Low Sodium Cotransport in Red Cells with Physiological Internal Sodium Concentration in Essential Hypertension

ALBERTO MONTANARI, EMILIO SANI, MAURO CANALI, ISABELLA SIMONI, PAOLO SCHIANCHI, ALBERICO BORGHETTI, AND ALMERICO NOVARINI

SUMMARY Ouabain-resistant Na and Li effluxes in erythrocytes from 18 normal subjects and 19 hypertensive subjects were studied in fresh cells that contained about 9 mmol Li and 2.5 or 6.5 mmol Na per liter of erythrocytes after intact cells had been incubated for 5 hours in 110 mM Li, 40 mM Na medium, with or without ouabain 10\(^{-4}\) M. Outward Na cotransport was estimated at both internal Na concentrations as the furosemide-sensitive unidirectional \(^{22}\)Na efflux from erythrocytes into a Na free-medium containing 75 mM MgCl\(_2\). The changes in furosemide-sensitive outward Na transport between the two levels of internal Na were considered as a measure of the response of Na cotransport to the changes in internal Na within its physiological range. At both levels of internal Na, outward Na cotransport was reduced in the majority but not in all of the patients with essential hypertension (p < 0.05 at 2.5 mmol; p < 0.001 at 6.5 mmol). The ratio of the changes in Na cotransport to those in internal Na was lower in the hypertensive patients than in the control subjects (17.2 \(\mu\)mol/liter red blood cells/hr/1 mmol in internal Na increase vs 42.2, p < 0.001). The Li-Na countertransport was increased in a few patients with essential hypertension, with no relationship to cotransport. We conclude that, in essential hypertension, the outward Na + K cotransport is impaired in fresh erythrocytes not treated with PCMBS (2,5 p-chloromercuribenzene sulfonate) or nystatin, even when internal Na is around its physiological range. (Hypertension 6: 826-831, 1984)

KEY WORDS • erythrocytes • cation fluxes • essential hypertension

ABNORMALITIES in the Na + K cotransport and Na-Na countertransport of red blood cells (RBCs) have been identified in human essential hypertension (EH). The maximal rate of Li-Na countertransport has been found to be increased, although the values have varied widely and have overlapped in normotensive and hypertensive subjects. Conflicting results have also been reported in furosemide-sensitive Na + K cotransport (COT). Garay et al., using PCMBS-treated, Na-loaded cells, found a COT value lower than normal in EH. Subsequently, they showed that COT was reduced in the RBCs, with an intracellular Na concentration (RBC Na) of 20 to 30 mM/liter RBCs; they attributed this defect to the reduced affinity of Na for the internal site of the cotransport system. Recently, Garay et al. demonstrated an increased K\(^+\) (internal Na concentration that ensures a half-maximal stimulation) with a low maximal rate of cotransport in the majority of their EH patients. In contrast, Adragna et al. found an elevated cotransport in PCMBS-treated RBCs, with a mean RBC Na content of 50 mM/liter RBCs.

Furosemide-sensitive Na and K fluxes have been previously measured in RBCs loaded with PCMBS or nystatin to contain 20 to 50 mM/liter of Na. Working at such levels of RBC Na, the authors studied the cotransport system at its maximal rate. However, Garay et al. found that this system was maximally sensitive to the changes of RBC Na around its physiological range. This may imply that COT participates in the regulation of RBC Na, because of its ability to produce outward Na fluxes against an electrochemical gradient. Although RBC Na in fresh cells has been suggested to be high in some EH patients, it is generally accepted that RBC Na as an average is within the normal range. On the other hand, the hypothesis has been developed that EH may somehow be related to a generalized defect in transmembrane Na transport. Blaustein has pointed out that an abnormality in the Na pump, which increased the intracellular Na by only 5%, may induce a significantly higher smooth muscle tension in the resistance vessels through a hampered Ca\(^++\) extrusion by the Na\(^+\) - Ca\(^++\) exchange system. Thus, it is reasonable that, if a defect in RBC COT rather than in the Na pump is involved in EH, it should be studied with a view of detecting a subtle impairment in its activity. Information is not available in EH patients on the function of the Na + K cotransport in fresh RBCs with the RBC Na around its narrow physiological range.

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For these reasons, we studied furosemide-sensitive Na efflux and its response to small changes in RBC Na within the physiological range. We developed a simple method to measure the outward furosemide-sensitive Na flux in fresh RBCs, in which preincubation in a Li-Na medium with or without ouabain caused the RBC Na to reach two different levels, both closely around the physiological range. We also simultaneously measured the Li-Na countertransport in the same RBCs.

**Materials and Methods**

**Patients**

We studied 37 subjects: a control group (Group 1) of 18 normotensive subjects who had a mean blood pressure (MAP) of 99 ± 2 mm Hg, ± SEM and a study group (Group 2) of 19 hypertensive inpatients with a MAP of 129 ± 2 mm Hg. Group 1 had 11 men and seven women aged 37 ± 2 years (range 18-53 years) who weighed 68 ± 1 kg (range, 61-77 kg) and who had no personal or family history of high blood pressure (BP). Medical examination and laboratory tests excluded any disease known to be associated with ion transport abnormalities. Group 2 had 14 men and five women aged 37 ± 2 years (range 18-62 years) who weighed 69 ± 2 kg (range 59-80 kg). None of the Group 2 patients had a diastolic BP lower than 95 mm Hg. The diagnosis of essential hypertension (EH) was primary, and laboratory tests to rule out any form of secondary hypertension. All EH patients had a family history of EH. None was under drug treatment at the time of the study.

**Loading of Red Blood Cells with Sodium and Lithium**

After the subjects had fasted overnight, blood was drawn into heparin tubes and immediately processed. Plasma and buffy coat were removed by centrifugation for 20 minutes at 4000 g. Aliquots were taken for mean cell volume (MCV) determination.

The RBCs were washed once at 3°C with LiCl 110, NaCl 40, glucose 10, TRIS-HCl 10 mM, with the pH adjusted to 7.40 ± 0.02 at 3°C and the osmolality to 290 ± 3 mOsm/kg H2O (Solution A). Washed RBCs were divided into two portions: the first was incubated at 30% hematocrit (Htc) at 37°C for 5 hours in Solution A, pH 7.40, at 37°C, which contained 22NaCl (10 μCi/ml RBC). The second portion was incubated in the same solution but with the addition of ouabain 0.1 mM. By means of this technique, we obtained two pools of RBCs. The first pool (incubated without ouabain) had a cellular Na of about 2.5 mmol/liter RBC (low RBC Na); the second pool (incubated with ouabain) had an internal Na of about 6.5 mmol/liter RBCs (high RBC Na). The intracellular Li concentration (RBC Li) was the same between the two pools of RBCs (about 9 mmol/liter RBC). The K concentration was slightly lower in cells with high Na (see results).

After incubation, the RBCs were washed five times at 3°C with MgCl2 75, sucrose 85, glucose 10, ouabain 0.01, TRIS-HCl 10 mM, pH 7.40 at 3°C, 290 mOsm/kg H2O (Solution B). After washing and resuspending at about 50% Htc, an aliquot of each RBC pool was taken for MCV determination. Another aliquot was packed by centrifugation at 7000 g for 20 minutes, 3°C, with a Fisher microcentrifuge (Gianni, Milan, Italy) by using 1 ml tubes. Preliminary experiments with 22Na albumin had shown that contamination by the extracellular fluid entrapped among packed RBCs was less than 1.2% of total volume. This small contamination was neglected, and the Htc of these maximally packed RBCs was considered equal to 100%. The 50 μl aliquots of packed RBCs were hemolyzed with deionized water; the hemolysates were allowed to stand at room temperature with frequent mixing for 2 hours, then centrifuged at 3000 g for 20 minutes. In the resulting supernatants, we measured the RBC Na, Li, and K concentrations and RBC radioactivity; in so doing, we measured the radioactivity both per volume of RBCs and per μCi of RBC Na.

**Lithium and Sodium Efflux**

Aliquots of both pools of RBCs (high and low RBC Na) were divided into three portions. The first portion was resuspended at 37°C in a water bath in Solution B, pH 7.40, at 37°C. The second portion was resuspended in NaCl 120, MgCl2 15, sucrose 17, glucose 10, ouabain 0.1, TRIS HCl 10 mM, pH 7.40 at 37°C, 290 mOsm/kg H2O (Solution C). The third portion of RBCs was resuspended in Solution B with furosemide 1 mM (Solution D). The Htc was approximately 10%.

At 0 time and after 60 minutes, samples of the three suspensions were taken and centrifuged at 4000 g at 3°C for 20 minutes. The supernatants were separated for 22Na and Li measurements. An aliquot of each whole suspension was also taken, hemolyzed, and diluted for the radioactivity count. The exact fractional Htc (FHtc) of each efflux suspension was calculated as the ratio between the radioactivity counts of equal amounts of hemolyzed, whole, efflux suspensions and of packed RBCs. Preliminary experiments had shown that the Htc calculated in this way was identical to that derived from the ratio between the hemoglobin content (optical density of hemolyzate at 415 nm) of the cell suspension and that of packed RBCs. Spontaneous hemolysis of efflux suspension was detected by reading the optical density of supernatants; it was less than 0.1% and neglected in calculations.

**Sodium and Lithium Efflux Calculations**

Preliminary studies had shown that under our experimental conditions the efflux of Na from RBCs was linear up to 90 minutes. This is in agreement with the previous observations of Garay et al. Therefore, unidirectional Na efflux was calculated as follows: Na efflux Omol/liter RBC x hr = 10°1-H+ x RC/ml supern. 60 min - RC/ml supern. 0 time/FHtc/(RC/xmol of RBC Na), where FHtc was the fractional Htc of the whole efflux suspension and RC the radioactivity counts. The Li efflux was calculated as the difference between the Li concentration at 60 minutes and 0
time referred to the FHtc, as previously described. The Li-Na countertransport (Li-Na T) was calculated as the difference between the Li efflux in Solution C and that in Solution B. Na cotransport (Na COT) was estimated by calculating the ouabain-resistant, furosemide-inhibited Na efflux into Na-free medium (the difference between unidirectional Na efflux into Solution B and that into Solution D). The difference in Na COT between the values obtained with high RBC Na and those with low RBC Na was calculated for each subject.

Analysis Techniques

Concentrations of Li, Na, and K in the hemolysates and Li in the supernatants were measured by atomic absorption spectrophotometry (Model 300, Perkin-Elmer Corporation, Norwalk, Connecticut). Standard solutions were used for the hemolysates, which contained comparable amounts of Li, Na, and K. For supernatants, standard solutions were prepared by dissolving LiCl in Solution B or C. The MCV was measured with a Coulter Counter. The MCV of the washed RBCs was measured in duplicate in 10 control subjects and eight EH patients in order to calculate the coefficient of variation (CV%) by the following formula:

$$CV\% = \frac{\sqrt{\sum (x_i - \bar{x})^2}}{2n} \times 100$$

where \(x_i\) and \(\bar{x}\) are the greater and the smaller of the duplicate values and \(n\) the number of considered subjects; CV% for MCV was 1.11%. Radioactivity was detected in 1 ml samples of supernatants of hemolyzed whole efflux suspensions or hemolyzed and diluted packed RBCs with the use of a Packard gamma counter. The LiCl, NaCl, MgCl₂ sucrose, TRIS, and HCl were from Carlo Erba, Milan, Italy; ouabain was from Merck Sharp & Dohme. Furosemide was a gift from Hoechst Italia. The NaCl was purchased from Amersham, England. Results are expressed as mean ± standard error of the mean (SEM) and compared by Student t test.

Table 1 shows the mean cell volume of the RBCs before and after 5 hours of incubation in Solution A with or without ouabain and the resulting concentrations of Li, Na, and K in the RBCs. No significant changes in MCV or in the total cation content were induced by such a procedure. This may be of importance since it has been shown that cell swelling may reduce Na⁺ + K⁺ cotransport. The RBC Li reached a mean value of over 9 mmol/liter RBCs, which is generally considered to be an amount able to saturate the internal sites of Li Na T.

Table 1 also shows that the two different incubation media affected the RBC Na as follows: 1) the mean values of RBC Na were different by at least 2.5 mmol/liter RBCs between low and high Na; 2) the changes between low and high RBC Na were similar in the control subjects and EH patients; 3) both the mean value (± SEM) and the range of variation of RBC Na in low as well as in high RBC Na were very close in controls and EHP.

Thus, it is conceivable under such experimental conditions that measuring furosemide-sensitive Na efflux gives reliable information on the activity of the Na + K cotransport in the physiological range of RBC Na. It is worth noting that the incubation of fresh RBCs in a medium containing 110 mM Li, 40 mM Na medium (Solution A) induced a net efflux of Na, with a final RBC Na of 4 to 6 mmol/liter RBCs below an initial value of 7.7 ± 0.3 mmol/liter RBCs. This net flux of Na seemed to be partly, but not completely, abolished by ouabain. We did not perform a specific study on the cation fluxes during RBC incubation with this type of external medium. However, the net Na efflux against its electrochemical gradient may take place through the Na-Li countertransport and ouabain sensitive Na pump.

Table 2 summarizes the values of Na cotransport in the controls and EH subjects at the two levels of RBC Na. It is shown that Na COT in EH patients is lower than in control subjects at both low and high RBC Na and that the ratio between the difference in Na COT to the corresponding difference in RBC Na between low

Table 1. Mean Cell Volume (MCV), Sodium, Potassium, and Lithium Concentrations and MCV in Red Blood Cells (RBCs) after 5 hours of Incubation in Li-Na Medium with or without Ouabain (0.1 mM), and the Difference Between High and Low RBC Na (A RBC Na)

<table>
<thead>
<tr>
<th></th>
<th>RBC MCV (M³)</th>
<th>RBC Na (mmol/liter RBCs)</th>
<th>ARBC Na (mmol/liter RBCs)</th>
<th>RBC K (mmol/liter RBCs)</th>
<th>RBC Li (mmol/liter RBCs)</th>
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<tbody>
<tr>
<td>Controls (n=18)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Initial</td>
<td>90.4 ± 0.3</td>
<td>2.70 ± 0.17</td>
<td>4.20 ± 0.31</td>
<td>104.3 ± 0.6</td>
<td>9.79 ± 0.37</td>
</tr>
<tr>
<td>Ouabain — (Low</td>
<td>89.9 ± 0.6</td>
<td>(1.60 - 4.38)</td>
<td>(2.51 - 6.57)</td>
<td>100.8 ± 0.7</td>
<td>9.15 ± 0.24</td>
</tr>
<tr>
<td>RBC Na)</td>
<td>90.9 ± 0.4</td>
<td>7.02 ± 0.31 (4.32 - 9.45)</td>
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<tr>
<td>Ouabain + (High</td>
<td>91.3 ± 0.7</td>
<td>2.31 ± 0.16 (1.03 - 4.62)</td>
<td>4.01 ± 0.26</td>
<td>103.6 ± 0.7</td>
<td>9.77 ± 0.48</td>
</tr>
<tr>
<td>RBC Na)</td>
<td>91.2 ± 0.6</td>
<td>6.29 ± 0.33 (4.20 - 9.45)</td>
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<tr>
<td>EH patients (n= 19)</td>
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<tr>
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TABLE 2. Sodium Contransport (Na COT; /nmol/lRBC • hr) in Control and Hypertensive Subjects at Two Distinct Levels of RBC Na Around Its Physiological Range

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Low RBC Na</th>
<th>High RBC Na</th>
<th>A COT/A RBC Na</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n=18)</td>
<td>91±9</td>
<td>253±18</td>
<td>42.2±4.6</td>
</tr>
<tr>
<td>p</td>
<td>0.05</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>EH patients (n=19)</td>
<td>61±10</td>
<td>136±15</td>
<td>17.2±2.3</td>
</tr>
</tbody>
</table>

The changes in RBC Na cotransport (A COT, /nmol/lRBC • hr) between low and high RBC Na were also calculated and divided for the correspondent variation of cell Na (A COT/A RBC Na).

and high RBC Na was significantly lower in EH patients. Figure 1 represents (as a straight line) the relationship between changes in Na COT (ANa COT) and in RBC Na (ARBC Na) in control and hypertensive subjects in the physiological range of RBC Na. Statistical comparison showed that the two lines not only were significantly parallel (slopes, 0.025 in hypertensive patients and 0.032 in control subjects, p > 0.05), but also that they were significantly different (not confounded); the line in EH patients was considerably below that of the control subjects (p < 0.001).

Table 3 depicts the results of the Li-Na T measurement. Li-Na T in EH patients was significantly higher in controls; no significant differences were detected in Groups 1 and 2 between the values at low RBC Na and high RBC Na.

Figure 2 shows the distribution and the lower and upper limits of COT and Li-Na T in the hypertensive and in 90% of the normotensive subjects. For COT, the limits (160 and 370 /nmol/liter RBCs • hr) were calculated in the 18 control subjects, while for Li-Na T (100-400 /umol/liter RBCs • hr) they were calculated by combining the present values with those of 46 other previously studied normotensive controls (26 men, 18 women, aged 38 ± 2 years, body weight, 72 ± 2 kg).

Discussion

The main object of this study was to measure the ouabain-resistant, furosemide-sensitive outward Na cotransport in RBCs at an internal Na concentration close to its physiological range. Our findings demonstrate that the furosemide-sensitive Na efflux was reduced in EH patients when the internal Na was between 2.5 and 6.5 mmol/liter RBC. Comparison of the cotransport between EH and control subjects has been previously made only in PCBMS or nystatin-treated RBCs. The present data clearly show that reduced cotransport in EH patients is also detectable in RBCs not treated with SH-reagents or ionophores.

The incubation of PCMBS-treated cells with different media allows the RBCs to reach widely different levels of RBC Na, ranging from 0 to 80 mmol/RBCs. In this way, the activation curve of the cotransport by the internal Na has been studied carefully in both normotensive and EH subjects.

However, at only two levels of RBC Na in each subject, which was all that was possible to obtain in cells not treated with PCBMS or nystatin, it might be difficult to reliably evaluate the sensitivity of cotransport to the changes in RBC Na by representing cotransport as a simple function of RBC Na. For this purpose,
we compared for each subject the difference in furosemide-sensitive Na efflux (ACOT) with the corresponding difference in internal Na between high and low RBC Na (ARBC Na). The ratio between the two was actually lower in EH patients than in the control subjects (Table 2).

A more impressive representation of these findings is obtained by plotting ACOT against ARBC Na in controls and hypertensives (Figure 1); this figure shows that any given, small elevation in RBC Na in its physiological range results in an increase in outward NaCOT significantly lower in EH patients than in control subjects. In other words, an increase in RBC Na greater in EH patients than in controls is required to obtain any given change in furosemide-sensitive Na efflux. A reduced sensitivity of the cotransport system in the low range of concentration of its substrate would depend mainly on the affinity of the system for this substrate. Therefore, our data are in agreement with those of Garay et al., 3, 8 of a low apparent affinity of cotransport for internal Na and of a high K for its activation by the internal Na. The experimental evidence that cotransport is able to produce net fluxes of Na against an electrochemical gradient,8, 15 and that its K is close to the physiological range of RBC Na,8 has led to the hypothesis that this system plays a physiological role in the regulation of RBC Na.

According to this view, our findings might suggest that the regulation of RBC Na is impaired at least in the RBCs of apart of hypertensives. As recently reviewed by Garay et al., 3, 8 the frequency of low cotransport or high countertransport in hypertensive patients is enormously variable among case-control studies from different laboratories.

Several variables other than BP have been found to be related to ouabain-resistant cation fluxes; they include overweight,23, 24 sex,2, 8, 14 race,1, 2, 28 age,24, 31 and plasma renin activity.25 Furthermore, both cotransport and countertransport have shown familial aggregation,1, 2, 3, 29 and their values in normotensive and hypertensive subjects seem to be related to the family incidence of hypertension.2, 14, 27 Regarding this point, recent data of Cooper et al. in a population of high school students have shown an increased maximal rate of net Na efflux by the Na/K pump plus Na-K cotransport in subjects with a family history of hypertension. Population-based studies24, 31 have also established that the relationship between hypertension and high countertransport is significant even when most possible confounding variables are taken into account; nevertheless, it is conceivable that the different incidence of these comitant factors may contribute to the wide variability in countertransport and cotransport among the case-control studies. However, as the main explanation for this variability, Canessa et al.13, 27 have advanced the hypothesis that hypertensive patients are heterogeneous with regard to erythrocyte Na transport.

Studies with simultaneous measurements of cotransport and countertransport have supported this view. Cusi et al., 3 Adragna et al., 3 and Garay et al. 8 have demonstrated that at least two independent subgroups, one with low cotransport (and normal countertransport), another with high countertransport (and normal or even elevated maximal rate of cotransport), are distinguishable among hypertensive patients. The relative size of the two subgroups seems to be different according to both racial and geographical factors. Indeed, low cotransport appears to be very frequent in European whites,8, 31 while elevated cotransport and countertransport is prevalent in North American whites.2, 26, 28 On the other hand, both American and African blacks may have low cotransport and normal countertransport irrespective of blood pressure. 29, 29 In addition, blacks show reduced furosemide-sensitive fluxes of Na but not of K, which suggests that the apparent Na:1 K stoichiometry of these fluxes is lacking in blacks. Further experimental evidence about the heterogeneity of hypertensives has been given by Levy et al., 32 who have been able to divide hypertensives into two subgroups according to the temperature-dependence of cotransport.

It may be difficult to distinguish subgroups within a small number of patients such as that of the present study. However, in Figure 2 we show that 11 of 19 EH patients had low cotransport, while countertransport, although it was higher than normal as the mean value (Table 3), was elevated over 400 nmol/1 RBCs • hr only in four of 19 hypertensives. In addition, only one EHP showed simultaneously high LiNaT and low CO. Thus, these findings seem to indicate an essential lack of association between low COT and high LiNaT in EH patients.

Conclusions

From our data we conclude that the furosemide-sensitive Na efflux is reduced in the RBCs of hypertensive patients when internal Na is around its physiological values; small variations in RBC Na induce parallel changes in NaCOT, which are blunted in hypertensive patients. This effect is detectable in RBCs not treated with PCMBS or nystatin. The activity of the one-one cation countertransport is increased as the mean value in EH patients. However, both high countertransport and low cotransport are present in some, but not all, of the EH patients. Furthermore, the lack of association in the same subjects between low count transport and high countertransport gives further support to the view that hypertensive patients are heterogeneous in terms of abnormalities of the ouabain-resistant cation fluxes.

References

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