The Effect of Galactose Metabolic Disorders on Rat Brain Na^+,K^+-ATPase Activity

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To evaluate the effect of galactose metabolic disorders on the brain Na^+,K^+-ATPase in suckling rats. Separate preincubations of various concentrations (1–16 mM) of the compounds galactose-1-phosphate (Gal-1-P) and galactitol (galtol) with whole brain homogenates at 37 °C for 1 h resulted in a dose dependent inhibition of the enzyme whereas the pure enzyme (from porcine cerebral cortex) was stimulated. Glucose-1-phosphate (Glu-1-P) or galactose (Gal) stimulated both rat brain Na^+,K^+-ATPase and pure enzyme. A mixture of Gal-1-P (2 mM), galtol (2 mM) and Gal (4 mM), concentrations commonly found in untreated patients with classical galactosemia, caused a 35% ($p < 0.001$) rat brain enzyme inhibition. Additionally, incubation of a mixture of galtol (2 mM) and Gal (1 mM), which is usually observed in galactokinase deficient patients, resulted in a 25% ($p < 0.001$) brain enzyme inactivation. It is suggested that: a) The indirect inhibition of the brain Na^+,K^+-ATPase by Gal-1-P should be due to the presence of the epimer Gal and phosphate and that the pure enzyme direct activation by Gal-1-P and Glu-1-P to the presence of phosphate only. b) The observed brain Na^+,K^+-ATPase inhibitions in the presence of toxic concentrations of Gal-1-P and/or galtol could modulate the neural excitability, the metabolic energy production and the catecholaminergic and serotoninergic system.

Introduction

Na^+,K^+-ATPase is the enzymatic basis of univalent cation transport (Sweadner and Goldin, 1980). It is implicated in neural excitability (Sastry and Phillis, 1977), metabolic energy production (Mata *et al.*, 1980), uptake and release of catecholamines (Bogdanski *et al.*, 1968) and Na^+-dependent tryptophan uptake system (Herrero *et al.*, 1983).

Three enzyme deficiencies have been described in association with galactose metabolic disorders, galactose-1-phosphate uridyl transferase (EC 2.7.1.12) (classical galactosemia), galactokinase (EC 2.7.1.6) and galactose-4-epimerase (EC 5.1.3.2) (Segal, 1995). The first enzyme catalyses the formation of uridyl-diphosphate (UDP)-galactose (Gal) from glucose-1-phosphate (Glu-1-P) and UDP-glucose. As a consequence of this block in the sugar nucleotide pathway, galactose-1-phosphate (Gal-1-P) accumulates in the lens, liver, kidney and brain, whereas Gal accumulates in liver, brain and kidney (Waggoner *et al.*, 1990). Galactitol (dulcitol) (galtol), the sugar alcohol of Gal, also accumulates in brain and lens, because of the action of a non-specific reduction on Gal (Wells *et al.*, 1965). The cataracts, so common in this disorder, appear to be the result of intralenticular accumulation of galtol (Hayman and Shimamoto, 1965). The metabolite Gal-1-P in galactosemia is believed to play a toxic role (Segal, 1995; Schweitzer, 1995; Schulpis *et al.*, 1997) resulting in the degeneration of liver and in a probable brain cholinergic implication (Tsakiris and Schulpis, 2000). The infants with deficiency of Gal-1-P uridyl transferase present a severe clinical picture. They develop diarrhea, vomiting, dehydration, hyperbilirubinemia, hepatic dysfunction, prolongation of clotting times and they are at risk for developing severe mental retardation and seizures (Segal, 1995).

Furthermore, patients with galactokinase deficiency galactosemia, in whom no Gal-1-P accumulates, galtol builds up cataracts and increases the

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intracerebral osmolarity (Wells *et al.*, 1965; Segal, 1995).

Additionally, decreased nerve conduction has been reported in galactosemic rats (Mirisin *et al.*, 1986; Calcutt *et al.*, 1990) and brain cholinergic dysfunction in rat brain *in vitro* galactosemia (Tsakiris and Schulpis, 2000). The peripheral neuropathy is associated with high Gal diets in rats and is characterized by endoneural edema (Calcutt *et al.*, 1990), increased tissue pressure and diminished nerve blood flow with demyelinization of nerve fibers (Myers and Powell, 1984). The edema and the increased endoneural sodium were related to Schwann cell injury attenuated by aldose reductase inhibition (Segal *et al.*, 1984).

Therefore, the aim of this study was the evaluation of Na^+,K^+-ATPase activity in the rat brain homogenates in relation to various concentrations of Gal and its derivatives.

Materials and Methods

Animals

For the experiments conducted on homogenised rat brain, 21 day old Albino Wistar rats of both sexes (Saint Savvas Hospital, Athens, Greece) were used. The suckling rats with their mother were housed in a cage at constant room temperature (22 ± 1 °C) under a 12hL:12hD (light 08.00–20.00 h) cycle. Animals were cared for in accordance with the principles of the *"Guide to the Care and Use of Experimental Animals"* (Committee on Care and Use of Laboratory Animals, 1985).

Tissue preparation

Rats were sacrificed by decapitation. Whole brains from five rats were rapidly removed, weighed and thoroughly washed with isotonic saline. They were homogenized in 10 vol. ice-cold (0–4 °C) medium containing 50 mM Tris (hydroxymethyl) aminomethane-HCl (Tris-HCl), pH 7.4 and 300 mM sucrose using an ice-chilled glass homogenizing vessel at 900 rpm (4–5 strokes). Then, the homogenate was centrifuged at 1,000 × g for 10 min to remove nuclei and debris. In the resulting supernatant, the protein content was determined according to the method of Lowry *et al.* (1951) and then Na^+,K^+-ATPase activities were measured. The enzyme incubation mixture was kept at 37 °C.

Preincubation of GAL and its derivatives

Various concentrations (1–16 mM) of Gal (Sigma), Gal-1-P (Sigma), and galtol (Sigma) were preincubated with 100 μg protein of whole brain homogenates or with 40 μg protein of pure Na^+,K^+-ATPase from porcine cerebral cortex (Sigma) for 1 h and then Na^+,K^+-ATPase activities were measured. The preincubation medium (about 1 ml) contained 50 mM Tris-HCl, pH 7.4 and 240 mM sucrose in the presence of 120 mM NaCl. For comparison, glucose (Glu) (Sigma) as well as glucose-1-phosphate (Glu-1-P) (Sigma) were also tested as above.

Determination of Na^+,K^+-ATPase activities

Na^+,K^+-ATPase activity was calculated as the difference between total ATPase activity (Na^+,K^+,Mg^{2+}-dependent) and Mg^{2+}-dependent ATPase activity. Total ATPase activity was assayed in an incubation medium consisting of 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 240 mM sucrose, 1 mM ethylenediamine tetraacetic acid K_2-salt (K^+-EDTA), 3 mM disodium ATP and 80–100 μg protein of the homogenate, in a final volume of 1 ml. Ouabain (1 mM) was added in order to determine the activity of the Mg^{2+}-ATPase. The values of Mg^{2+}-dependent ATPase were similar in the presence of ouabain in the reaction mixture as also in ouabain absence and without NaCl and KCl (in the presence of $MgCl_2$ only). The reaction was started by adding ATP and was stopped after a 20 min incubation period by the addition of 2 ml of a mixture of 1% lubrol and 1% ammonium molybdate in 0.9 M H_2SO_4 (Atkinson *et al.*, 1971; Bowler and Tirri, 1974; Tsakiris, 2001). The yellow colour which developed was read at 390 nm.

Statistical analysis

The data were analyzed by two-tailed ANOVA followed by Student's *t*-test. P values < 0.05 were considered statistically significant.

Results

Figure 1 presents the effects of Gal-1-P or galtol concentrations on Na^+,K^+-ATPase activity in whole brain homogenates or on pure enzyme (from porcine cerebral cortex). Gal-1-P (2 mM) inhibited the brain Na^+,K^+-ATPase (A) about 30% ($p < 0.01$). The above concentration is usually found in the blood and brain of untreated patients with classical galactosemia (Segal, 1995). Concentrations higher than 2 mM, e.g. 8 mM, inhibited the enzyme activity up to 80% ($p < 0.001$). On the contrary, Gal-1-P extremely stimulated the pure enzyme (B) up to 400% of the control with 1 mM, and 900–4100% with 2–8 mM. Galtol inhibited the brain Na^+,K^+-ATPase (C) about 25% ($p < 0.01$) with a concentration 2 mM, which is also commonly found in the untreated galactosemic patients (Schulpis *et al.*, 1997). Concentrations higher than 2 mM were not able to inhibit the enzyme anymore. However, galtol (1 mM) stimulated the pure enzyme (D) about 40% ($p < 0.01$) and up to 120% ($p < 0.001$) with higher concentrations (8 and 16 mM).

Figure 2 illustrates the effects of Glu-1-P or Gal concentrations on Na^+,K^+-ATPase activity in brain

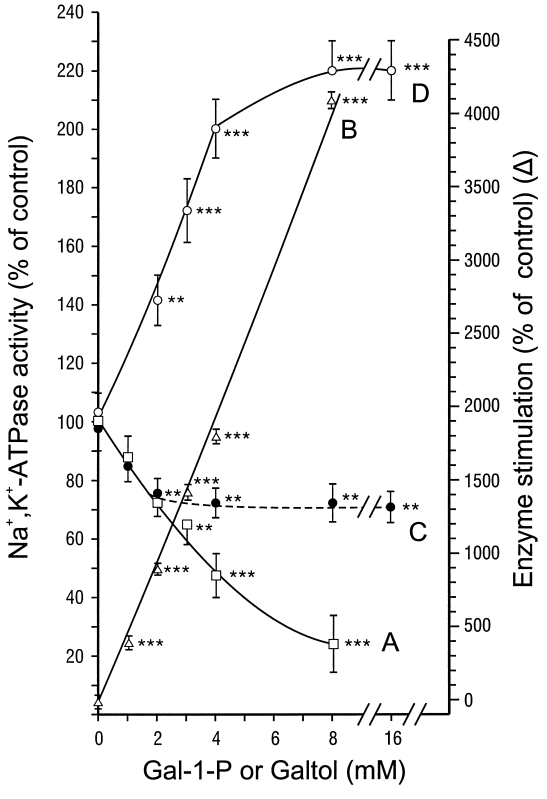

Fig. 1. Effects of different concentrations of galactose-1-phosphate (Gal-1-P) or galactitol (galtol) on Na^+,K^+-ATPase activity in whole brain homogenates or on pure enzyme activity. Gal-1-P was preincubated for 1 h with rat brain homogenates (A, □) or with pure enzyme (B, △). Galtol was preincubated for 1 h with rat brain homogenates (C, ●) or with pure enzyme (D, ○). Control value of brain Na^+,K^+-ATPase activity was 1.87 ± 0.17 μmol Pi/h × mg protein and that of the pure enzyme 14.80 ± 1.60 μmol Pi/h × mg protein. Values represent means ± SD of four experiments. The average values of each experiment arised from three determinations of the enzyme activity. ** $p < 0.01$; *** $p < 0.001$; as compared to control value.

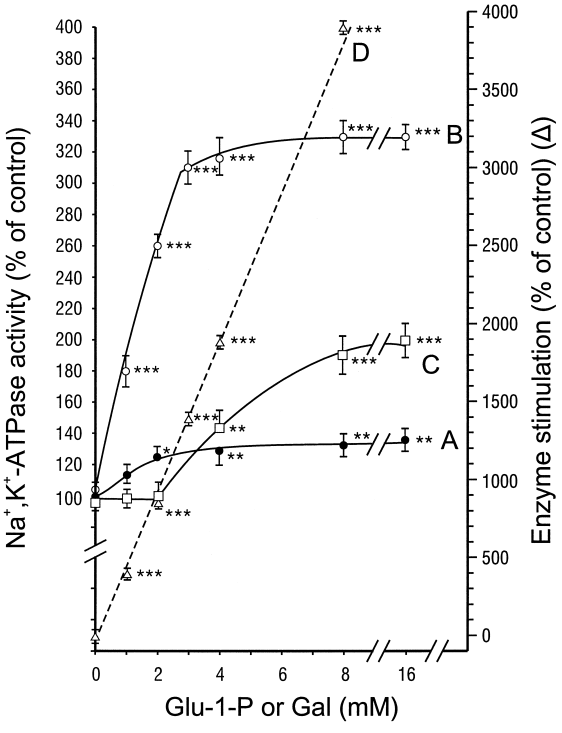

Fig. 2. Effects of different concentrations of glucose-1-phosphate (Glu-1-P) or galactose (Gal) on Na^+,K^+-ATPase activity in whole brain homogenates or on pure enzyme activity. Glu-1-P was preincubated for 1 h with rat brain homogenates (C, □) or with pure enzyme (D, △). Gal was preincubated for 1 h with rat brain homogenates (A, ●) or with pure enzyme (B, ○). Control value of brain Na^+,K^+-ATPase activity was 1.87 ± 0.17 μmol Pi/h × mg protein and that of the pure enzyme 14.80 ± 1.60 μmol Pi/h × mg protein. Values represent means ± SD of four experiments. The average values of each experiment were calculated from three determinations of the enzyme activity. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; as compared to control value.

homogenates or on pure enzyme activity. Gal increased the brain Na^+,K^+-ATPase activity (A) up to 35% ($p < 0.01$) at concentrations higher than 4 mM. Concentration 4 mM of the substance is also found in the untreated patients with classical galactosemia (Segal, 1995) and that of 1 mM in those of patients with galactokinase deficiency galactosemia (Segal, 1995). Moreover, Gal enormously increased the pure enzyme activity (B) about 80% ($p < 0.001$) with 1 mM and up to 230% ($p < 0.001$) with concentrations higher than 4 mM. Glu-1-P (> 2 mM) stimulated the brain Na^+,K^+-ATPase (C). Concentrations higher than 8 mM resulted in an 100% ($p < 0.001$) increase of the enzyme activity. Additionally, Glu-1-P stimulated the pure enzyme (D) up to 400% of the control with 1 mM and 900–3900% with 2–8 mM. On the contrary, Glu tested over the range of 1–16 mM, had not any statistically significant effect on the activity of both rat brain Na^+,K^+-ATPase and pure enzyme ($p > 0.05$).

In order to evaluate the brain Na^+,K^+-ATPase in *in vitro* conditions similar to those of patients with classical galactosemia (A) and with galactokinase deficiency galactosemia (B), we measured the brain enzyme activity after preincubation with a mixture of metabolites commonly found in these disorders (see Table I). Thus, Gal-1-P (2 mM), galtol (2 mM) and Gal (4 mM) (A) resulted in an 35% inhibition ($p < 0.001$) of the rat brain enzyme activity. Additionally, a mixture of galtol (2 mM) and Gal (1 mM) (B) decreased the enzyme activity about 25% ($p < 0.001$). For comparison, Gal-1-P (2 mM) or galtol (2 mM) alone resulted in an 22–30% enzyme inhibition ($p < 0.01$). Gal of 1 mM was not capable to affect the enzyme activity, whereas Gal of 4 mM alone stimulated the enzyme about 30% ($p < 0.01$).

Table I. Effect of Gal-1-P, galtol and Gal on Na^+,K^+-ATPase activity in suckling rat brain homogenates.

Treatment	Na^+,K^+-ATPase activity (μmol Pi/h × mg protein)
Control	1.87 ± 0.17
Gal-1-P (2 mM)	1.31 ± 0.05***
galtol (2 mM)	1.46 ± 0.05**
Gal (1 mM)	2.09 ± 0.10
Gal (4 mM)	2.43 ± 0.12**
(A) Gal-1-P+galtol+Gal (2 mM) (2 mM) (4 mM)	1.25 ± 0.06***
(B) galtol + Gal (2 mM) (1 mM)	1.40 ± 0.05***

** $p < 0.01$; *** $p < 0.001$; as compared to control value. The preincubation time of galactose-1-phosphate (Gal-1-P), galactitol (galtol) and galactose (Gal) with suckling rat whole brain homogenate (0.1 mg protein/ml) was 1 h, after which the enzyme activity was measured. Values represent means ± SD of five experiments. The average value of each experiment produced from three determinations.

Discussion

The effect of small compounds on the activity in some membrane bound enzymes (such as Na^+,K^+-ATPase) was suggested to alter the membrane fluidity causing functional changes in allosteric properties of enzymes (Ballantyne and Marro, 1992).

Gal-1-P, galtol, Glu-1-P and Gal concentrations had completely different effects on the activities of brain Na^+,K^+-ATPase and on the pure enzyme acting directly and indirectly. The indirect inhibition by Gal-1-P (Fig. 1A) may be due to the presence of both the epimer Gal and phosphate, because Glu-1-P or Gal caused a brain enzyme stimulation (Fig. 2C and A). The inhibitory effect of Gal-1-P could be due to the presence of phosphate group in position 1 of the sugar. The inhibitory effect of Gal-1-P seems to represent a different type of interaction than a simple phosphorylation of Na^+,K^+-ATPase, since Glu-1-P had a different action on the brain enzyme. Thus, the observed statistically significant different effect on Gal-1-P or Glu-1-P on brain Na^+,K^+-ATPase (Fig. 1A and 2C) could be the result of a different configuration induced by Gal and Glu. Gal as an epimer of Glu at C-4 differs in configuration only at one asymmetric center. Additionally, the ester Gal-1-P has been also considered a pathogenic agent inhibiting other enzymes, such as glucose-6-phosphate dehydrogenase, phosphoglucomutase, glycogen phosphorylase (Nordie, 1991) as well as acetylcholinesterase (Tsakiris and Schulpis, 2000), but the evidence remains presumptive. The increase of brain enzyme activity by Gal (Fig. 2A) and the absence of an effect by Glu, show the different indirect effect of these epimers on the enzyme. On the other hand, the similar pure enzyme activation by Gal-1-P or Glu-1-P (Fig. 1B and Fig. 2D) suggests that phosphate may stimulate the pure Na^+,K^+-ATPase directly.

Additionally, galtol (up to 2 mM) resulted in a concentration dependent indirect inhibition of brain Na^+,K^+-ATPase (Fig. 1C). On the contrary, Gal (4 mM) activated the brain enzyme, whereas a lower concentration of the carbohydrate (1 mM) did not cause any effect (Fig. 2A). Therefore, the enzyme indirect inhibition in our *in vitro* classical galactosemia (Table IA) should be due to Gal-1-P and galtol, whereas in galactokinase deficiency galactosemia (Table IB) to galtol alone.

If these *in vitro* findings come into the *in vivo* reality, e.g. classical galactosemia and galactokinase deficiency galactosemia, brain Na^+,K^+-ATPase inhibition in these patients may modulate the neural excitability (Sastry and Phillis, 1977), the metabolic energy production (Mata *et al.*, 1980) and the catecholaminergic and serotoninergic system (Bogdanski *et al.*, 1968; Herrero *et al.*, 1983). Nevertheless, the measurement of Na^+,K^+-ATPase activity represents a way to evaluate toxic effects and their consequences regarding central nervous system symptomatology, especially in metabolic inherited diseases as classical galactosemia where Gal-1-P and galtol are found considerably elevated.

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