Characterisation of the Potassium Influx in Rat Erythrocytes

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Abstract. In the rat erythrocyte membrane five different transport pathways for K^+ are present In addition to the well characterised K^+ transport via the Na^+ pump, the Na,K,Cl cotransport and the Ca^{2+}-activated K^+ channel, there are a K,Cl cotransport and a residual (leak) K^+ transport The K,Cl cotransport is already present under physiological conditions, and can be stimulated by N-ethylmaleimide treatment but not by a cell volume increase A low ionic strength stimulated increase of the residual K^+ influx can be demonstrated in rat erythrocytes after suppressing the K Cl cotransport pathway

Between 11 and 19 weeks of age, rats show significant differences in all transport pathways of the erythrocyte potassium influx Using influx data from individual rats a significant correlation between the total K^+ influx and the ouabain-sensitive K^+ influx has been found

Maintaining the rats on a diet poor in essential fatty acids leads to a significant change of the linoleic acid content of the erythrocyte membrane phospholipids However, no significant effect on the various K^+ transport pathways has been found An analysis of the fatty acid composition of the erythrocyte membrane phospholipids showed significant correlations between the content of oleic acid and arachidonic acid and the ouabain-sensitive K^+ influx (as well as the total K^+ influx)

Key words: Cation transport Erythrocyte (rat) Ionic strength — Membrane lipids

Introduction

For K^+ there are five known transport systems in erythrocytes of various species (i) active transport via the Na^+ pump (ouabain-sensitive) (ii) Na,K,Cl cotransport (bumetanide-sensitive), (iii) K Cl cotransport (specific for Cl^-[and Br^-] stimu-

lated by N-ethylmaleimide treatment, volume-dependent), (iv) Ca^{2+}-activated K^+ channel (Gardos channel, inhibited by chinin), and (v) residual (leak) K^+ transport (e.g. Bernhardt et al. 1988).

The active K^+ transport, the Na,K,Cl cotransport and the Ca^{2+}-induced K^+ transport via the Gardos channel have been described in detail for rat erythrocytes (Jenkins and Lev 1973; Duhm and Göbel 1984; Orlov et al. 1989). In addition, N-ethylmaleimide stimulation of K^+ influx has been reported for rat erythrocytes (Ellory et al. 1982). However, it cannot be concluded from these experiments whether the N-ethylmaleimide effect is based on K,Cl cotransport stimulation. Further information is needed to identify and characterise the K,Cl cotransport pathway (e.g. anion dependence, volume sensitivity) to decide whether the N-ethylmaleimide stimulation is due to a stimulation of the K,Cl cotransport (as in low K^+ type (LK) sheep erythrocytes (Lauf and Theg 1980; Dunham and Ellory 1981)) or to the activation of a latent K,Cl cotransport system under special experimental conditions (as in mature human erythrocytes (Dunham et al. 1980)).

Human and LK sheep erythrocytes show a significant increase of residual K^+ influx in low ionic strength media. This effect is absent in high K^+ (HK) type sheep and bovine erythrocytes (Bernhardt et al. 1991; Erdmann et al. 1991). The low ionic strength effect in rat erythrocytes has been an additional focus of the present experiments.

In different cells including red blood cells the various ion transport pathways are influenced by the lipid composition of the membrane (Gruber and Deuticke 1973; Solomonsen et al. 1976; Kirk 1977; Laird et al. 1986; Uratani et al. 1987; Erdmann et al. 1990). In this respect, the phospholipid head groups as well as the phospholipid fatty acids seem to be of importance (Marsh 1987). To investigate the influence of the lipid composition on erythrocyte membrane transport, cells from different species with different natural lipid compositions (Nelson 1967; Wessels and Veerkamp 1973) or cells of one species after artificial modifications may be used. Besides direct modification of the lipid composition by means of phospholipid exchange proteins (Kuypers et al. 1984), the lipid composition can be varied using special diets (Vajreswari et al. 1983; Iritani and Narita 1984; Croft et al. 1985). The present experiments were also designed to study K^+ transport pathways in rat erythrocytes and their dependence on the membrane phospholipid fatty acid composition. Cation fluxes in human and chicken erythrocytes have been shown to be dependent on the age of the individuals investigated (Shanbaky et al. 1987; Weder et al. 1987). Therefore, the age of the animals has been also considered.

Materials and Methods

Male 7-weeks-old rats of the Shoe:Wist (Ico) strain (VTP Schönwalde) were held in wire cages at 20 °C and 12 h light/dark cycle (conventional conditions). The animals received

standard pellet food "R-Rehbrücke" for 4 weeks. In the diet study, the rats of the control group were maintained on standard pellet food for another 5 or 8 weeks. The experimental (diet) group had the sunflower oil in the pellets completely replaced by pork fat. For age related experiments, rats were kept in five parallel groups (4 to 12 weeks) on standard food.

Blood was drawn by retro-orbital puncture under ether anaestesia. Heparin was used as anticoagulant. The blood was stored on ice until investigated (within 9 h of sampling). For fatty acid analysis, erythrocytes were washed three times in isotonic NaCl and hemolysed in distilled water. To prepare hemoglobin-free ghosts membranes were washed three times in distilled water. Lipids were extracted from the membranes using chloroform/methanol (2:1, v/v), then distilled water was added. The organic solvents were evaporated in a stream of N_2. Lipids were split using KOH/CH_3OH. CH_3OH/BF_3 was used for methylation. A GC 18.3 chromatograph with 30 m capillaries (XE-60) was used for chromatographic measurements.

For K^+ influx measurements, the erythrocytes were separated from plasma and buffy coat by three washing steps including centrifugation (2000 × *g*, 8 min), removal of the supernatant by aspiration, and resuspension of the cells in a solution containing (in mmol/l): NaCl 145; glucose 10; Na_2HPO_4/NaH_2PO_4 5.8 (pH 7.4) at room temperature (Cl^--containing physiological ionic strength solution). For experiments in Cl^--free media, the erythrocytes were washed seven times (2000 × *g*, 8 min) according to Dunham and Ellory (1981) in a solution containing (in mmol/l): $NaCH_3SO_4$ 165; glucose 10; Na_2HPO_4/NaH_2PO_4 5.8 (pH 7.4) ($CH_3SO_4^-$-containing physiological ionic strength solution, same tonicity as the Cl^--containing physiological ionic strength solution – 300 mOsm, measured with a vapor pressure osmometer). In some experiments the erythrocytes were treated with 2 mmol/l N-ethylmaleimide (15 min) in the Cl^--containing physiological ionic strength solution at 37 °C (pH 7.4). Subsequently, the cells were washed twice in the same solution without N-ethylmaleimide. The erythrocytes were washed once in a medium of the same composition as the flux medium (except for K(^{86}Rb)Cl or K(^{86}Rb)CH_3SO_4 and transport inhibitors). The cells were suspended at about 5% hematocrit in a total volume of 0.95 ml of flux solution. The Cl^--containing physiological ionic strength solution or a low ionic strength solution containing (in mmol/l): sucrose 250; glucose 10; Na_2HPO_4/NaH_2PO_4 5.8 (pH 7.4) (same tonicity as the physiological ionic strength solution, 300 mOsm) were used as flux solutions. Hypoosmotic solutions used in some experiments contained 115 mmol/l and 200 mmol/l NaCl and sucrose, respectively. In experiments with the major anion Cl^- replaced by $CH_3SO_4^-$ (see above), $CH_3SO_4^-$-containing physiological ionic strength solution or the low ionic strength solution were used in flux measurements. In each experiment 1 mmol/l EGTA was used to prevent K^+ flux via Ca^{2+}-induced K^+ channel. Total $^{86}Rb^+$ influx was measured in the absence of any other specific transport inhibitors. Ouabain (Serva), at a final concentration of 10 mmol/l was used to inhibit the Na^+ pump (Willis and Ellory 1983). The Na,K,Cl cotransport pathway was blocked with 0.1 mmol/l bumetanide (Lovens Kemise Fabr. Kobenhavn) (Palfrey and Greengard 1981).

The suspensions for flux measurements were pre-incubated for 5 min at 37 °C. Then, 0.05 ml of radioactive stock solution (^{86}RbCl + KCl or ^{86}RbCl + KCH_3SO_4) were added to each sample tube to give a final concentration of 7.5 mmol/l. The labelled suspensions (radioactivity about 50 kBq/ml) were incubated for another 30 min at 37 °C in a shaking water bath. The $^{86}Rb^+$ influx was stopped by quick centrifugation (15,000 × *g*, 10 s) and replacing the extracellular solution with 1 ml of icecold $MgCl_2$ solution composed of

(mmol/l): $MgCl_2$, 107; MOPS, 10 (pH 7.4). Additionally, the samples were washed four times with this $MgCl_2$ solution.

The cell pellet was lysed with 0.5 ml (v/v) Triton X-100 and the protein precipitated by adding 0.5 ml of 5% (w/v) trichloric acid. After 5 min centrifugation ($15,000 \times g$) the $^{86}Rb^+$ activity was determined in the supernatant by Cerenkov counting in a liquid scintillation counter. The specific activity of $^{86}Rb^+$ solutions was determined by counting a suitable sample of the radioactive stock solution. Hemoglobin content of the erythrocyte suspensions was determined as cyanhemoglobin content using Drabkins reagents (Sigma) (Drabkin and Austin 1935).

$^{86}Rb^+$ influxes were calculated assuming linear dependence of flux on time (Dunham and Ellory 1981) and expressed as mmol K^+ per l cells per h. Each triplicate experiment was carried out at least three times with blood from different animals. The results are given as mean ± S.D. The significance of differences was determined by Student's or Welch's test.

Results

Using ouabain and bumetanide to inhibit the K^+ influx via the Na^+ pump and the Na,K,Cl cotransport the partial fluxes were investigated in rat erythrocytes. The results are presented in Table 1. In addition, flux measurements in the absence or presence of specific inhibitors in low ionic strength solutions are included in Table 1. The total K^+ influx can be divided into an ouabain-sensitive part, a bumetanide-sensitive part, and an (ouabain and bumetanide)-insensitive part. In low ionic strength solution the total and the ouabain-sensitive K^+ influx are found

Table 1. K^+ influx of rat erythrocytes in solutions of different ionic strength in dependence on inhibitor sensitivity. K^+ influx was measured in the absence of any transport inhibitors (except 0.1 mmol/l Na_2-EGTA), in the presence of 10 mmol/l ouabain or 0.1 mmol/l bumetanide and in the presence of both ouabain and bumetanide in physiological and low ionic strength media. Results are mean ± S.D. from nine independent experiments. Values obtained in low ionic strength solution are significantly different from those measured in physiological ionic strength solution ($P < 5\%$).

	Influx [mmol K^+/(l cells per h)]	
	physiol. ionic strength	low ionic strength
Total	9.94 ± 1.39	5.50 ± 0.64
Ouabain-sensitive	6.01 ± 1.09	4.76 ± 0.56
Bumetanide-sensitive	2.57 ± 0.60	0.06 ± 0.33
(Ouabain and bumetanide)-insensitive	0.94 ± 0.60	1.00 ± 0.40

Table 2. (Ouabain and bumetanide)-insensitive K^+ influx of rat erythrocytes in solutions of different ionic strength under various conditions. K^+ influx was measured in the presence of 10 mmol/l ouabain and 0.1 mmol/l bumetanide in physiological and in low ionic strength media. Control values were obtained in Cl^--containing solutions (300 mOsm). Cl^--free physiological and low ionic strength media contained 165 mmol/l and 7.5 mmol/l $CH_3SO_4^-$, respectively. Treatment of the cells with 2 mmol/l N-ethylmaleimide was carried out for 15 min. Hypoosmotic physiological and low ionic strength media contained 115 mmol/l NaCl and 200 mmol/l sucrose, respectively. Results are mean ± S.D. from three independent experiments. Asteriscs indicate values significantly different from the control value ($P < 5\%$).

	Influx [mmol K^+/(l cells per h)]	
	physiol. ionic strength	low ionic strength
control	0.94 ± 0.60	1.00 ± 0.40
Cl^--free media	0.39 ± 0.01*	1.21 ± 0.11
after N-ethyl-maleimide pre-treatment	2.39 ± 0.42*	0.86 ± 0.06
hypoosmotic media	1.32 ± 0.36	0.89 ± 0.07

to be significantly decreased in comparison to the values obtained in physiological ionic strength solution. The bumetanide-sensitive K^+ influx disappears in low ionic strength medium. On the other hand, the (ouabain and bumetanide)-insensitive K^+ influx remains unaffected upon decreasing the external ionic strength.

To decide whether there is a participation of the K,Cl cotransport in the (ouabain and bumetanide)-insensitive K^+ influx, this partial flux was investigated in more detail. Table 2 shows the (ouabain and bumetanide)-insensitive K^+ influx under various experimental conditions. Replacement of the major anion Cl^- by $CH_3SO_4^-$ resulted in a decrease of the (ouabain and bumetanide)-insensitive influx in physiological ionic strength solution. In low ionic strength solution no significant differences between the (ouabain and bumetanide)-insensitive K^+ influxes in Cl^- and $CH_3SO_4^-$ media could be found. N-ethylmaleimide (2 mmol/l) treatment stimulated the (ouabain and bumetanide)-insensitive K^+ influx in physiological ionic strength solution. In low ionic strength solution no stimulating effect of N-ethylmaleimide treatment on this influx could be detected. The influx values obtained in hypotonic media of both ionic strengths did not significantly differ from the control values (isoosmotic Cl^--containing solution).

Figures 1 and 2 illustrate the dependence of K^+ influxes in erythrocytes on the age of rats maintained on standard food. All flux values obtained for rats aged 11 weeks significantly differed ($P < 1\%$) from those obtained for rats aged 19 weeks. In physiological as well as in low ionic strength solution the total and

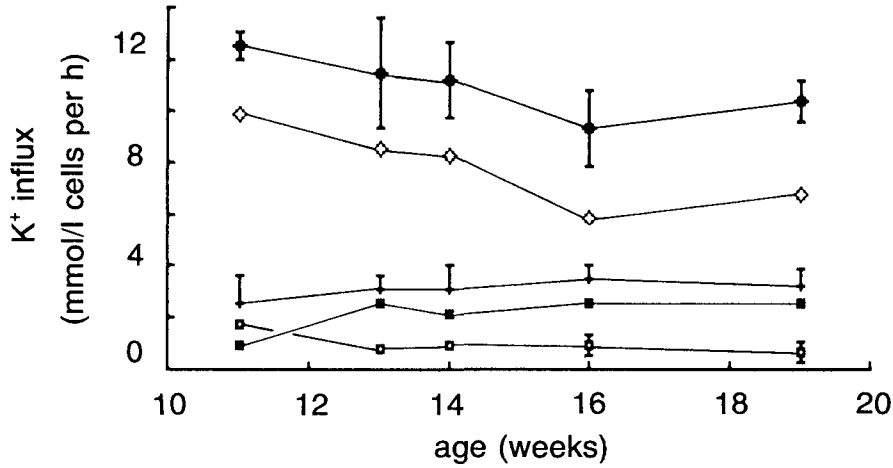

Figure 1. Age-dependence of K^+ influx of rat erythrocytes in Cl^-- containing physiological ionic strength solution (mean ± S.D.). ♦ – total K^+ influx, + – K^+ influx in the presence of 10 mmol/l ouabain, □ – K^+ influx in the presence of 10 mmol/l ouabain and 0.1 mmol/l bumetanide, ◊ – the ouabain-sensitive K^+ influx (difference between total K^+ influx and K^+ influx in the presence of ouabain), ■ – the bumetanide-sensitive K^+ influx (difference between the K^+ influx in the presence of ouabain and the K^+ influx in the presence of both ouabain and bumetanide).

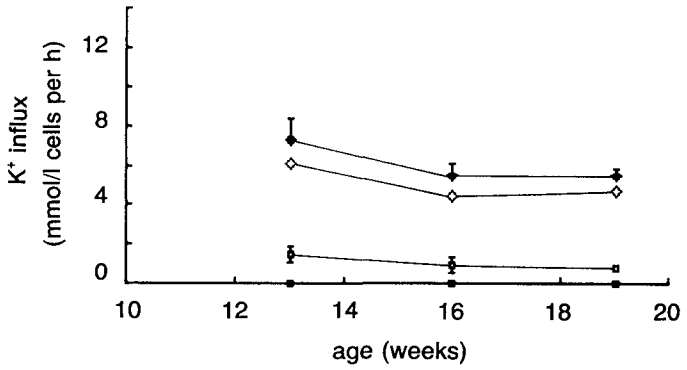

Figure 2. Age-dependence of K^+ influx of rat erythrocytes in low ionic strength solution (mean ± S.D.). ♦ – total K^+ influx, □ – K^+ influx in the presence of 10 mmol/l ouabain and 0.1 mmol/l bumetanide, ◊ the ouabain-sensitive K^+ influx (difference between total K^+ influx and K^+ influx in the presence of ouabain), ■ – the bumetanide-sensitive K^+ influx (difference between the K^+ influx in the presence of ouabain and the K^+ influx in the presence of both ouabain and bumetanide).

the ouabain-sensitive K^+ influx decreased with increasing age of the animals In solution of physiological ionic strength the bumetanide-sensitive and the (ouabain and bumetanide)-insensitive K^+ influx were significantly changed with the sum of these influxes remaining almost constant In low ionic strength solution no bumetanide-sensitive influx could be observed at any time In addition to the above age-related measurements, individual K^+ influx data were analysed for 35 animals For Cl^--containing physiological ionic strength solutions a correlation ($r = 0.75$, $P < 1\%$) was found between the total and the ouabain-sensitive K^+ influx

In another set of experiments, the fatty acid composition of the erythrocyte membrane was investigated cells from rats maintained on standard food and from animals maintained on a diet poor in essential fatty acids were used The contents of palmitic, oleic, linoleic, stearic and arachidonic acids in erythrocyte membrane phospholipids were determined After feeding periods of 5 and 8 weeks the content of linoleic acid (60% of control value ($P < 1\%$)) but not of any other fatty acids analysed was decreased The K^+ influxes of erythrocytes from rats maintained on essential fatty acids poor diet were the same as values obtained for erythrocytes from control animals (standard food, data not shown)

Analysing the individual data for 18 animals (control and special diet group) correlations were found between K^+ influx values and the fatty acid content of the membrane phospholipids (total influx to oleic acid $r = 0.63$, total influx to arachidonic acid $r = -0.69$ ouabain-sensitive influx to oleic acid $r = 0.56$, and ouabain-sensitive influx to arachidonic acid $r = -0.63$ ($P < 5\%$)) No correlations with the contents of the other fatty acids investigated could be observed Either no significant correlations were detected between bumetanide sensitive and/or the (ouabain and bumetanide) insensitive K^+ influxes and the fatty acid content of the membrane phospholipids

Discussion

Based on the results presented at least four components can be distinguished in the K^+ influx in rat erythrocytes in physiological solution, characterised by different sensitivities to effective transport inhibitors (Table 1) Another important K^+ transport pathway (Ca^{2+}-stimulated K^+ channel) was not investigated in this study, being eliminated by the presence of EGTA in all flux solutions With a share of about two thirds on total K^+ influx, the Na^+ pump mediated K^+ influx (ouabain-sensitive K^+ influx) is the most powerful inward translocation system for this cation in rat erythrocytes A decrease of K^+ influx via Na^+ pump observed in low ionic strength solution as compared with the value obtained in physiological ionic strength medium is an effect also reported for human erythrocytes (Bernhardt et al 1989) A more or less direct influence of ionic composition of the solution

on the Na^+ pump (possibly due to changing of the protein conformation) should be taken into account. Factors important in this regard could be the changed ionic strength of the solution, changed transmembrane potential and/or increased intracellular pH.

A bumetanide-sensitive component of the K^+ influx has been observed in rat erythrocytes; it can be interpreted as transport via a Na,K,Cl cotransport system (Palfrey and Greengard 1981). This influx disappears in low ionic strength solution. Two factors might be responsible: (i) zero external Na^+ concentration leads to the disappearance of the driving force (Scott 1987), and/or (ii) the extracellular Cl^- concentration (7.5 mmol/l) is too low with respect to the requirement of external Cl^- for the Na,K,Cl cotransport system (Chipperfield 1980). In addition, factors which could be of importance in influencing the Na^+ pump (see above) could also play a regulatory role in the Na,K,Cl cotransport.

In Cl^--containing physiological ionic strength solution the (ouabain and bumetanide)-insensitive K^+ influx represents about 10% of the total K^+ influx. This flux is not significantly changed upon reducing the ionic strength of the solution. The question arises whether there is a participation of the K,Cl cotransport in the (ouabain and bumetanide)-insensitive K^+ influx in rat erythrocytes. Although there is no specific inhibitor for this flux available, it can experimentally be separated due to its absolute requirement for chloride. The experiments in Cl^--free media (replacement by $CH_3SO_4^-$) demonstrated the presence of a Cl^--dependent component of the (ouabain and bumetanide)-insensitive K^+ influx in rat erythrocytes under physiological ionic strength conditions. On the other hand, in low ionic strength solution replacement of Cl^- by $CH_3SO_4^-$ was without any effect on the (ouabain and bumetanide)-insensitive K^+ influx. Furthermore, pre-treatment of the cells with 2 mmol/l N-ethylmaleimide resulted in a stimulation of the (ouabain and bumetanide)-insensitive K^+ influx in physiological but not in low ionic strength solution.

These findings (Table 2) can be interpreted in terms of the presence of a composed (ouabain and bumetanide)-insensitive K^+ influx: (i) a K,Cl cotransport (Cl^--dependent, stimulated by N-ethylmaleimide) and (ii) the residual K^+ influx (Cl^--independent and unaffected by N-ethylmaleimide). The K,Cl cotransport mediates about two thirds of the (ouabain and bumetanide)-insensitive K^+ influx in physiological ionic strength solution. In low ionic strength media, this K,Cl cotransport is absent since the extracellular Cl^- concentration is very low (Dunham and Ellory 1981). However, it cannot be completely ruled out that the increased intracellular pH of cells in low ionic strength media (Bernhardt et al. 1991) inhibits the K,Cl cotransport (Brugnara and Tosteson 1987). Taking into account the presence of the K,Cl cotransport in rat erythrocytes a threefold increase of the residual K^+ transport can be observed after reducing the ionic strength of the extracellular solution. Low ionic strength-induced stimulation of the residual K^+

influx has been described for human and LK sheep erythrocytes, whereas this effect was absent in bovine and HK sheep erythrocytes (Bernhardt et al. 1991, Erdmann et al. 1991).

Furthermore, the characteristics of the K,Cl cotransport in rat erythrocytes are different from those reported for erythrocytes of other mammalian species. The K,Cl cotransports in LK sheep erythrocytes, "young" human erythrocytes and erythrocytes of some other species, are Cl^--dependent and can be stimulated by cell swelling, N-ethylmaleimide treatment and high hydrostatic pressure (Lauf and Theg 1980; Dunham and Ellory 1981; Lauf 1985; Hall and Ellory 1986). In mature human erythrocytes the K,Cl cotransport is apparently latent under physiological conditions and can be activated by the above mentioned manoeuvres but not by a cell volume increase (Dunham et al. 1980; Hall and Ellory 1986; Ellory et al. 1987). The activated K,Cl cotransport in mature human erythrocytes, however, is Cl^--dependent and can be further stimulated by cell swelling (Lauf et al. 1984; Ellory et al. 1985; Brugnara and Tosteson 1987). A third possibility of the K,Cl cotransport was found in mammalian red blood cells: in rat erythrocytes, the K,Cl cotransport already present under physiological conditions is Cl^--dependent but does not show a significant change after cell volume increase.

It is interesting to note that, as compared to human erythrocyte also rat erythrocytes show a different volume dependence of another K^+ transport system. It was shown by Gurlo et al. (1991) that the K^+ influx via the Na,K,Cl cotransport after cell volume decrease is enhanced in rat erythrocytes, whereas there is no significant change in human erythrocytes.

A dependence of erythrocyte cation transport on the age of donor individuals has been reported for the Na,K,Cl cotransport system in human erythrocytes (Weder et al. 1987) and in chicken erythrocytes (Shanbaky et al. 1987). The "individual age of donors" is taken as a possible interim value reflecting unknown relevant physiological changes of the erythrocyte population during lifetime of the organism. Attention should be drawn to the fact that age-related changes for the bumetanide-sensitive K^+ influx (Na,K,Cl cotransport) and the (ouabain and bumetanide)-insensitive K^+ influx (sum of K,Cl cotransport and residual transport) in physiological ionic strength solution (Fig. 1) are inversely related resulting in a nearly unaffected ouabain-insensitive K^+ influx (sum of the three mentioned influxes). This could be a hint to a connection of Na,K,Cl cotransport, K,Cl cotransport and residual K^+ transport with respect to the physiological regulation of these monovalent cation transport pathways.

A homoeostase mechanism seems to exist in rat erythrocytes maintaining the fatty acid pattern relatively independent of fatty acid uptake (Gibson et al.1984). Changes in this pattern caused by a diet deficient in essential fatty acids were limited to the linoleic acid. However, a change in linoleic acid content of the erythrocyte membrane phospholipids did not lead to any changes in the K^+ influxes

via the four different pathways investigated Therefore, no significant importance of this fatty acid for the K^+ transport should be assumed On the other hand, using individual fatty acid and K^+ influx data, there were significant correlations between the Na^+ pump mediated K^+ inward transport and the contents of oleic and arachidonic acids in the membrane phospholipids ($r = 0\ 75$, $P < 1\%$) This is in accordance with the observation that Na^+,K^+-ATPase activity can be modulated by the fatty acid composition of the surrounding membrane lipids (Solomonsen et al 1976)

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