Increased inward passive permeability in vitro to sodium in uraemic erythrocytes

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1. We have reported a normal sodium (Na) pump, but decreased loop-diuretic-sensitive Na efflux in erythrocytes from patients with chronic renal failure on haemodialysis, suggesting a different mode of co-transport in uraemia.

2. The present work extends these findings and examines in vitro simultaneous unidirectional and radiolabelled Na and K fluxes through the Na/K/Cl co-transport and the Na/K pump in washed erythrocytes from seven subjects with chronic renal failure and seven controls. Erythrocyte cytosolic calcium was also examined.

3. Ouabain-sensitive 86Rb influx was similar in patients and controls (1.76 ± 0.19 versus 1.72 ± 0.13 mmol h⁻¹ litre⁻¹ of erythrocytes) as was ouabain-sensitive 22Na efflux (3.62 ± 0.36 versus 4.04 ± 0.39 mmol h⁻¹ litre⁻¹ of erythrocytes).

4. Bumetanide-sensitive 86Rb and 22Na influx and 22Na efflux were measured at three concentrations (4, 8 and 12 mmol/l) of external K. In chronic renal failure, mean bumetanide-sensitive 22Na efflux was decreased at all external K concentrations compared with controls, and at physiological concentrations (4 mmol/l) external K was lower than controls (0.14 ± 0.01 versus 0.38 ± 0.05 mmol h⁻¹ litre⁻¹ of erythrocytes, P < 0.01). Mean bumetanide-sensitive 86Rb influx was also reduced in chronic renal failure at all external K concentrations, and at 4 mmol/l external K was lower than controls (0.13 ± 0.04 versus 0.34 ± 0.04 mmol h⁻¹ litre⁻¹ of erythrocytes, P < 0.01). Conversely, bumetanide-sensitive 22Na influx was markedly increased at all external K levels in chronic renal failure, and at 4 mmol/l external K values were elevated compared with controls (0.64 ± 0.18 versus 0.34 ± 0.04 mmol h⁻¹ litre⁻¹ of erythrocytes, P < 0.001). The mean cytosolic calcium concentration was higher in erythrocytes in chronic renal failure than controls (134.4 ± 8.6 versus 63.7 ± 5.8 mmol/l, P < 0.001).

5. Thus, in washed erythrocytes incubated in artificial media there is a markedly increased ouabain-insensitive Na influx in subjects with chronic renal failure which might be explained in part by the higher levels of cytosolic calcium. In vivo, this cell defect combined with suppression of the Na/K pump could lead to intracellular Na accumulation and play a role in uraemic complications.

INTRODUCTION

In chronic renal failure (CRF), a number of defects in transmembrane cation transport have been reported [1]. Firstly, a decrease in the activity of the Na,K pump, leading to accumulation of intracellular Na, has been reported in erythrocytes from uraemic subjects [2]. The defect was found to be reversible after initiation of chronic intermittent haemodialysis [3], except in a subset of patients with raised cell Na [4]. Other reports have described a decrease in furosemide-sensitive Na efflux (co-transport) in erythrocytes from subjects with CRF [5, 6]. In a further kinetic analysis, we reported normal intracellular Na and ouabain-sensitive Na efflux in uraemic erythrocytes washed and incubated in artificial media, but a uniformly reduced Vmax, for furosemide-sensitive Na efflux [7]. To further delineate the relationship of co-transport to total cation transport in erythrocytes from subjects with CRF, we now measure simultaneous unidirectional Na and Rb fluxes through the Na,K pump and the loop-diuretic-sensitive Na,K,Cl co-transport. As cytosolic calcium ([Ca²⁺]i) may alter furosemide-sensitive Na efflux, we also measured [Ca²⁺]i in erythrocytes from controls and subjects with CRF.

METHODS

Subjects

Seven Caucasian male patients with end-stage renal disease, receiving chronic intermittent haemodialysis, were studied. The age range was from 30 to 73 years (mean 55 ± 12.8 years). The aetologies of
end-stage renal disease and the number of patients (in parentheses) were as follows: chronic glomerulonephritis (3), polycystic kidney disease (2), chronic interstitial nephritis (1), Alport syndrome (1). Pertinent predialysis clinical parameters were: systolic blood pressure 153.6 ± 8.6 mmHg, diastolic blood pressure 82.6 ± 4.6 mmHg, serum creatinine 1025 ± 53 μmol/dl, blood urea nitrogen 24.3 ± 3.4 mmol/dl, serum albumin 3.9 ± 0.1 g/dl, serum calcium 2.4 ± 0.1 mmol/dl, serum phosphorus 1.7 ± 0.1 mmol/dl, serum sodium 136.8 ± 1.7 mmol/l, chloride 105 ± 2.9 mmol/l, serum potassium 4.8 ± 0.3 mmol/l and carbon dioxide 19.1 ± 0.8 mmol/l. Mean time on dialysis was 8.2 ± 1.3 years. Patients received three treatments per week and were dialysed against a bath with the following composition: sodium 135 mmol/l, calcium 3.5 mmol/l, potassium 2 mmol/l, magnesium 1.5 mmol/l, chloride 105 mmol/l, bicarbonate 35 mmol/l and dextrose 2 g/l. The controls included seven Caucasian male subjects aged 24 to 40 years (mean 33.3 ± 6.1 years). They were normotensive with no medical problems. Informed consent was obtained from all subjects.

Preparation of erythrocytes

Fresh venous blood was drawn between 08.00 and 10.00 hours in control subjects and in patients just before starting dialysis. Blood was collected into heparinized tubes and the plasma and buffy coat were discarded after separation by a 10min centrifugation at 3000 g. The packed erythrocytes were then washed four times in a choline washing solution containing: choline chloride 150 mmol/l, MgCl₂ 1 mmol/l, Tris-Mops 10 mmol/l, pH 7.4, at 4°C. The erythrocytes were used the same day.

Modification of erythrocyte Na content

The procedure for nystatin-loading described by Cass and Daimark [8] and revised by Canessa et al. [9] was used to modify the intracellular Na concentration and achieve a uniform content of 14 mmol/l erythrocytes in order to minimize intra-individual variations. Changes in erythrocyte volume during nystatin-loading were determined by measuring the haemoglobin per litre erythrocytes in fresh and Na-loaded erythrocytes.

Cation determination

Cation concentration in erythrocytes was determined on an atomic absorption spectrophotometer (Model 5000; Perkin–Elmer Corp., Norwalk, CT, U.S.A.) using standards in double-distilled water. Aliquots (0.2 ml) of a 50% erythrocyte suspension were taken and lysed completely in 9.8 ml of 0.02% Acationox for intracellular Na measurement in fresh and Na-loaded erythrocytes. A further dilution of 1/10 in double-distilled water was used for K and haemoglobin determinations.

Measurement of unidirectional radioactive efflux

Measurements of unidirectional fluxes were performed according to modified procedures described by Brugnara et al. [10] and Canessa et al. [11]. For ^22Na efflux measurements through the pump, 1 ml of erythrocyte suspension was added to 10 ml of flux medium. The medium contained: NaCl 130 mmol/l, KCl 4 mmol/l, choline chloride 16 mmol/l, MgCl₂ 1 mmol/l, glucose 10 mmol/l, Tris–Mops 10 mmol/l, pH 7.4 at 37°C, with or without 0.1 mmol/l ouabain. The same medium containing 0.1 mmol/l ouabain but with 4, 8 or 12 mmol/l KCl were used for Na efflux measurements through the co-transport system, in the presence or absence of 0.01 mmol/l bumetanide. In these solutions, the choline chloride concentration was adjusted to keep the sum of K plus choline equal to 20 mmol/l. Each flux suspension was then distributed into six previously chilled tubes. After capping, three tubes were incubated for 5 min and three for 25 min in a shaking bath at 37°C for determination of the ouabain-sensitive fluxes. The time course of bumetanide-sensitive Na fluxes was 5 and 65 min. The reaction was stopped by cooling for 2 min in ice and then centrifuging at 1000 g for 5 min at 4°C. Supernatant (0.8 ml) was removed and counted in a gamma counter (Beckman, Model 4000).

Five aliquots of 1 ml of a 1/50 dilution of the initial suspension in choline washing solution were counted for determination of initial specific activity. The efflux in mmol h⁻¹ litre⁻¹ of erythrocytes was calculated from: efflux = I/erythrocyte specific activity × V s × 1000 × F × c.p.m./h, where erythrocyte specific activity is counted per min per mmol, V s is the volume in litres of supernatant counted, F is ml of flux medium per ml of erythrocyte and c.p.m./h is the c.p.m. in the supernatant of the sample taken after a 25 (or 65) min incubation, minus c.p.m. in the sample taken after a 5 min incubation.

Measurement of unidirectional radioactive influx

Ten millilitres of medium was chilled and mixed with 10 μCi of ^22Na or 30 μCi of ^86Rb. The radioactivity in five aliquots of 20 μl of medium was measured for determination of initial specific activity. To the 10 ml of medium, 0.8 ml of packed erythrocytes was added. The influx medium, containing 4 mmol/l K for ouabain-sensitive ^86Rb influx determination and 4, 8 and 12 mmol/l K for bumetanide-sensitive ^86Rb influx, was processed as described for the efflux. The supernatant of the samples collected after 5 and 25 (or 65) min incubation at 37°C was discarded and the cells were washed four times with 4 ml of cold Na washing solution containing: NaCl 150 mmol/l, MgCl₂ 1 mmol/l and Tris–Mops 10 mmol/l, pH 7.4, at 4°C. The erythrocyte pellets were lysed with 1 ml of 0.02% Acationox in double-distilled water. The
tubes were vortexed and spun for 20 min at 3000 g. Aliquots of 0.8 ml were counted in a gamma counter. Aliquots of 50 μl were diluted 50 times with an automatic diluter and the concentration of haemoglobin was determined by absorbance at 540 nm. The influx in mmol h⁻¹ litre⁻¹ of erythrocytes was calculated from: influx = c.p.m./l erythrocyte [25 (or 65) − 5 min incubation]/initial specific activity (c.p.m./mmol), where c.p.m./l erythrocyte = c.p.m. in the lysate × 1/VI × 100/haematocrit and where VI is the volume in litres of lysate counted. The amount of cells present in the lysate was calculated using the haematocrit and concentration counter. Aliquots of 50111 were diluted 50 times with 540nm. The influx in mmol h⁻¹ litre⁻¹ of erythrocyte was determined by absorbance at 540nm. The influx in mmol h⁻¹ litre⁻¹ of erythrocyte and the concentration of haemoglobin in the lysate. The same methodology but without exposure to nystatin or changes in intracellular Na.

**Intracellular calcium determination**

Erythrocyte [Ca²⁺]<sub>i</sub> was measured by fluorometry according to a method modified from Merritt et al. [12]. Fresh venous blood was collected in heparinized tubes and after separation by ultracentrifugation, erythrocytes were incubated for 1 h at 37°C in glucose containing Hepes-buffered saline in the presence of 1μmol/l of Fluo-3/AM and 1 μl of pluronic acid. The cells loaded with Fluo-3/AM were then centrifuged through a 5% BSA pad to remove the free probe. Erythrocytes were resuspended in the loading buffer containing CaCl₂ (1 mmol/l) at 1% haematocrit suspension. Fluorometry measurements were done in a stirred cuvette at 37°C using a F-2000 Hitachi spectrofluorometer (Hitachi, Japan). Erythrocytes were excited at 506 nm and the emission recorded at 526 nm. Auto-fluorescence of a parallel aliquot of fresh erythrocytes that had not been exposed to Fluo-3 was taken for each sample as a measure of F<sub>min</sub> [12]. Digitonin (30 μmol/l) was added to lyse the cells and the obtained measurements were taken as F<sub>max</sub>. values. [Ca²⁺]<sub>i</sub> was calculated according to the formula: Ca (nmol/l) = K<sub>d</sub> × (F − F<sub>min</sub>)/(F<sub>max</sub> − F) where K<sub>d</sub> was 865. The results were expressed in nmol/l.

**Statistical analysis**

All data are reported as mean ± SEM. Statistical significance was evaluated using unpaired Student's t-test. The null hypothesis was rejected for a P value less than 0.05.

**RESULTS**

Intracellular electrolyte concentration and cell volume determination of haemoglobin concentration in fresh and in Na-loaded erythrocytes showed mild shrinkage with recovery of 98 ± 3.5% of the initial volume. Mean intracellular Na and K concentrations were not different in fresh erythrocytes from uraemic patients and normal controls (7.7 ± 0.8 versus 8.5 ± 0.3 mmol/l erythrocyte and 95.2 ± 5.0 versus 92.03 ± 6.9 mmol/l erythrocyte, respectively) and both cell populations achieved a similar intracellular Na and K content after Na-loading using nystatin (13.9 ± 0.6 versus 14.6 ± 0.8 mmol/l erythrocyte and 89.6 ± 5.4 versus 84.9 ± 7.1 mmol/l erythrocyte, respectively).

**Na,K pump unidirectional fluxes**

Figure 1 shows mean rates of ouabain-sensitive ²²Na efflux in dialysis patients and controls and simultaneous ouabain-sensitive ⁸⁶Rb influx when erythrocytes were incubated in media containing 130 mmol/l NaCl and 4 mmol/l KCl. Mean levels of ⁸⁶Rb influx in dialysis patients and control patients (1.76 ± 0.19 versus 1.72 ± 0.13 mmol h⁻¹ litre⁻¹ of erythrocytes) were nearly identical in dialysis patients and controls (1.76 ± 0.19 versus 1.72 ± 0.13 mmol h⁻¹ litre⁻¹ of erythrocytes, and the values for ²²Na efflux (3.62 ± 0.36 vs 4.04 ± 0.39 mmol h⁻¹ litre⁻¹ of erythrocytes) were also similar (P not significant). When results were expressed as efflux rate constants, no difference was found in uraemic compared with control erythrocytes (0.30 ± 0.04 versus 0.34 ± 0.04/h).

**Na,K co-transport unidirectional fluxes**

Bumetanide-sensitive ²²Na efflux after incubation in media containing 130 mmol/l NaCl and 4 mmol/l KCl was reduced in uraemic compared with control erythrocytes (0.14 ± 0.01 versus 0.38 ± 0.08 mmol h⁻¹ litre⁻¹ of cells, P < 0.01). Efflux rate constants were respectively 0.011 ± 0.003 and 0.024 ± 0.004/h, P < 0.001). Na influx through the Na,K,CI co-transport pathway was markedly higher in uraemic erythrocytes than in controls (0.64 ± 0.18 versus 0.37 ± 0.04 mmol h⁻¹ litre⁻¹ of cells, P < 0.05). Bumetanide-sensitive ⁸⁶Rb influx was lower in uraemic erythrocytes than in controls (0.13 ± 0.04 versus 0.34 ± 0.04 mmol h⁻¹ litre⁻¹ of erythrocytes).

Figures 2 and 3 depict mean levels for simul-
Residual unidirectional fluxes

At 4 mmol/l K\textsubscript{o} ouabain- and bumetanide-resistant \textsuperscript{22}Na efflux was not significantly different between uraemic erythrocytes and controls (0.5 ± 0.05 versus 0.63 ± 0.009 mmol h\textsuperscript{-1} litre\textsuperscript{-1} of erythrocytes). However, the residual \textsuperscript{22}Na influx measured in erythrocytes previously exposed to both ouabain and bumetanide and 4 mmol/l K\textsubscript{o} was markedly increased in uraemic subjects compared with controls (3.53 ± 0.09 versus 1.98 ± 0.09 mmol h\textsuperscript{-1} litre\textsuperscript{-1} of erythrocytes, \(P < 0.05\)) (Fig. 4). These experiments, when performed in fresh erythrocytes, yielded similar results (4.92 ± 0.75 versus 1.87 ± 0.09 mmol h\textsuperscript{-1} litre\textsuperscript{-1} of erythrocytes, \(P < 0.01\)).

Intracellular calcium

Figure 5 shows that mean free [Ca\textsuperscript{2+}]\textsubscript{i} was significantly higher in uraemic erythrocytes than in controls (134.4 ± 8.6 versus 63.7 ± 5.8 nmol/l, \(P < 0.001\)).

DISCUSSION

The present study using washed erythrocytes incubated in artificial media found similar unidirectional Na and Rb fluxes through the Na,K pump in dialysis patients and controls, confirming our previous results [5, 7]. In order to assess cation transport function through the Na,K pump in humans, researchers have used erythrocytes to
measure ouabain-sensitive fluxes, examining either the Na efflux component or the K influx component [2–7]. Since the now historical work by Welt et al. [2] showing reduced pump activity in uraemia, several other studies have reported alterations of the Na,K pump. Kaji and Khan [1], in a review of the literature, discussed the evidence for Na,K pump inhibition in CRF. From their compilation, it was concluded that erythrocyte Na,K pump abnormalities such as reduced Na efflux, decreased K influx and low number of pump units were reported only in erythrocytes from severely uraemic patients before the initiation of chronic haemodialysis. A reduced Na,K pump has also been noted in a subset of haemodialysed patients who maintain a consistently high erythrocyte intracellular Na concentration [4]. Our data show that in uraemic erythrocytes with normal K and Na content, Na,K pump activity as measured by unidirectional cation transport is indistinguishable from controls. Moreover, our results indicate a K to Na 'gear ratio' of 1 to 2 which, as described by Maizels [13], is an important indicator of normal pump function. Our results obtained in vitro in stable and adequately dialysed patients do not exclude the existence of a dialysable inhibitor in patients with end-stage renal failure who have not been dialysed or who have been dialysed sub-optimally. Likewise, they do not exclude the possibility of an ouabain-like factor reducing the pump activity in vitro. They only attest that the transport protein, when subtracted from the uraemic milieu, functions in the normal range.

Although outward passive permeability of Na is thought to be normal in erythrocytes from patients with CRF [4], inward permeability has been found to be increased in erythrocytes stored in a physiological medium at 4°C [14]. Our data also show a markedly increased residual Na influx in erythrocytes from patients with CRF. This increased ouabain- and bumetanide-resistant Na influx could be mediated through one or both of the ouabain-insensitive Na transport systems, including the 4,4'-diisothiocyanostilbene-2,2'-disulphonate (DIDS)-sensitive Cl⁻/NaCO₃⁻ anion exchanger and the Na/H antiporter. In previous studies, we found increased Na/H antiporter activity and abundance in erythrocytes from patients with CRF whereas the activity of the Cl⁻/NaCO₃⁻ DIDS-sensitive anion exchanger was similar in uraemic and control erythrocytes [15].

On the other hand, the simultaneous inward and outward unidirectional fluxes through the Na/K/Cl co-transport system have not been studied in CRF. We first found an uncoupling of the normal 1:1 relationship between furosemide-sensitive internal Na-activated Na and K efflux rates in human erythrocytes [7]. We are now reporting a similar uncoupling defect for the external K-activated, bumetanide-sensitive inward fluxes of Na and K. Under basal conditions in human erythrocytes, the Na/K/Cl co-transport system mediates an equal bidirectional flux of Na and K [16]. In our study, the bumetanide-sensitive Na/K/Cl co-transport system functions with comparable efficiency in both the inward and outward directions when measured in erythrocytes from controls. In uraemic erythrocytes, however, bumetanide-sensitive Na influx is markedly higher than either ⁸⁶Rb influx or ²²Na efflux through the same pathway.

The physiological role of the co-transport pathway in non-nucleated erythrocytes is not precisely known, but it has been implicated in the regulation of the Na and K gradients across cell membranes. This pathway may then function as a 'second pump' to help in extruding Na against its electrochemical gradient [17].

The relevance of the decreased outward erythrocyte Na/K co-transport in CRF described several years ago [5, 6] remains uncertain. We first hypothesized [5] that in CRF an endogenous circulating furosemide-like substance could inhibit the transporter. However, in a subsequent study [18], we found that furosemide-sensitive Na efflux was not decreased in normal human erythrocytes after incubation in uraemic plasma. This finding suggests that the abnormalities observed are not mediated by plasma-borne inhibitors but more probably reflect an acquired membrane or cellular defect which alters the mode of the transporter. Garay [19] has reported that both an increased internal calcium concentration and an increased accumulation of cyclic AMP can inhibit furosemide-sensitive Na efflux. In our study, erythrocytes from CRF patients displayed an almost 2-fold higher content of calcium than controls and it is likely that the high intracellular calcium explains at least in part the low bumetanide-sensitive Na efflux.

Regardless of the mechanism, if widespread in other cells this defect could be of important physiological significance. Changes in Na fluxes through the ouabain-insensitive pathways have not been found to affect the final intracellular Na concentration [20, 21]. This property is probably due to a secondary upregulation of the Na/K pump since it is known that in human erythrocytes the Na/K pump and facilitated transport are not independent [22]. In CRF, the cells are exposed in vivo to ouabain-like factors which suppress the pump. This inhibition occurs in the presence of a Na/K co-transport pathway which is unable to compensate and should lead in vivo to Na intracellular accumulation. Such an increase in intracellular Na would then be of importance in tissues such as vascular smooth muscle and could explain the hypertension seen in end-stage renal disease before initiation of dialysis as well as more delayed cardiovascular complications of the uraemic state.

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