Lactoferrin Stimulates Erythrocyte Na^+/K^+-Adenosine Triphosphatase: Effect of Some Modulators of Membrane Phosphorylation

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We studied the effect of some modulators of signal transduction on the erythrocyte Na^+/K^+-ATPase. Go6976 and Go6983 (protein kinase C inhibitors) showed a stimulatory effect and calyculin A (protein phosphatase inhibitor) exerted an inhibitory effect on the Na pump activity. Some of the tested modulators of cell-signaling [protein phosphatase(s), phosphodiesterase, calmodulin and some protein kinases] interfered with the lactoferrin (Lf) stimulatory effect on the sodium pump. Lf itself was able to modulate the effect of some agents upon the pump activity. Moreover, an additive effect of stimulation was found when Lf and some agents were used simultaneously. The summarized results showed that: (i) Lf upregulates the Na^+/K^+-ATPase in erythrocytes and facilitates the K^+ influx into the erythrocytes; (ii) the effect of pump stimulation is mediated by phosphorylation processes. These results suggest a potential opportunity for using Lf alone or together with other agents as a stimulator of the erythrocyte Na^+/K^+-ATPase.

Key words: Lactoferrin, Erythrocytes, Na^+/K^+-ATPase

Introduction

Na^+/K^+-ATPase is a transmembrane enzyme, simultaneously involved in cell signal transduction in response to the extracellular stimuli while maintaining a normal ion gradient. The enzyme possesses a catalytic (α) and regulatory (β) subunit and an additional γ subunit, which modulates the pump affinity for monovalent cations (Hoffman *et al.*, 2002).

In the cells, having mitochondria and a nucleus, the cell signals from the Na pump lead to the stimulation of free oxygen radicals (FORs) generation in mitochondria and to gene expression control in the nucleus. Cell signals mediated by Na^+/K^+-ATPase probably provide particularly the erythrocyte's functions or, otherwise, might be common for other cell types in general (Minetti and Low, 1997). We suggested that erythrocyte Na^+/K^+-ATPase might be a signal transductor and also an object of cell regulation via endogenous cardiac glycosides, hormones and growth factors, which also use various cell signaling pathways (Therien and Blostein, 2000).

Binding of various ligands to their receptors on the erythrocyte cell membrane leads to conformational changes (Zhou and Low, 2001) and induction of cell signals that change the erythrocyte functional activity (Feschenko and Sweadner, 1994). Erythrocyte membrane contains various signal molecules, that may participate in the signal pathways, controlling the Na^+/K^+-ATPase: G-proteins (Escribá *et al.*, 2003), PKA (Montoliu *et al.*, 2004), serine/threonine protein phosphatases (Bize *et al.*, 1998), tyrosine protein phosphatases (Zipser and Kosower, 1996), casein kinase 2 (CK2) (Wei and Tao, 1993) and PKC (Escribá *et al.*, 2003). A signalosome CO39 has been isolated and purified from erythrocytes in which protein kinase D (PKD) and CK2 were found by immune precipitation (Uhie *et al.*, 2003).

Lactoferrin (Lf), a multifunctional cytokine, combines the properties of an ion transport regulator (Sun *et al.*, 1991) with antioxidative activity (Cohen *et al.*, 1992). Our previous studies showed that Lf stimulates glycolysis and the antioxidative activity of the erythrocyte (Maneva *et al.*, 2003).

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Having in mind the proved connection between glycolysis and the Na pump in the erythrocyte (Fossel and Solomon, 1978), here we aimed to check whether Lf, as a stimulator of glycolysis, exerts control on the Na^+/K^+-ATPase, and if so, whether or not its effect interferes with those of the erythrocyte membrane proteins, acting as modulators of the Na pump activity. To address these questions we determined: 1) the effect of Lf on the erythrocyte Na^+/K^+-ATPase; 2) the possible relation between the effects of Lf and other signal transduction modulators using PKC inhibitors (Go6976 and Go6983), caffeine as a phosphodiesterase inhibitor, serine/threonine protein phosphatase inhibitors (okadaic acid and calyculin A), a calmodulin inhibitor (W-7) and a CK2 activator (spermine); 3) that the maintaining of a high K^+ level inside the cells is one of the basic life phenomena (Bogner *et al.*, 1998). Red blood cell Na^+/K^+-ATPase plays a central role in the regulation of intra- and extracellular cation homeostasis. Positive correlation between the Na^+/K^+-ATPase activity and the intracellular K^+ has been reported (Ling, 1998). Decreased Na^+/K^+-ATPase activity in erythrocytes from patients with diabetes was also found (Okegbile *et al.*, 1997). We were interested to find out whether Lf, as a possible stimulator of the Na^+/K^+-ATPase activity, would lead to an increase of the K^+ content in patients with insulin-independent diabetes.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. The below mentioned concentrations were chosen according the producer's prescription and data existing in the literature about their optimal effect: Go6976 (50 nM) and Go6983 (50 nM) – inhibitors of protein serine kinase PKC. Go6976 is an inhibitor of the classic isoforms of PKC and PKD, while Go6983 inhibits only the classic isoforms; both inhibitors were used simultaneously to exclude the effect of PKD (Gschawendi *et al.*, 1996). Caffeine (20 mM) was used as a phosphodiesterase inhibitor, okadaic acid (20 nM) and calyculin A (50 nM) as inhibitors of serine/threonine protein phosphatases, *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7) (30 μM) as a calmodulin antagonist, and spermine (50 nM) as an activator of CK2.

Isolation of erythrocytes

Heparinized fresh blood from healthy donors was centrifuged at $2{,}000 \times g$ for 5 min at 4 °C and the pellet was resuspended in 4 volumes of phosphate buffered saline (PBS) (0.144 g/l KH_2PO_4, 9.0 g/l NaCl, and 0.795 g/l $Na_2HPO_4 \cdot 7H_2O$), pH 7.4. After three times washing at 1,800, 1,500, $1{,}300 \times g$ the erythrocytes were isolated by density separation (Cohen *et al.*, 1976). The erythrocyte fraction was resuspended in PBS, pH 7.4 to obtain a cell concentration of 2×10^7/ml, counted in a Burker's camera by a Standard UF2 microscope (Carl Zeiss, Jena, Germany). The suspension did not contain other cell types.

Preparation of the erythrocyte membranes

5 ml of packed red blood cells (RBCs) were mixed with 15 ml cold PBS, pH 7.4, and centrifuged at $5{,}900 \times g$ for 10 min at 4 °C. The supernatant was discarded, and cells were washed with 15 ml cold PBS, centrifuged as above, and resuspended in 5 ml of 5 mM Na_2HPO_4, pH 8.0, for hypotonic cell lysis. Lysed cells were centrifuged at $25{,}000 \times g$ for 15 min at 4 °C, after which the supernatant was gently aspirated and discarded. The RBC membrane pellet was repeatedly washed (4–5 times) with 5 mM Na_2HPO_4 until the pellet appeared white (indicating the removal of hemoglobin), and membranes were used for further experiments. The protein content of hemoglobin-free pellets was determined according to Bradford (1976) with human serum albumin as a calibrator. Samples were diluted to a protein content of 1.5 g/l.

Erythrocyte membrane Na^+/K^+-ATPase activity

Samples (50 μl) were added to 450 μl of the following mixture: 100 mmol of NaCl, 20 mmol of KCl, 2.5 mmol of $MgCl_2$, 0.5 mmol of EGTA, 50 mmol of Tris [tris(hydroxymethyl) aminomethane] hydrochloride buffer, pH 7.4, 1.0 mmol of ATP, 1.0 mmol of phosphoenolpyruvate, 0.16 mmol of NADH, 5 kU of pyruvate kinase, 12 kU of lactate dehydrogenase (all purchased from Sigma). Finally, the test modulators were added to the samples in the above mentioned concentrations. After 5 min, 5 μl of 10 mmol/l ouabain was added to inhibit the ouabain-sensitive ATPase activity. The change in absorbance was measured at 340 nm by a twin test (*i.e.*, combination of two assays in one

cuvette); rate A (*i.e.*, slope of total ATPase activity), 80–280 s; rate B (*i.e.*, slope of ouabain-resistant ATPase activity), 400–600 s. The difference between the two slopes was proportional to the Na^+/K^+-ATPase activity (Vásárhelyi *et al.*, 1997).

Erythrocyte K^+ content

Intracellular K^+ content was measured by an AVL analyzer with a K^+-selective electrode. 16 Patients with insulin-independent diabetes were divided into 2 groups according to their blood glucose levels: 8 patients with normal and 8 with elevated levels of blood glucose, *i.e.* (5.3 ± 0.75) and (9.1 ± 1.45) mmol/l glucose, respectively. Hematocrite was measured in the samples of fresh heparinized blood from each patient. Quadruplicates of the samples containing 0.5 ml blood were washed three times in 10 ml PBS, pH 7.4, and centrifuged at $2000 \times g$ for 10 min. The sediment was resuspended in 0.5 ml PBS. To the samples 50 nM Lf and 20 mM KCl were added (Table I). The control sample contained only PBS. After 24 h of incubation and three times washing under the conditions described above, the cells were lyzed by addition of 2 ml double distilled water and freezing at −70 °C. After centrifugation at $6{,}000 \times g$ for 30 min the sediment was discarded and K^+ content was measured in the supernatant.

Statistics

Statistical calculation of the results was made by variation analysis using Student's t-test.

Results

Lf (50 nM) stimulated with 47% the erythrocyte Na-pump activity. When Lf was not present in the media the ATPase activity was (4.44 ± 0.98) U/g protein, and in the presence of 10 nM, 50 nM and 100 nM Lf, an augmentation was found: (4.92 ± 0.83) U/g protein ($p > 0.1$), (6.54 ± 0.90) U/g protein ($p < 0.001$) and (5.84 ± 0.87) U/g protein ($p < 0.001$), respectively.

Lf influenced differently the intracellular K^+ content depending on the blood glucose levels in patients with diabetes (Table I).

Patients with diabetes having normal blood glucose levels

After incubation of the erythrocytes with Lf alone the K^+ content was 16% higher than in the control samples without Lf ($p < 0.05$). Lf facilitated the K^+ influx into the erythrocytes. When the erythrocytes were incubated simultaneously with Lf and KCl the K^+ content elevated by 23% ($p < 0.001$) compared to the control samples. When erythrocytes were incubated with KCl alone, the K^+ content enhanced by 18% (Table I).

Patients with diabetes having high blood glucose levels

The incubation with Lf did not affect the erythrocyte K^+ content. The simultaneous application of Lf and KCl enhanced the erythrocyte K^+ content by 9% ($p < 0.01$) (Table I).

Both Go6976 (with 194%) and Go6983 (with 84%) stimulated the ATPase activity. The effect was more pronounced for Go6976. Calyculin A in-

Table I. K^+ content in erythrocytes from patients with insulin-independent diabetes.

Agent (n = 8)	K^+ [mmol/(l Er Ht)]	Effect (%)	p
Without agents (controls)			
Normal blood glucose	66.61 ± 3.96	–	–
High blood glucose	63.97 ± 3.19	–	–
Lf			
Normal blood glucose	77.31 ± 5.63	+16	<0.05
High blood glucose	61.18 ± 2.57	– 4	>0.1
Lf + KCl			
Normal blood glucose	81.96 ± 5.98	+23	<0.001
High blood glucose	70.00 ± 4.00	+17	<0.01
KCl			
Normal blood glucose	78.36 ± 4.62	+18	<0.05
High blood glucose	74.00 ± 5.23	+18	<0.05

n, Number of patients; Ht, hematocrite.

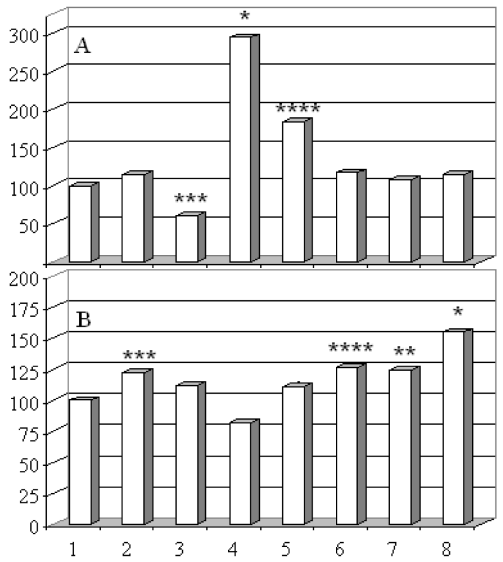

Fig. 1. (A) Effect of modulators of phosphorylation on the erythrocyte Na^+/K^+-ATPase; 1, control without Lf or agents (100%); 2, with okadaic acid; 3, with calyculin A; 4, with Go6976; 5, with Go6983; 6, with caffeine; 7, with W-7; 8, with spermine. (B) Lf stimulatory effect in the presence of modulators of phosphorylation; 1, control with 50 nM Lf but without estimated agents (100%); 2, okadaic acid + Lf; 3, calyculin A + Lf; 4, Go6976 + Lf; 5, Go6983 + Lf; 6, caffeine + Lf; 7, W-7 + Lf; 8, spermine + Lf. * $p < 0.001$; ** $p < 0.025$; *** $p < 0.01$; **** $p < 0.05$.

hibited reliably the ATPase activity by 39% ($p < 0.001$), but okadaic acid had no significant effect on ATPase activity. Caffeine enhanced the pump activity by 17%, but the effect was not statistically reliable. The calmodulin antagonist W-7 did not show a reliable effect on the Na pump activity. Spermine enhanced slightly and not reliably the ATPase activity by 14% ($p > 0.1$) (Table II and Fig. 1A).

Some of the tested agents acted as modulators of the pump stimulation exerted by Lf. When they were in simultaneous use with Lf the estimated values were reliable higher than the values obtained with Lf alone. This increase concerned the results obtained with okadaic acid (22%, $p < 0.05$), caffeine (26%, $p < 0.025$), W-7 (24%, $p < 0.01$) and spermine (55%, $p < 0.001$) (Table III and Fig. 1B).

The ATPase activity increased reliably by 55% for okadaic acid, 191% for calyculin A, 45% for caffeine, 69% for W-7 and 127% for spermine (Table II and Fig. 1B), when Lf was added to the media, in comparison to the effect of the same agents when used alone (Table II).

Lf has exerted an inhibitory effect on the stimulation of the sodium pump with both Go6976 and Go6983; Lf decreased this effect of stimulation by 65% ($p < 0.001$) and 18% ($p > 0.1$), respectively (Table II). Go6976 inhibits both the classic isoforms of PKC, and PKD also. Go6983 is used for

Table II. Effects of some modulators of phosphorylation on the Na^+/K^+-ATPase activity.

Agent ($n = 6$)	Na^+/K^+-ATPase [U/g protein]	p	Difference vs. control (%)
Without agents or Lf (control)	4.44 ± 0.98	–	–
Okadaic acid	5.18 ± 0.61	>0.1	+14
Okadaic acid + Lf	8.01 ± 0.75	<0.001	+80
Calyculin A	2.71 ± 0.98	<0.02	–39
Calyculin A + Lf	7.89 ± 1.36	<0.001	+78
Go6976	13.04 ± 2.11	<0.001	+194
Go6976 + Lf	4.57 ± 2.59	>0.1	+3
Go6983	8.18 ± 3.24	<0.05	+84
Go6983 + Lf	7.26 ± 3.03	>0.1	+16
Caffeine	5.68 ± 1.57	>0.1	+17
Caffeine + Lf	8.26 ± 1.34	<0.001	+86
W-7	4.81 ± 0.86	>0.1	+8
W-7 + Lf	8.14 ± 0.49	<0.001	+83
Spermine	5.06 ± 0.61	>0.1	+14
Spermine + Lf	10.11 ± 2.59	<0.001	+127

n, Number of samples.

Table III. Some modulators of the protein phosphorylation change the Lf stimulatory effect.

Agent (n = 6)	Na^+/K^+-ATPase [U/g protein]	p
Lf	6.54 ± 0.90	–
Okadaic acid + Lf	8.01 ± 0.75	<0.02
Calyculin A + Lf	7.89 ± 1.36	>0.1
Go6976 + Lf	4.57 ± 2.59	>0.1
Go6983 + Lf	7.26 ± 3.03	>0.1
Caffeine + Lf	8.26 ± 1.34	<0.05
W-7 + Lf	8.14 ± 0.49	<0.01
Spermine + Lf	10.11 ± 2.59	<0.001

n, Number of samples.

excluding the effect of PKD, because it inhibits only the classic forms of PKC (Gschawendi *et al.*, 1996). Because Lf interferes with the effect of Go6976 (inhibits it) it may be presumed a synergic participation in signals with PKD.

Discussion

Our previous studies showed that Lf binds erythrocyte specifically (Taleva *et al.*, 1999) and that Lf receptor interaction leads to the stimulation of glycolysis and antioxidative protection of the erythrocyte (Maneva *et al.*, 2003). The established mutual connection in the glycolysis control and Na^+/K^+-ATPase (Fossel and Solomon, 1978) brought us to the suggestion that Lf, as a stimulator of glycolysis (Maneva *et al.*, 2003), would affect the Na^+/K^+-ATPase activity. Our results pointed Lf as a stimulator of the Na pump: 50 nM of Lf enhanced about 50% the enzyme activity.

Lf receptor interaction might intervene in short-term effects of regulation, involving processes of changes in the association, phosphorylation and the oxidation level of membrane proteins. Erythrocyte membrane contains most of the signal molecules in the beginning of the cell transduction, which may conduct regulatory stimuli induced by Lf.

Several mechanisms for the activation of the Na pump by Lf during the binding to its membrane receptors are possible:

(i) Lf receptor interaction may cause conformational changes, altering the enzyme access to the sites for phosphorylation in the pump: PKA and PKC may phosphorylate the α-subunit in definite conformation dependent on the cell type (Feschenko and Sweadner, 1994).

(ii) It was found that Lf activates CK2 and is a phosphate acceptor of the enzyme (Maekawa *et al.*, 2002). CK2 phosphorylates and regulates a wide spectrum of erythrocyte membrane proteins, associated in multiprotein complexes with the Na pump (Wei and Tao, 1993).

(iii) Lf also stimulates tyrosine phosphorylation of membrane proteins (Kobayashi *et al.*, 2005). It has been found protein tyrosine kinase activity associated with the erythrocyte membrane (Zylinska *et al.*, 2002).

(iv) The stimulatory effect of Lf could be indirect, because Lf activates the Na^+/H^+ antiport (Sun *et al.*, 1991).

(v) The molecular structure of the erythrocyte membrane receptor for Lf is still unknown. It has been reported that some bacterial receptors for Lf (Tanaka *et al.*, 2004) are glyceraldehyde-3 phosphate-dehydrogenase (GAPD). GAPD in the erythrocyte is a transmembrane enzyme, bound with band 3 (Campanella *et al.*, 2005). Lf, as a final electron acceptor from glycolysis (Maneva *et al.*, 2003) might receive the electron direct from the GAPD, since it directly binds to it. Furthermore, Lf might transduct signals leading to the conformational change of band 3 and its association with the Na pump.

(vi) Lf, as an antioxidant, protects the erythrocyte membrane proteins from oxidative disturbances and facilitates the pump function.

We suppose that, as a consequence of the Lf stimulatory effect on the Na pump activity, a reliably higher K^+ content retains in the erythrocytes of patients with diabetes and normal blood glucose level (Table I). It may be supposed that a favourable effect is due also to the Lf antioxidative activity that sustains the thiol status of the erythrocyte maintaining the intracellular glutathione (Maneva *et al.*, 2003). The deep and hardly reversible changes in the membrane structures, engaged with maintaining of the ion balance, is one possible explanation for the absence of protective function of the K^+ content in the erythrocytes of patients with high blood glucose levels (Babu and Singh, 2004) (Table I).

Some of the tested modulators of cell signaling are also modulators of the Lf stimulatory effect on the erythrocyte Na^+/K^+-ATPase (Table III and Fig. 1B). Lf itself is able to modulate the effect of some agents upon the pump activity (Table II). Moreover, an additive effect of stimulation was

found when Lf and some agents are used simultaneously (Table II).

Lf and OA (an inhibitor of PP2A) are activators of CK2 (Bordin *et al.*, 1993; Maekawa *et al.,* 2002) and the MAPK signal pathway to the Na^+/H^+ antiport activation (Sartori *et al.*, 1999; Dhenan-Duthille *et al.*, 2000). Probably PP2A is an negative regulator of the Lf stimulatory effect on the Na^+/K^+-ATPase. (Tables II and III, Fig. 1A and B).

Caffeine is an inhibitor of the phosphodiesterase activity, but data exist that it does not have an effect on the erythrocyte cAMP-dependent protein kinase (Biovin *et al.*, 1988). The binding of caffeine to hydrophobic domains of erythrocyte proteins (Sato *et al.*, 1990) may lead to changes in the erythrocyte protein complexes, facilitating or holding up the functions of enzymes, participating in cell signals induced by Lf (Table III and Fig. 1B).

Data exist that CaM is an inhibitor of the erythrocyte Na^+/K^+-ATPase (Okafor *et al.*, 1997). It may be supposed that W-7 facilitates the regulatory effects of Lf by limiting the CaM access as a substrate of CK2 (Meggio *et al.*, 1987) (Table III and Fig. 1B). Lf functions as a phosphate acceptor from CK2 (Hatomi *et al.*, 2000).

It is possible to suggest the existence of crosstalk in signal transduction pathways of Lf and spermine leading to the stimulation of the Na^+/K^+-ATPase (Table III and Fig. 1B)**.** Such cell signals include tyrosine phosphorylation of membrane proteins (Khan *et al.*, 1990a; Tamaka *et al.*, 1998) and/or cAMP-dependent regulatory signals with CK2 participation (Khan *et al.*, 1990b; Maekawa *et al.*, 2002).

Lf could modulate the effect of caffeine on the ATPase, involving in cell signals with the participation of caffeine. Such a common effector is CK2 activated by Lf (Hatomi *et al.*, 2000). It may be supposed that Lf weakens the inhibitory effect of caffeine on the CK2 (Lecomte *et al.*, 1980) (Table II).

Lf eliminates the stimulatory effect of PKC inhibitors on the ATPase activity (Table II). Binding of Lf to the membrane receptors on the erythrocyte might lead to conformational changes in the membrane, and the inhibitor's binding sites (Swannie and Kaye, 2002) on the enzyme may become hard for access.

Our results show that Go6976 (an inhibitor of the classic isoforms and PKD) and also the "excluding" PKD inhibitor Go6983 (Martiny-Baron *et al.*, 1993) activate significantly the ATPase activity (Table II and Fig. 1A). The effect of Go6976 stimulation is almost twice that of Go6986. That probably means a special role of PKD as a negative regulator of the Na pump. PKD has a catalytic domain that possesses more similarities with the CaM-dependent kinases than with the isoforms of PKC. PKD effectively phosphorylates synthetic substrates of Ca^{2+}/CaM-dependent kinase II, but does not catalyze the phosphorylation typical for PKC pseudo-substrates (Ron and Kazanietz, 1999).

Go6983 does not limit the stimulation with Lf, while Go6976 eliminates it (Table III and Fig. 1B). It might be supposed that Lf participates in the ouabain-dependent signal pathway in which PKD is involved. It is probably different from the control exerted by Lf on the functions of Na^+/K^+-ATPase as a regulator of the ion transport.

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