acute phase of porphyria large amounts of δ-aminolaevulinic acid are excreted in the urine, as high as 100 mg a day[28,29] or up to 410 μmoles a day[30]; the amounts found in blood have been infrequently studied. CHISHOLM[12] found plasma concentrations of δ-aminolaevulinic acid from 0.2 to 2.2 μg/ml in seven adults. By contrast, SWEENEY *et al.*[31], in a single case of acute intermittent porphyria during an acute attack, reported a plasma δ-aminolaevulinic acid level of 24 μg/ml, with a cerebrospinal fluid level of 2.8 μg/ml.

Barbiturates precipitate acute attacks in cases of latent porphyria and seriously aggravate the neurological involvement. They may act as porphyria inducers or inhibitors of oxidative mechanisms of brain tissue. Barbiturates have a direct effect on cell membrane and possibly on transmembrane flux[32]. No effect of barbiturates was demonstrable on the $(Na^+ + K^+)$-dependent ATPase under the present experimental conditions, which would suggest that if inhibition of the $(Na^+ + K^+)$-dependent fraction of ATPase by δ-aminolaevulinic acid is the mechanism for the neurological disturbance in porphyria, this is not the site of action of barbiturates.

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