Ion Transport Systems in Erythrocytes From Paramyotonia Patients

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Abstract. To test the hypothesis of a general defect in the ion transport systems in the cell membranes of patients with *paramyotonia congenita*, we measured the ouabain-inhibitable and the furosemide-inhibitable K^+ influxes in erythrocytes from two patients. In the temperature range examined (15—40 °C), the ouabain-inhibitable flux did not differ significantly from control. Also the furosemide-inhibitable flux was well in the rather large control range. The dependence of these fluxes on the external K^+ concentration was normal. Energy depletion of the erythrocytes by preincubation with desoxy-glucose led to reduced K^+ influxes that were not significantly different in patients and controls. The results do not support the hypothesis of a general defect in the ion transport systems in paramyotonia; they do, however, not rule out the possibility that the ion transport systems in paramyotonic skeletal muscle are defect.

Key words: Erythrocytes — Na^+/K^+ Pump — Ouabain — Furosemide — Paramyotonia

Introduction

The dominantly inherited muscle disease *paramyotonia congenita* (Eulenburg) is characterized by a transient muscle stiffness which occurs whenever a patient uses his muscles in the cold, and by a severe weakness which develops in the musculature during the exposure to cold. The symptomatology shows a certain variability among afflicted families so that almost centainly the disease is genetically heterogeneous. As yet, it cannot be excluded that the basic defect is different in the different types of the disorder. The most likely mechanism for the muscle stiffness is that of a transient depolarization-induced contracture (Ricker et al. 1986). The state of muscle weakness usually exceeds the cooling period for many hours, disappearing very slowly after the normal muscle temperature has

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been restored. The immediate cause of the muscle weakness is a progressive depolarization of the muscle fiber membranes, so that at a muscle temperature of 27 °C the resting membrane potential is only about −40 mV. This depolarization makes the fibers inexcitable; it has been explained as being caused by an abnormal temperature dependence of the membrane sodium conductance (Lehmann-Horn et al. 1981). The reason for the persistance of muscle weakness is not known. A possible explanation for it is that sodium channels, once they have been opened in the cold, do not close again, even when brought back to physiological temperature; however, other reasons for the long-lasting depolarization have not been excluded.

For instance, when a normal mammalian muscle is kept at a low temperature, the fibers take up sodium from the extracellular space and depolarize (Akaike 1975). When the muscle temperature is set to normal again, the Na^+/K^+ pump extrudes the excessive intracellular sodium, re-establishing a high resting membrane potential. It is well known that this repolarization is inhibited in the presence of ouabain, a substance which blocks the Na^+/K^+ pump. Thus, a defect of this physiological mechanism could be responsible for the slow recuperation from cold-induced weakness in paramyotonia. The hypothesis concerning the presence of such a defect might be tested in studying the temperature dependence of the ion transport systems in muscle fibers from paramyotonia patients.

Since human muscle tissue is difficult to obtain, we decided to perform preliminary investigations of membrane transport systems in better accesssible cells, i.e. erythrocytes from paramyotonia patients. Also, this was based on a further hypothesis that the temperature-dependent membrane defect in paramyotonia congenita is generalized. (The reason why it is noticed in the patients' musculature only, could be that muscle is the only tissue likely to fall below the critical temperature of about 32 °C.) It fact, an earlier study (Szymanska et al. 1986) has shown that the lipid composition of erythrocytes from paramyotonia patients is abnormal as far as the saturated/unsaturated fatty acids ratio is concerned. This finding encouraged us to undertake the present investigation.

Materials and Methods

The study was carried out with erythrocytes of 2 unrelated patients and of a 28-year-old control male person who all gave informed consent. Patient A was a 30-year-old man with symptoms of both *paramyotonia congenita* and hyperkalemic periodic paralysis (Ricker et al. 1983). His muscle properties had been evaluated electrophysiologically (Lehmann-Horn et al. 1981). Patient B, a 43-year-old woman, has had no other symptoms than those of paramyotonia (Lehmann-Horn et al. 1984). Although the patients thus seemed to have two different types of *paramyotonia congenita*, the typical cold-induced muscle stiffness and weakness were very pronounced in both of them. Neither the patients nor their relatives suffered from hypertension. The control person was also normotensive

which is of importance since the condition of essential hypertension can be associated with abnormal Na^+/K^+ transport (Garay et al. 1983). The plasma K^+ concentration was in the upper third of the normal range for patient A, and was normal for patient B.

Blood samples were taken by venipuncture. They were treated strictly identically and in parallel throughout. The blood was immediately heparinized and transported to the laboratory at 4 °C. There, the samples were centrifuged, the plasma and the buffy coat were carefully removed, and the cells were washed 3 times in a K^+-free buffer (see below). Erythrocyte counts, hematocrit and hemoglobin concentrations were obtained using a Coulter Counter. For the determination of the Na^+ and K^+ contents, aliquots of erythrocytes were lysed and, after protein precipitation with 10 % tricholoroacetic acid, the hemolysates were analysed with an Eppendorf flame photometer. For the influx measurements a hematocrit of 2 % was prepared in a K^+-free buffer solution and the suspension was stored at 4 °C for later use. The solution was changed every 12 hours. Under these conditions, the erythrocyte volume increased by less than 1.5 % within 3 days.

The K^+ influx was measured according to the filtration method of Werkheiser and Baetley (1957), using ^{86}rubidium as a tracer. The cells were placed in the various test solutions given below, stored for 15 min at 37 °C and cooled again to 4 °C. Then, ice cold $^{86}Rb^+$ solution was added to the cell suspensions so that the final hematocrits were about 2 %. The suspensions were divided into aliquots of 200 μl. A "zero time" aliquot was obtained by immediate centrifugation of the cells through 75 μl silicone oil (Beckman microfuge, 12.000 × g, 1 min). Then, the influx was started in the remaining aliquots by placing them in a shaking waterbath, set to various temperatures (range 15—40 °C). The incubation period was limited to 30, 60, 120 or 240 min, then the influx was stopped by centrifugation of the aliquots.

Unidirectional K^+ influxes were calculated from the amount of $^{86}Rb^+$ taken up by the cells over a certain period of time, considering the specific activity of the $^{86}Rb^+/K^+$ solution according to the method of Sachs and Welt (1967). No correction was made for backflux since under the conditions chosen it is < 2 % of the ouabain-inhibitable K^+ influx (Sachs 1977). The ouabain-inhibitable K^+ influx was calculated as the difference of fluxes measured in the absence and presence of ouabain. The furosemide-inhibitable K^+ influx was calculated as the difference between influxes measured in media containing ouabain and in media containing both ouabain and furosemide. The K^+ influx in the presence of both ouabain and furosemide was taken as the K^+ leak flux.

Statistics: All influx measurements were carried out in duplicate and averaged. Errors are given as SEM. In the Eadie and in the Hill plots, the curves were fitted to the data points by a linear regression program. To assess the significance of differences between mean test and control values, Student's t-test was used. In the Arrhenius plot, straight lines were fitted to the data points by a linear regression program, but here, the errors of the slopes of the fitted lines (proportional to the activation energies) are given as confidence limits for $p < 0.05$.

Solutions: The standard solution had the following compositon (in mmol/l): NaCl 135, KCl 5, $MgCl_2$ 3, Hepes 10, glucose 10, (pH 7.2 at 37 °C). For the preparation of the cells a similar solution was used in which KCl was replaced by NaCl. For the study of influxes after the depletion of the cellular energy stores a solution was used in which glucose was replaced by desoxy-glucose. To study the dependence of the K^+ influx on the extracellular potassium concentration, we varied this parameter in the range 0.5—20 mmol/l, keeping the sum of NaCl and KCl constant at 140 mmol/l. The associated variation of the extracellular Na^+ concentration is known to have no effect on the K^+ influx (Dunham et al. 1980). The final concentrations of ouabain and furosemide, when added, were 0.5 and 1.0 mmol/l, respectively. The specific activity of $^{86}Rb^+$ was 0.5—3.8 mCi/mg (18.5—140 MBq/mg), the activity in the final solutions was 0.3 μCi/ml (11.1 kBq/ml).

All reagents were of analytical grade. Ouabain and furosemide were from Sigma, München, and $^{86}Rb^+$ from NEN, Dreieich, West Germany both.

Results

Temperature dependence of K^+ influx

The experiments were carried out on the same day the blood samples were drawn. For both patients and the control person, and for every temperature in the range of 15—40 °C, the ^{86}Rb count per minute was a linear function of the incubation time, i.e. the K^+ influx was constant. The fluxes were not different for the two patients, but were lower than for the control person. This is illustrated in Figure 1, where the fluxes were plotted as a function of the temperature.

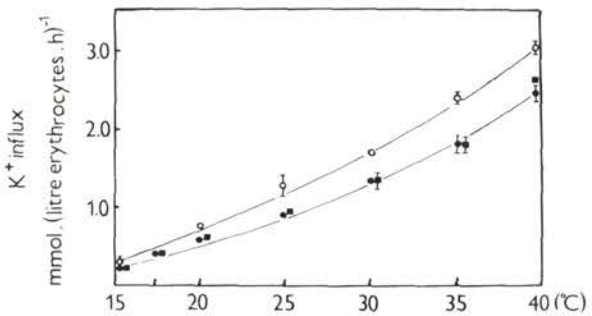

Fig. 1. Temperature dependence of the K^+ influx in erythrocytes freshly drawn from 2 paramyotonia patients (patient A: solid circles, patient B: solid squares) and a simultaneously investigated healthy control (open circles). Measurements in 5 mmol/l K^+ and 135 mmol/l Na^+.

To investigate the components of the transport system, we measured the fluxes in the presence of ouabain and in the presence of both ouabain and furosemide. For both patients, the ouabain-inhibitable K^+ influx had about the same value (1.2 mmol/l erythrocytes × hour at 37 °C) and was not different from control. The Na^+ content of the erythrocytes was 5.9 mmol/l erythrocytes (SEM 0.8, $n = 4$) and 6.7 mmol/l erythrocytes (SEM 0.5, $n = 4$) for patients A and B, respectively, and 6.3 mmol/l erythrocytes (SEM 0.4, $n = 4$) for the control person. These results do not suggest an alteration of the Na^+/K^+ pump in paramyotonia erythrocytes.

In contrast, the K^+ influx inhibited by the addition of furosemide was lower for both patients than for the control person, and this accounted entirely for the lower total values of the K^+ influx in the patients. The significance of this result was checked by additional measurements on erythrocytes of 6 other normotensive control persons (see below). The residual K^+ influxes were not significantly different from control for either patient (about 0.2 mmol/l erythrocytes/hour at 37 °C).

The ouabain- and the furosemide-inhibitable fluxes are plotted as a function of the inverse temperature in Figure 2. From the slopes in the Arrhenius plots, the activation energies for the K^+ influx can be calculated. This has been done separately for temperature ranges of 15—20 and 20—40 °C; the results are presented in Table 1. The activation energies were significantly different below and above 20 °C for both the ouabain-inhibitable and the furosemide-inhibitable influx, but did not reveal any abnormalities in the temperature of range 25—37 °C which is critical for the symptoms of paramyotonia. In no case was there a significant difference between the activation energies obtained for the patients and the control.

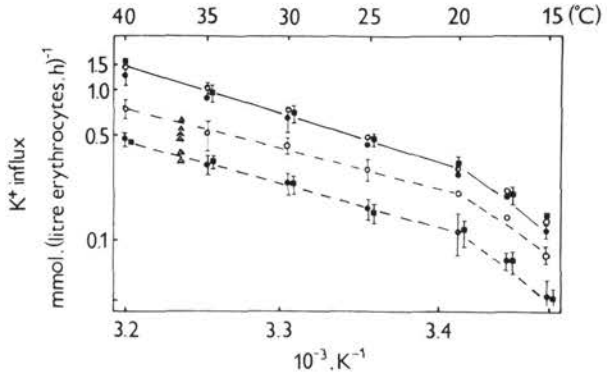

Fig. 2. Arrhenius plot of the ouabain-inhibitable (solid line) and the furosemide-inhibitable (dashed lines) K^+ influxes for the 3 individuals investigated (symbols as in Fig. 1). Open triangles represent the furosemide-inhibitable K^+ influxes determined for 6 further normotensive controls at 37 °C only.

Table 1. Apparent energies of activation (E_a) of the ouabain- and furosemide-inhibitable K^+ influxes in fresh erythrocytes. Figures give E_a in kJ/mol ± the confidence limits for $p < 0.05$. ($n = 6$ and $n = 10$ for the low and high temperature ranges, respectively)

°C	Ouabain-inhibitable K^+ influx		Furosemide-inhibitable K^+ influx	
	15—20	20—40	15—20	20—40
Patient A	134 ± 29	67 ± 17	122 ± 39	50 ± 9
Patient B	124 ± 17	60 ± 8	124 ± 30	42 ± 8
Control	119 ± 19	69 ± 12	125 ± 15	43 ± 9

Energy dependence of the K^+ influx

The experiments were carried out on erythrocytes stored in the K^+-free buffer for 1 day at 4 °C. The cells were then ATP-depleted by incubation in the desoxyglucose buffer for 6 hours at 37 °C. Cells incubated under the same conditons

in the standard buffer served as undepleted controls. The energy depletion led to an abolition of the ouabain-inhibitable K^+ influx in both patient and control erythrocytes. The furosemide-inhibitable K^+ influx was reduced to the same level for the patient and control erythrocytes, i.e. the energy depletion abolished the difference in the furosemide-inhibitable K^+ inluxes of the erythrocytes of the patients and the control.

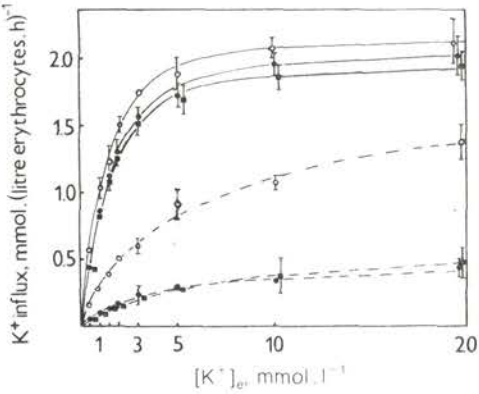

Fig. 3. Dependence of K^+ influxes in erythrocytes on the extracellular potassium concentration, $[K^+]_e$. The cells had been stored for 2 days in a K^+-free, 140 mmol/l Na^+-containing solution at 4 °C. Solid lines: ouabain-inhibitable fluxes, dashed lines: furosemide-inhibitable fluxes. For symbols see Fig. 1. The dotted column indicates the physiological range of control values as reported by Duhm and Göbel (1984).

Potassium dependence of the K^+ influx

The dependence of the K^+ influx in erythrocytes on the external K^+ concentration, $[K^+]_e$, was of particular interest, because *paramyotonia congenita* is sometimes associated with hyperkalemic periodic paralysis, a disease in which this dependence might be abnormal. Indeed, our patient A suffered from this combined condition. We tested this dependence in a $[K^+]_e$ range of 0.5—20 mmol/l. The experiments were carried out with erythrocytes stored for 2 days. The Na^+ concentration of the erythrocytes from patient A and the control person had by then increased to 8.9 (SEM 0.8, $n = 4$) and 10.6 (SEM 1.3, $n = 4$) mmol/l erythrocytes, respectively (for patient B not determined). The results are illustrated in Figure 3. The furosemide-inhibitable K^+ influx as a function of $[K^+]_e$ follows the Michaelis-Menten kinetics, so that the maximal K^+ influx, V_{max}, at a saturating $[K^+]_e$, and the apparent Michaelis-Menten constant, K_m, can be obtained from the intercept and the slope, respectively, of a plot of

$$\frac{V}{[K^+]_e} = \frac{V_{max}}{K_m} - \frac{1}{K_m} \cdot V,$$

where V is the K^+ influx at a given $[K^+]_e$. Such a plot is illustrated in Figure 4. Table 2 gives the V_{max} and K_m values for both patients and the control. For both patients, V_{max} was only about 30 % of control, while K_m was not significantly different from control.

The $[K^+]_e$ dependence of the ouabain-inhibitable K^+ influx in erythrocytes with a high internal K^+ concentration and at high external Na^+ concentrations does not follow the Michaelis-Menten kinetics (Sachs 1977; Garrahan and Glynn 1967). Rather, it is characterized by a slightly sigmoid course (which is not very obvious in the curves shown in Fig. 3). The best description of such a relationship is given by the transformed Hill equation (Sachs 1967)

$$\ln \frac{V}{V_{max} - V} = n \,.\, \ln [K^+]_e - \ln K_s$$

where n is the Hill coefficient and K_s is a constant describing the affinity of the Na^+/K^+ pump for external potassium. A plot of the data contained in Figure 3 according to this equation shows that the kinetic properties of the ouabain--inhibitable K^+ influxes for the patient and control erythrocytes were not significantly different (Figure 5).

Table 2. Ouabain-inhibitable and turosemide-inhibitable K^+ influxes (in mmol/l erythrocytes × × hour) in erythrocytes stored for 1 day in a K^+-free, 140 mmol/l Na^+-containing solution at 4 °C. Measurements at 37 °C in the presence of glucose and desoxy-glucose.

	Ouabain-inhibitable K^+ influx		Furosemide-inhibitable K^+ influx	
	glucose	desoxy-glucose	glucose	desoxy-glucose
Patient A	1.20	0.16	0.58	0.26
Patient B	1.25	0.00	0.66	0.29
Control	1.38	0.13	1.10	0.31

Table 3. Kinetic parameters of the ouabain- and furosemide-inhibitable K^+ influxes in erythrocytes stored for 2 days in a K^+-free, 140 mmol/l Na^+-containing solution at 4 °C. (V_{max} is the maximum transport number in mmol/l erythrocytes × hour, n is the Hill coefficient, K_s and K_m are the apparent binding constants in mmol/l)

	Ouabain-inhibitable K^+ influx			Furosemide-inhibitable K^+ influx	
	V_{max}	n	K_s	V_{max}	K_m
Patient A	2.08	1.36	1.42	0.52	4.1
Patient B	1.96	1.20	1.36	0.57	5.2
Control	2.10	1.40	1.22	1.60	4.2

Furosemide-inhibitable K^+ influx in normotensive controls

Since in this study the erythrocytes from the paramyotonia patients and the control person differed only in their V_{max} values of the potassium-dependent

furosemide-inhibitable K^+ influx, we studied this dependence further in erythrocytes from 6 normotensive individuals. V_{max} values were determined at 37 °C on the day of venipuncture and 2 days later. In freshly isolated cells, we found a rather low V_{max} of 0.8 mmol/l erythrocytes/hour (SEM 0.28, $n = 6$), and the inter-individual variation of V_{max} was smaller than reported (Duhm and Göbel 1984; Wolowyk and Slosberg 1982). The Na^+ content was normal (6.6 mmol/l erythrocytes, SEM 0.5, $n = 6$). In cells stored for 2 days in a K^+-free solution at 4 °C, V_{max} was increased by a factor of 1.7 and the Na^+ content was increased to 10.8 mmol/l erythrocytes (SEM 1.3, $n = 6$). The potassium dependence of the furosemide-inhibitable K^+ influx in erythrocytes from paramyotonia patients was studied after 2 days of storage. The V_{max} values obtained from these stored paramyotonia cells were 0.52 and 0.57 mmol/l erythrocytes/hour, i.e. lower than the mean control values, although the Na^+ content was normal (see above).

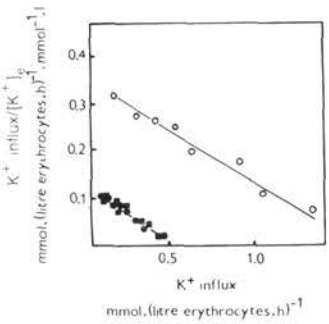

Fig. 4. Eadie plot of the dependence of the furosemide-inhibitable K^+ influxes on $[K^+]_e$ in erythrocytes stored for 2 days in a K^+-free, 140 mmol/l Na^+-containing solution at 4 °C. Symbols as in Fig. 1.

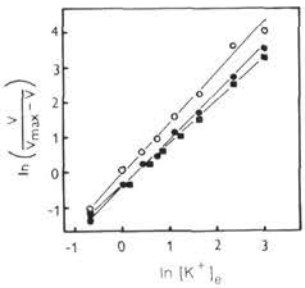

Fig. 5. Hill plot of the dependence of the ouabain-inhibitable K^+ influxes on $[K^+]_e$ in erythrocytes stored for 2 days in a K^+-free, 140 mmol/l Na^+-containing solution at 4 °C. Symbols as in Fig. 1.

There was, however, one control V_{max} value that was even lower than the paramyotonia values.

The K_m values for the furosemide-inhibitable K^+ influxes were about 5 mmol/l erythrocytes for both freshly isolated and stored cells and were not significantly different for patients and controls.

Discussion

The main result of this study is the finding that the potassium transport mechanisms in erythrocytes from paramyotonia patients were not abnormal. All the Na^+/K^+ pump properties investigated were similar as reported previously

(Sachs 1977; Wolowyk and Slosberg 1982; Brod et al. 1984). In particular, the apparent energies of activitation of the ouabain-inhibitable (Charnok et al. 1971) and the furosemide-inhibitable (Ellory et al. 1983; Stuart et al. 1980) K^+ influxes were normal. The apparent energy of activation of both the ouabain- and the furosemide-inhibitable K^+ influxes did not show any anomalous temperature dependence in a range of 25—40 °C, the range in which paramyotonic muscle cells have an abnormal temperature dependence of the Na^+ and Cl^- conductances. This result indicates that the defect in paramyotonia does not affect the transport mechanisms studied and/or is not expressed in the erythrocytes.

The transport activity of the furosemide-inhibitable K^+ influx was smaller in both patients than in the control person. However, it is well known that this parameter is subject to interindividual differences (Garay et al. 1983; Duhm and Göbel 1984; Wolowyk and Slosberg 1982). Both patients happened to have their values in the lower third of the large physiological range (Duhm and Göbel 1982 and our additional controls), while the control person happened to have it in the upper third. Until we have a chance to investigate more patients from other families — which is difficult, because the disease is so rare — we do not feel justified to attribute any significance to the low V_{max} values of the furosemide-inhibitable K^+ influx found in our paramyotonia patients.

The increase in the ouabain-inhibitable K^+ fluxes during the 2-day storage of the cells is readily explained by the increased intracellular Na^+ concentrations, but we have no explanation for the simultaneously occurring increase in V_{max} of the furosemide-inhibitable K^+ influxes.

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