Increased Na$^+$/Mg^{2+} Antiport in Erythrocytes of Patients with Cystic Fibrosis

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Summary: Na$^+$/Mg^{2+} antiport and Na$^+$-independent Mg^{2+} efflux were investigated in erythrocytes of 41 patients with cystic fibrosis and 26 controls. Na$^+$-independent Mg^{2+} efflux was unchanged in cystic fibrosis, but a significantly increased activity of Na$^+$/Mg^{2+} antiport was detected (control: 0.16 ± 0.02, cystic fibrosis: 0.39 ± 0.06, Mg^{2+} efflux, mmol/30 min × l cells, mean ± SEM, p < 0.01). An increased activity of Na$^+$/Mg^{2+} antiport was only found in patients with severe clinical symptoms. There was no correlation of the increased Na$^+$/Mg^{2+} antiport to the dF508 genotype. In a patient with increased Na$^+$/Mg^{2+} antiport, the capacity of this transport system was unchanged 14 weeks after double lung transplantation but reached control values after 53 weeks. The sweat of cystic fibrosis patients with severe clinical symptoms showed a significantly increased Mg^{2+} concentration (control (n = 12): 0.053 ± 0.08, cystic fibrosis (n = 9): 0.123 ± 0.016 mmol/l, mean ± SEM, p < 0.001).

Introduction

In patients with cystic fibrosis the cystic fibrosis transmembrane conductance regulator is mutated. A deletion of phenylalanine at codon 508 is found in about 70% of Caucasian cystic fibrosis patients; hundreds of different mutations of the cystic fibrosis gene have been described in the remaining 30% of patients (1). Besides the defect in cAMP-dependent chloride transport, other defects have been observed in cystic fibrosis, such as increased Na$^+$ absorption across airway epithelia, reduced mucin secretion from salivary glands and increased sulphatation of glycoconjugates secreted by airway epithelia (as reviewed in l.c. (2)). It has been suggested that these defects are secondary to the reduced Cl$^-$ conductance, or that they may represent additional regulatory functions of cystic fibrosis transmembrane conductance regulator. The latter not only functions as a Cl$^-$ channel but also seems to influence the activity of other transport proteins by leading to a cystic fibrosis transmembrane conductance regulator-associated protein kinase A activation, for example, of the outwardly rectifying chloride channel (3). In cystic fibrosis, re-

duced Cl$^-$ transport via both Cl$^-$ channels contributes to the disturbed Cl$^-$ secretion.

During the last few years Mg^{2+} efflux systems of erythrocytes have been characterised. Mg^{2+} efflux from Mg^{2+}-loaded erythrocytes proceeds via an electroneutral Na$^+$/Mg^{2+} antiport which transports two Na$^+$ for one Mg^{2+} and can be reversibly inhibited by amiloride (4). This transport, therefore, represents an amiloride inhibitable Na$^+$ influx pathway. Amiloride is currently being used as a therapeutic agent in cystic fibrosis, inhibiting Na$^+$ (and secondary H$_2$O) uptake into the bronchial epithelial cells, thereby leading to a decrease of mucus viscosity (5). Additionally to Na$^+$/Mg^{2+} antiport, erythrocytes possess a Na$^+$-independent Mg^{2+} efflux system. This Mg^{2+} efflux is accompanied by Cl$^-$ efflux for charge compensation. Reduction of the Cl$^-$ efflux by high extracellular Cl$^-$ or Cl$^-$ channel blockers like 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid also diminish Mg^{2+} efflux across this system, an effect which can be overcome by the lipophilic cation tetraphenylphosphonium (6). As Mg^{2+} efflux is coupled either to Cl$^-$ or to Na$^+$ transport, investigations were performed

to determine whether these Mg^{2+} transport systems might be changed in cystic fibrosis. In a preliminary report it was shown that the Mg^{2+} content of erythrocytes was not changed in cystic fibrosis, but the Mg^{2+} efflux capacity of erythrocytes was increased in some patients (7). Here it is reported that Na$^+$/Mg^{2+} antiport is significantly increased in cystic fibrosis patients with severe clinical symptoms, that it is accompanied by an increased Mg^{2+} concentration in sweat, and that it is not coupled to the dF508 genotype of cystic fibrosis.

Materials and Methods

Patients

Cystic fibrosis patients (n = 41; 23 female, 18 male) and 26 age matched controls (11 female, 15 male) not affected with cystic fibrosis were included in the study. Age (in years), mean ± SD: patients 19.4 ± 10.3, controls 21.4 ± 14.4. All patients had chronic *Pseudomonas aeroginosa* infection of the lungs, suffered from pancreas insufficiency, and had not been treated with amiloride. The patients were routinely genotyped. Patients were divided into three groups based on the severity of their clinical symptoms, according to the *Shwachman* score without X-ray examination (8): mild (CF1) > 55, medium (CF2) 55−41, severe (CF3) < 41 points.

Sweat test

Sweat tests were performed on the forearm with pilocarpine iontophoresis with a Webster sweat inducer system (Wescor, Logan, Utah, USA). For measurement of the Mg^{2+} concentration in sweat 10 µl samples were diluted with 1 ml 100 g/l trichloroacetic acid/ 1.75 g/l LaCl$_3$ and Mg^{2+} was measured by atomic absorption spectrophotometry (AAS, Philips SP9).

Mg^{2+} efflux

Heparinized blood was taken from controls and cystic fibrosis patients. Blood was centrifuged at 1000 g for 10 min. The erythrocytes were taken for the measurement of Mg^{2+} efflux, as already described (4). Briefly, the cells were loaded with Mg^{2+} by incubating a cell suspension (volume fraction 0.1) for 30 min at 37 °C in KCl medium (in mmol/l: 140 KCl, 12 MgCl$_2$, 50 sucrose, 5 glucose, 30 Hepes/Tris, pH 7.4). With the addition of 6 µmol/l of the cation ionophore A23187 (Boehringer Mannheim, Germany) dissolved in dimethyl sulphoxide. For removal of the ionophore, the cells were incubated four times in KCl medium plus 10 g/l bovine serum albumin for 10 min at 37 °C. The KCl medium was removed by washing the cells twice with cold (4 °C) choline chloride medium (in mmol/l: 140 choline chloride, 5 glucose, 30 Hepes/Tris, pH 7.4).

Mg^{2+} efflux was measured by reincubating a cell suspension (volume fraction 0.1) at 37 °C in Mg^{2+}-free NaCl medium (substitution of KCl in KCl medium by 140 mmol/l NaCl) or Mg^{2+}-free choline chloride medium (substitution of KCl in KCl medium by 140 mmol/l choline chloride). At the beginning of reincubation and after 30 min, 0.5 ml aliquots of the cell suspensions were centrifuged for 1 min at 10 000 g. Aliquots (100 µl) of the supernatants were diluted with 1 ml 100 g/l trichloroacetic acid/1.75 g/l LaCl$_3$, and Mg^{2+} was measured by AAS. Mg^{2+} efflux was calculated from the increase of Mg^{2+} in the reincubation media and was related to cell volume. Cell volume was determined by measuring the haematocrit and the haemoglobin content of a cell lysate. Na$^+$-dependent Mg^{2+} efflux (Na$^+$/Mg^{2+} antiport) was determined by subtracting Mg^{2+} efflux in choline chloride medium (which represents Na$^+$-

independent Mg^{2+} efflux at physiological extracellular Cl$^-$ concentration) from Mg^{2+} efflux in Na$^+$ medium.

Results

Table 1 shows that Na$^+$-independent Mg^{2+} efflux, which is accompanied by Cl$^-$ efflux, is not changed in cystic fibrosis. The mean Na$^+$/Mg^{2+} antiport, however, is more than doubled in cystic fibrosis patients. The elevated mean is due to high values in some patients, whereas other patients had normal antiport rates. By dividing the patients into three groups according to the severity of their disease, it was obvious that particularly patients with severe clinical symptoms (CF3) showed an increased Na$^+$/Mg^{2+} antiport capacity, whereas none of the other patients (CF1 or CF2) showed any change in transport capacity (tab. 2, fig. 1). In all patients with increased Na$^+$/Mg^{2+} antiport, this transport was inhibited by 1 mmol/l amiloride (fig. 1), which also inhibited Na$^+$/Mg^{2+} antiport in controls and cystic fibrosis patients with normal transport rates. Figure 2 shows the Na$^+$/Mg^{2+} antiport rates of patients with the homozygous dF508 mutation, classified according to the *Shwachman* score. The increased Na$^+$/Mg^{2+} antiport could not be attributed to a specific mutation of the cystic fibrosis gene as some patients with the homozygous dF508 mutation showed a normal Na$^+$/Mg^{2+} antiport, while in others it was increased. Also in the remaining

Tab. 1 Na$^+$/Mg^{2+} antiport and Na$^+$-independent Mg^{2+} efflux from Mg^{2+}-loaded erythrocytes from controls and cystic fibrosis patients. Mean ± SEM. Significant difference between control and cystic fibrosis patients by *Mann-Whitney* test; **, p < 0.01.

	n	Na$^+$/Mg^{2+} antiport	Na$^+$-independent Mg^{2+} efflux
		(Mg^{2+} efflux, mmol/30 min × 1 cells)	
Control	26	0.16 ± 0.02	0.12 ± 0.01
Cystic fibrosis	41	0.39 ± 0.06**	0.13 ± 0.01

Tab. 2 Na$^+$/Mg^{2+} antiport from Mg^{2+}-loaded erythrocytes of controls and cystic fibrosis patients. The patients were grouped according to the severity of their disease, CF1 (mild), CF2 (medium), CF3 (severe), for details see Methods. Mean ± SEM. Significant difference between control and cystic fibrosis patient groups by *Mann-Whitney* test; ***, p < 0.001.

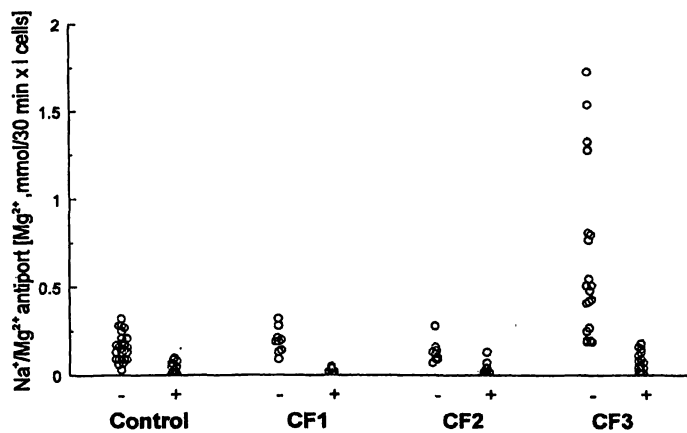|	n	Na$^+$/Mg^{2+} antiport (Mg^{2+} efflux, mmol/30 min × 1 cells)
Control	26	0.16 ± 0.02
CF1	9	0.19 ± 0.02
CF2	10	0.13 ± 0.02
CF3	22	0.63 ± 0.10***

Fig. 1 Na⁺/Mg²⁺ antiport in Mg²⁺-loaded erythrocytes from controls and patients with cystic fibrosis. Incubation without (−) or with (+) 1 mmol/l amiloride (Sigma, München, Germany). Na⁺/Mg²⁺ antiport is determined as Mg²⁺ efflux in NaCl medium minus efflux in choline chloride medium. CF1, CF2 and CF3 are cystic fibrosis patients subdivided into groups according to the severity of their clinical symptoms (for details see Methods).
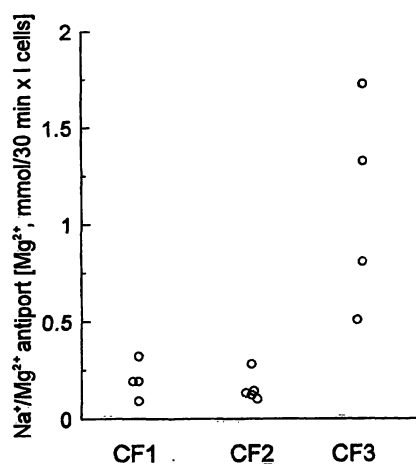

Fig. 2 Na⁺/Mg²⁺ antiport in Mg²⁺-loaded erythrocytes from cystic fibrosis patients with the homozygous dF508 mutation subdivided into groups according to the severity of their clinical symptoms.

patients (dF508 heterozygous with additional mutations, or homozygous for mutations of the cystic fibrosis gene other than dF508) no correlation between genotype and Na⁺/Mg²⁺ antiport could be detected.

One patient with increased Na⁺/Mg²⁺ antiport (Mg²⁺ efflux: 0.58 mmol/30 min × 1 cells) underwent double lung transplantation. Fourteen weeks after transplantation, Mg²⁺ efflux was almost the same (0.54 mmol/30 min × 1 cells), although the clinical symptoms had greatly improved. However, 53 weeks after lung transplantation Na⁺/Mg²⁺ antiport was normalised (Mg²⁺ efflux, 0.22 mmol/30 min × 1 cells).

Results for the Mg²⁺ concentration in the sweat of cystic fibrosis patients were similar to those obtained for Na⁺/

Mg²⁺ antiport (tab. 3), i. e. only the patients with the highest severity score (CF3) showed significantly increased sweat Mg²⁺ concentrations.

Discussion

Intracellular Mg²⁺ is needed for many physiological and metabolic functions, i. e. activation of enzymes, charge reduction of negatively loaded substances like DNA or RNA and regulation of ion channels (9). To fulfil these tasks, the intracellular concentration of free Mg²⁺ ([Mg²⁺]ᵢ) has to be kept within narrow limits. Only 5% of total intracellular Mg²⁺ is ionised, and the concentration range of [Mg²⁺]ᵢ is about 0.2−1.0 mmol/l, depending on cell type. [Mg²⁺]ᵢ increases when Mg²⁺ is released from intracellular ligands, i. e. after breakdown of ATP or intracellular acidification. Thereafter, it is normalised by activation of Mg²⁺ efflux systems, especially electroneutral Na⁺/Mg²⁺ antiport. Na⁺/Mg²⁺ antiport works only at increased intracellular free Mg²⁺ concentration. Under these conditions it represents a Na⁺ influx and Mg²⁺ efflux pathway which has been found in several cell types (erythrocytes, thymocytes, cardiomyocytes, hepatocytes, HL60 cells) of different species with different transport capacities. This transport system is not identical with any of the other Na⁺ influx systems (e. g. Na⁺/H⁺ or Na⁺/Ca²⁺ exchange) (review in l. c. (10)). As we report here, in human erythrocytes it is specifically increased in a sub-population of cystic fibrosis patients with a severe clinical condition.

The increased Na⁺/Mg²⁺ antiport in cystic fibrosis is not coupled to the dF508 genotype or any other known mutation of the cystic fibrosis gene, as patients with homozygous dF508 mutations display normal as well as increased antiport. It is likely that the increased Na⁺/Mg²⁺ antiport is caused by an as yet unknown effect of the disease that only develops in a subgroup of severely affected patients. In accordance with this result, the clinical severity of cystic fibrosis was also not correlated with the genotype (1). This also agrees with the findings after lung transplantation. Transplantation of the lung induced normalisation of Na⁺/Mg²⁺ antiport of the

Tab. 3 Mg²⁺ concentration in sweat of controls and cystic fibrosis patients, grouped according to table 2. Mean ± SEM. Significant difference between control and cystic fibrosis patient groups by *Mann-Whitney* test; ***, p < 0.001.

	n	[Mg²⁺] in sweat (mmol/l)
Control	12	0.053 ± 0.008
CF1	4	0.060 ± 0.015
CF2	10	0.070 ± 0.010
CF3	9	0.123 ± 0.016***

erythrocytes. The lag period may be explained by the turnover rate of the erythrocytes. In the transplanted patient all clinical problems remained unchanged except respiratory distress. Therefore, the increased Na^+/Mg^{2+} antiport must be caused by the affected lung. Another possibility might be a long term effect of the immunosuppressive therapy on the increased transport. A higher rate of oxygen radical production due to the lung infection in cystic fibrosis patients seems not to be the cause of the increased Na^+/Mg^{2+} antiport, as oxygen radicals induced a reduction and not an increase of Na^+/Mg^{2+} antiport (11).

Increased transport rates of Na^+/Mg^{2+} antiport should lead to higher transcellular Mg^{2+} fluxes. The increased Mg^{2+} content in the sweat of the CF3 group could be explained by higher transcellular Mg^{2+} fluxes due to increased Na^+/Mg^{2+} antiport. Due to the small amount of transported Mg^{2+}, the increased Na^+ uptake via Na^+/Mg^{2+} antiport would not significantly change the Na^+

content of sweat. Paunier et al. (12) reported similar values for the Mg^{2+} content of sweat in controls, but did not find a significant difference between controls and cystic fibrosis children. However, they did not divide their patients into subgroups according to the severity of disease and, therefore, might have overlooked increased Mg^{2+} concentrations by just comparing means, or might have had a population of patients with mild or modest clinical symptoms.

If Na^+/Mg^{2+} antiport is increased not only in erythrocytes but also in other cell types, then intracellular Mg^{2+} may be reduced in these other cell types. A high Mg^{2+} supply would then be beneficial to these cystic fibrosis patients.

Acknowledgement

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