Nongenomic Effect of Aldosterone on Ion Transport Pathways of Red Blood Cells

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Key Words

Red blood cell • Erythrocyte • Aldosterone • Nongenomic effect • Ion transport • Fluorescence imaging

Abstract

The direct influence of aldosterone on the human and avian red blood cell (RBC) transport systems, Na⁺/K⁺ pump, Na⁺,K⁺,2Cl⁻ symporter, and K⁺(Na⁺)/H⁺ exchanger, was investigated with tracer kinetics. The present work proved that aldosterone has no significant effect on these transport pathways. However, in young human RBCs containing reticulocytes aldosterone showed a significant inhibition of the Na⁺,K⁺,2Cl⁻ symporter. Investigations of the Li⁺ efflux via the Na⁺/Li⁺ exchanger using atom absorption spectroscopy revealed that aldosterone has no effect on this transporter. Studies of the effect of aldosterone on the Ca²⁺ content and the intracellular pH (pH_i) were carried out on single RBCs with a fluorescence imaging system. Both parameters are affected by aldosterone. The Ca²⁺ uptake in the presence of aldosterone, under conditions where the Ca²⁺ pump is inhibited, showed marked differences from the control. Since the effect is nifedipine-sensitive, it seems that aldosterone affects a Ca²⁺ channel. In addition, aldoster-

one leads to an acidification of the intracellular medium after an initial alkalisation due to an effect on the Na⁺/H⁺ exchanger.

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Introduction

Aldosterone plays an essential role in electrolyte and fluid homeostasis and thus the control of blood pressure. Due to its lipophilic nature, aldosterone enters the cell by diffusion through the cell membrane. In the cytoplasm of the target cells, it binds to an intracellular receptor, the mineralocorticoid receptor (MR), which is linked to several other proteins including heat shock proteins and immunophilins [1]. Some reports indicated that some aldosterone effects are not mediated through binding to the MR [2-5]. These effects were termed “nongenomic” to distinguish them from the “genomic” effects that occur as a result of gene transcription after activation of nuclear receptors. The nongenomic effects involve rapid actions of aldosterone, which are independent of transcription and translation. The nongenomic effects were suggested to be mediated by a distinct receptor, which is insensitive to inhibitors of the classical mineralocorticoid receptor [6, 7]. In many cell lines, aldosterone induces a

rapid (within 10 min) increase of inositol trisphosphate and intracellular Ca^{2+} , repression of protein kinase C activity, and activation of the Na^+/K^+ pump. These changes were observed in human mononuclear leukocytes, vascular smooth muscle cells, and rat cardiomyocytes [2, 8, 9]. Specific antagonists of MR and inhibition of transcription or protein synthesis did not block these actions. A double-blind placebo-controlled randomized study in humans showed that, after intravenous administration of a large dose of aldosterone, there were significant changes in systemic vascular resistance, cardiac output, and cardiac index compared with the placebo group. The effects appeared at 3 min and dissipated within 10 min, a time course that is too rapid to be explained by genomic effects [5]. Using the fission yeast *Schizosaccharomyces pombe* as a model, which does not contain nuclear receptors, it was demonstrated that aldosterone can cause changes of the proteome [10].

Already in 1958 Friedman and Friedman [11] investigated the effect of aldosterone on the blood plasma Na^+ and K^+ content during refrigeration or subsequent re-warming. The results showed an increase of the intracellular Na^+ concentration, possibly caused by hindering Na^+ efflux [11]. However, another study was unable to demonstrate any effect of aldosterone on Na^+ transport in red blood cells (RBCs) [12]. The retardation of Na^+ exchange in canine RBCs at physiological aldosterone concentration was reported by Spach and Streeten [13]. Such an effect of aldosterone in RBCs cannot be mediated by genomic mechanisms due to the lack of a nucleus in these cells. It has, however, to be taken into account that canine and human RBCs contain different ion transport pathways in the membrane. For instance, canine RBCs have a $\text{Na}^+/\text{Ca}^{2+}$ exchanger, which is absent in human RBCs but they do not have a Na^+/K^+ pump, which is present in other mammalian RBCs (except dog and cat) [14].

In general, the RBCs are a perfect model to study the nongenomic action of aldosterone because they do not contain a nucleus (only young RBCs contain a nucleus). It is not known so far whether a steroid hormone receptor is still present in RBCs. In human lymphocytes aldosterone but not glucocorticoids binds a 50-kDa membrane protein that was isolated by Eisen et al. [15].

In recent years many specific ion transport pathways were described in the RBC membrane [16]. Therefore, the aim of the present study was to investigate the effect of aldosterone on different ion transport pathways known to exist in the RBC membrane. This includes the Na^+/K^+ pump, the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ symporter, the Na^+/H^+ ex-

changer, and the K^+ transport under conditions where nearly all Na^+ and K^+ transporters are blocked (except the $\text{K}^+(\text{Na}^+)/\text{H}^+$ exchanger). In addition, possible effects of aldosterone on the intracellular pH (pH_i) and the Ca^{2+} content of RBCs were investigated.

Materials and Methods

Blood and solutions

Blood was used from healthy human donors and obtained from the blood bank of the University Hospital of the Saarland University (Homburg). Chicken blood was obtained from the chicken farm Presser (Illingen).

RBCs were separated by centrifugation ($2000 \times g$, 5 min) at room temperature. The cells were washed three times with a physiological solution containing (mM): NaCl 145, glucose 10, morpholinoethane sulfonic acid/Tris-(hydroxymethyl)aminomethane (MES/Tris) 10, for tracer kinetic measurements or HEPES/NaOH 10, for fluorescence microscopy measurements, pH 7.4 at room temperature. Plasma and buffy coat were aspirated. RBCs were then finally washed once in the appropriate medium used for the flux experiment, i.e. in physiological solution or in low ionic strength (LIS) solution containing (mM): sucrose 250, glucose 10, MES/Tris 10, pH 7.4.

To obtain a fraction of young RBCs containing reticulocytes, the cells were separated on the basis of their density (young cells have a lower density than old RBCs). The RBC suspension was centrifuged at $2,500 \times g$ for 1 h. The top and bottom layer (top for young cells, bottom for old cells, 20% each of all RBCs centrifuged) was taken and separately centrifuged again at $2,500 \times g$ for 1 h. The top and bottom layer was separated again and the same procedure was repeated once more (in total 3 h centrifugation). Young RBCs were used in comparison to old cells and 'normal' cells (not density separated) for flux measurements.

K^+ influx measurements

Washed RBCs were suspended at about 5% haematocrit in a total volume of 1 ml of flux medium (physiological or LIS solution) contained in an Eppendorf 1.5 ml microcentrifuge tube. To inhibit the Na^+/K^+ pump and the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ symporter, ouabain (0.1 mM) and bumetanide (0.1 mM) were added, respectively. EGTA (0.1 mM) was present in all flux media. To determine the (ouabain + bumetanide + EGTA)-resistant K^+ flux, all three substances were present. Aldosterone dissolved in ethanol was present in the flux solutions at different concentrations. For control measurements ethanol was added. The cell suspension was equilibrated at the flux temperature of 37°C for 5 min after which tracer solution ($[\text{KCl} + {}^{86}\text{RbCl}]$), 7.5 mM final concentration, was added to start the flux measurement. The duration of exposure of cells to isotope (i.e. the flux time) was 30 min. The isotope uptake was stopped by centrifugation at $15,000 \times g$ (10 s), and the supernatant removed by aspiration. The cells were then washed free of extracellular radioactivity by four successive re-suspensions and centrifugations ($15,000 \times g$, 10 s) in ice-cold medium consisting of (mM): MgCl_2 106,

N-morpholinopropane sulfonic acid (MOPS)/NaOH 10, pH 7.4. The cell pellet was lysed with 0.5 ml of 1% (v/v) Triton X-100 and the protein precipitated by adding 0.5 ml of 5% (w/v) trichloroacetic acid followed by centrifugation at $15,000 \times g$ for 5 min. The activity of ^{86}Rb in the supernatant was determined by Cerenkov counting in a liquid scintillation analyser (Packard, Tri-Carb 2900). Haemoglobin content of RBC suspensions was determined as cyanmethaemoglobin using Drabkin's reagent. The flux was calculated in $\text{mmol}/(\text{l}_{\text{cells}} \times \text{h})$.

Intracellular Ca^{2+} content measurement

Intracellular free Ca^{2+} levels of single cells were monitored using an inverted fluorescence microscope (Nikon) and the Ca^{2+} -sensitive fluorescent dye fluo-4 AM (4 μM , Molecular Probes). This indicator has a high-affinity binding for Ca^{2+} ($K_d = 345 \text{ nM}$) and a very large fluorescence intensity increase in response to Ca^{2+} binding (>100 fold). The application of fluo-4 for Ca^{2+} measurements in intact RBCs was reported by our group elsewhere [17]. Aliquots of fluo-4 AM were mixed with Pluronic F-127 (20 % DMSO in H_2O) and prepared to give a final concentration of 1 M (stock solution). The non-ionic detergent was used to assist in dispersion of the non-polar AM ester in aqueous media. Washed RBCs (1 % haematocrit) were incubated with the fluo-4 AM for 45 min at 37°C in the dark. For the experiments in the presence of nifedipine (0.1 mM), this substance was added 10 min before the end of the 45 min incubation. Cells were then washed in physiological solution (with HEPES/NaOH buffer) to remove the extracellular fluorescent dye and were re-suspended (0.015 % haematocrit) in the same buffer (in the presence of nifedipine, if required). Aldosterone (at different concentrations) or ethanol (control) was added 5 min before the start of the imaging procedure.

Our experimental approach was designed to provide a qualitative assessment of fluorescence intensity while maintaining identical imaging parameters under all conditions. This was accomplished by taking images with a CCD camera (Visitron System, Puchheim, Germany) using a 100×1.4 (NA) oil immersion lens with infinity corrected optics. An image was taken every 20 s (exposure time 500 ms) for a 30 min experiment.

Dye molecules were excited with a xenon lamp at a wavelength of 488 nm. The emission was recorded at 520 nm. The RBCs were put on a cover slip coated with poly-L-lysine (PLL) and the experiments were performed at room temperature in a dark room.

Intracellular pH measurements

The intracellular pH (pH_i) was measured using 2',7'-bis(carboxyethyl)-5(6)-carboxy-fluorescein tetraacetoxymethyl ester (BCECF-AM) [18]. Washed erythrocytes at a haematocrit of 1 % were suspended in a total volume of 0.5 ml in physiological solution in a 1.5 ml Eppendorf tube. The RBCs were then loaded with the fluorescent indicator BCECF-AM (6 μM) for 45 min at 37°C in the dark. For the experiments in the presence of amiloride (0.1 mM), this substance was added 10 min before the end of the 45 min incubation. After the incubation with the fluorescent dye, RBCs were washed with physiological solution three times (in the presence of amiloride, if required), in order to remove the extracellular fluorescent dye,

and were re-suspended (0.015 % haematocrit) in the same buffer.

The BCECF-AM loaded cells were put on a cover slip coated with poly-L-lysine (PLL) and the experiments were performed at room temperature in a dark room.

A calibration curve of the fluorescence intensity depending on pH was created, using the K^+/H^+ ionophore nigericin [19, 20]. For this reason 5 μM nigericin was added to RBCs suspended in the calibration buffer solutions containing (mM): KCl 135, NaCl 10, glucose 10 and HEPES/NaOH 10. The calibration curve was linear in the pH range 6.8 - 7.8.

Li^+ efflux measurements

Washed RBCs were added to a Li^+ loading solution containing (mM): LiCl 150, glucose 10, MOPS 10, pH 7.4 and incubated at 37°C for 3 h. After incubation, extracellular Li^+ was removed by washing the RBCs three times with a solution containing (mM): sucrose 85, MgCl_2 75, glucose 10, MOPS 10, pH 7.4. Then RBCs were washed ones with the flux medium (physiological solution, which contained additionally (mM): ouabain 0.1, bumetanide 0.1, EGTA 0.1). The Li^+ efflux was measured in the flux medium in the presence or absence of phloretin (0.1 mM) and aldosterone (0.01 mM) at 20% haematocrit and 37°C . If aldosterone was absent (control), ethanol was added. All solutions used for Li^+ efflux experiments had osmolarities of 290 - 295 mOsm/l (measured with an automatic semi-micro osmometer from Knauer, Germany).

The cell suspension was slightly vortexed and the first samples ($2 \times 1.5 \text{ ml}$) were taken within less than 1 min ($t = 0$) and centrifuged ($15,000 \times g$, 30 s). The supernatant of the two samples was acidified with 50 μl of HNO_3 . The same procedure was carried out after an incubation of 30 min. To determine the intracellular Li^+ concentration of the RBCs at the beginning of the efflux experiment 1.8 ml of the RBC suspension were taken and added to 18 ml de-ionised water. The intra- and extracellular Li^+ concentrations were measured using an atomic absorption spectrometer (Perkin Elmer 2100) and the Li^+ efflux was calculated in $\text{mmol}/(\text{l}_{\text{cells}} \times \text{h})$.

Reagents

All chemicals used (except the chemicals mentioned below) were purchased from Sigma-Aldrich (Muenchen, Germany). Nigericin (stock solution 250 mM), phloretin (stock solution 10 mM), nifedipine (stock solution 100 mM), and amiloride (stock solution 100 mM) were dissolved in ethanol. BCECF AM or fluo-4 AM was prepared in stock solutions (1 M) with Pluronic F-127 (20% DMSO in water). Aldosterone (Fluka Chemie GmbH, Buchs, Switzerland) was dissolved in ethanol so that the final concentration of ethanol was less than 0.1%. ^{86}Rb (in RbCl) was produced by Amersham Biosciences (Amersham, U.K.). Ouabain and MES were from SERVA (Heidelberg, Germany).

Statistical Treatment of Results

The data are presented as mean values $\pm$ SD of independent experiments. The significance of differences was tested by paired or unpaired Student's *t*-test or ANOVA as applicable. Differences were considered significant if $p < 0.05$. Each experiment was repeated at least 3 times on blood from different

Fig. 1. Effect of aldosterone (at different concentrations and incubation times) on the K⁺ influx of human RBCs in physiological solution mediated by the Na⁺/K⁺ pump and the Na⁺,K⁺,2Cl⁻ symporter. The (ouabain + bumetanide + EGTA)-resistant K⁺ influx (residual K⁺ transport) is also shown. ■ Na⁺/K⁺ pump, □ Na⁺/K⁺ pump with aldosterone, ▒ Na⁺,K⁺,2Cl⁻ symporter, ▤ Na⁺,K⁺,2Cl⁻ symporter with aldosterone, ▨ residual K⁺ influx, ▩ residual K⁺ influx with aldosterone.

Fig. 2. Effect of aldosterone (10 μM) on the K⁺ influx of young human RBCs containing reticulocytes in physiological solution mediated by the Na⁺/K⁺ pump, the Na⁺,K⁺,2Cl⁻ symporter, and the (ouabain + bumetanide + EGTA)-resistant K⁺ influx (residual K⁺ transport). ■ control (with ethanol, without aldosterone), □ with 10 μM aldosterone.

donors. For Ca²⁺ and pH_i determinations at least 3 measurements (with blood from 3 different donors and at least 10 single cells from each blood, i.e. 30 single cells) were carried out for each experimental condition.

Results

K⁺ influx measurements

To examine the influence of aldosterone on the K⁺ fluxes mediated by the Na⁺/K⁺ pump, the Na⁺,K⁺,2Cl⁻ symporter, and the (ouabain + bumetanide + EGTA)-resistant pathway (i.e. the K⁺ transport under conditions where nearly all K⁺ transporters are blocked, except the K⁺(Na⁺)/H⁺ exchanger), different steroid concentrations as well as pre-incubation times were chosen (Fig. 1). The aldosterone concentration varied between 100 nM and 10 μM, the pre-incubation times were 10 or 30 min before the K⁺(⁸⁶Rb⁺) was measured (in the presence of aldosterone). As one can see from Fig. 1, no significant differences of the K⁺ influx mediated by the 3 investigated transport pathways in physiological solution in the presence of aldosterone in comparison to the control were found. Please note that for each experimental condition (aldosterone concentration and pre-incubation time) separate control measurements were carried out. We have no explanation why the flux mediated by the Na⁺/K⁺ pump is close to 4 mmol/(l_{cells} × h) in the presence or absence (control) of 100 nM and 10 μM aldosterone,

which is significantly higher than the control value published earlier by our group. The flux mediated by the Na⁺/K⁺ pump for young RBCs containing reticulocytes (see below, Fig. 2) is in the expected range and fits to our previous data for mature RBCs. However, in Fig. 1 all K⁺ influxes mediated by the Na⁺,K⁺,2Cl⁻ symporter and the (ouabain + bumetanide + EGTA)-resistant pathway are in the normal range. In some experiments the pre-incubation time was extended to 60 min but without a significant effect (data not shown). Since the (ouabain + bumetanide + EGTA)-resistant K⁺ influx is relatively small, measurements of this flux were performed in LIS solutions. In LIS solution the (ouabain + bumetanide + EGTA)-resistant K⁺ influx is markedly increased. However, also in this medium no significant differences between aldosterone-containing samples and the control were found (same aldosterone concentrations and pre-incubation times as for physiological solution, n > 10, data not shown).

In separate experiments the K⁺ influx mediated by the Na⁺/K⁺ pump, the Na⁺,K⁺,2Cl⁻ symporter, and the (ouabain + bumetanide + EGTA)-resistant pathway were measured for young RBCs containing reticulocytes (see Methods). For this cell fraction no significant changes of the K⁺ flux via the Na⁺/K⁺ pump and the (ouabain + bumetanide + EGTA)-resistant pathway in the absence (control) and presence of aldosterone (10 μM) were found. The results are shown in Fig. 2. In contrast,

the addition of 10 μM aldosterone led to a significant reduction of the K^+ influx mediated by the $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ symporter (Fig. 2). For all conditions the aldosterone pre-incubation time was 10 min and aldosterone was present during the flux measurements.

For comparison adequate experiments were carried out with RBCs from chicken blood, which contain a nucleus. For these RBCs as for 'normal' human RBCs no significant differences were obtained for the investigated K^+ influxes (via the Na^+/K^+ pump, the $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ symporter, and the (ouabain + bumetanide + EGTA)-resistant pathway) in the absence or presence of aldosterone (10 μM , 10 min pre-incubation time, data not shown).

Ca^{2+} content measurements

Intact RBCs were loaded with fluo-4 AM in the presence of 1 mM o-vanadate, an inhibitor of the Ca^{2+} pump, and afterwards transferred in a physiological solution containing 2 mM Ca^{2+} immediately before starting the fluorescence measurements. It should be noticed that a time period of about 2 – 5 min was necessary to let the cells settle down to the cover slip surface and to start the experiment. Fig. 3 shows the results of the measurements of the Ca^{2+} content. In the absence of o-vanadate the intracellular Ca^{2+} concentration was too low to detect any significant change of the fluorescence signal for the control (shown in Fig. 3B) and aldosterone containing probes. The curves for the aldosterone containing probes in the absence of o-vanadate (not shown) did not differ from the control without o-vanadate. However, in the presence of o-vanadate a continuous increase of the intracellular Ca^{2+} concentration was observed for control cells (Fig. 3A,B). In the presence of 1 μM or 10 μM aldosterone the curves differ from the control, especially at the beginning (0 – 10 min) and at the end (20 – 30 min) of the experiment (Fig. 3B). The differences at the beginning remain unclear but overall it seems that the presence of aldosterone leads to a change of the intracellular Ca^{2+} content. A statistical analysis showed that after 18 min the obtained values for the aldosterone containing samples are significantly different from the control value. For visible reasons, the error bars are presented in Fig. 3B only for selected times (about 5, 20, and 25 min). Using a lower aldosterone concentration (100 nM), no significant difference from the control curve could be detected (not shown).

Comparable experiments with the Ca^{2+} channel blocker nifedipine (100 μM) in the presence of o-vanadate revealed that the fluorescence signal was very low and no significant change were detected over the time

period of 30 min (like in the absence of o-vanadate, see Fig. 3B). When nifedipine was present, the addition of aldosterone did not result in any changes (not shown).

Experiments on chicken RBCs were carried out to compare the aldosterone effect with human RBCs (Fig. 4A,B). Since chicken RBCs contain a nucleus, genomic aldosterone effects cannot be excluded in these cells. In addition, chicken RBCs have a bigger cell volume and respectively higher fluorescence intensity than human RBCs. From the fluorescence image (Fig. 4A) it can be recognised that the highest fluorescence signal comes from the nucleus. Like for human RBCs a continuous increase of the intracellular Ca^{2+} content was observed for control chicken RBCs in the presence of o-vanadate in physiological solution. In contrast, the addition of aldosterone (10 μM) led to a decrease of the intracellular Ca^{2+} content. The values for both curves are statistically significant different at the first 4 min and after 20 min of the experiment (Fig. 4B). For visible reasons, the error bars are presented in Fig. 4B only for selected times (1 and 28 min).

Intracellular pH measurements

The pH_i of RBCs was determined with the pH-sensitive fluorescent dye BCECF-AM. First, we confirmed that in the presence of nigericin, the response of the dye is a linear function of the pH of the solution (and hence also of pH_i) over the range investigated (cf. [19]). The calibration was carried out on single cells using an inverted fluorescence microscope as well as on cell suspensions using a fluorimeter. Based on the calibration a pH_i of 7.191 ± 0.004 ($n = 8$) was obtained, when the RBCs were suspended in the physiological solution ($\text{pH} = 7.4$). This measured pH_i is in agreement with the pH_i value reported in the literature [20] and did not change significantly during a 30 min measuring period in physiological solution (Fig. 5, control, without ethanol).

Investigating the effect of aldosterone on pH_i of single cells it was interesting to see that under control conditions, i.e. in the presence of ethanol, a small pH shift towards alkaline values ($\text{pH}_i \sim 7.3$) could be seen at the beginning of the experiment (at $t = 0$). Furthermore, in these control cells a pH_i shift can be seen over the 30 min measuring period (Fig. 5). There was also a pH_i shift in the presence of aldosterone (100 nM, 1 μM , 10 μM) at the beginning of the experiments but interestingly the curves declined 5 - 10 min after the start of the fluorescence measurements. A statistical analysis revealed that the pH_i values in the presence of aldosterone at different concentrations are significantly different from the

Fig. 4. Fluorescence kinetics of fluo-4 loaded chicken RBCs in physiological solution in the presence of 2 mM Ca^{2+} and 1 mM o-vanadate for a time period of 30 min. CaCl_2 and aldosterone (10 μM) or ethanol (control) were added before the start of the fluorescence measurements. A: Fluorescence image of single chicken RBCs, B: Ca^{2+} kinetic measurements. Each curve represents the average of measurement of 30 cells (from 3 different chickens). Representative error bars (S.D.) are shown at the times of about 1 and 28 min.

control value (in the presence of ethanol) after 30 min of the experiment (Fig. 5). For visible reasons, the error bars are presented in Fig. 5 only for selected times (about 5, 18, 25, and 30 min).

Comparable experiments in the presence of the inhibitor of the Na^+/H^+ exchanger amiloride (100 μM) revealed that the fluorescence signal in the presence of aldosterone (10 μM) did not differ significantly from the control (absence of aldosterone) as well as from the curve in the presence of aldosterone (10 μM) and the absence of amiloride (Fig. 6). For visible reasons, the error bars are presented in Fig. 6 only for selected times and in an alternating procedure (to avoid overlapping) for the curves in the presence of amiloride plus aldosterone and amiloride

Fig. 5. Fluorescence measurements of the intracellular pH (pH_i) of human RBCs in physiological solution in the presence or absence of aldosterone (100 nM, 1 μM or 10 μM). Aldosterone or ethanol (control) was added before the start of the fluorescence measurements. Each curve represents the average of measurement of 45 cells (taken from 3 different blood donors). Representative error bars (S.D.) are shown at the times of about 5, 18, and 25 min.

only (5, 10, 15, 20, 25, and 30 min). Representative error bars for the curve in the presence of aldosterone are shown in Fig. 5. The results demonstrate that in contrast to the control measurement of Fig. 5 (presence of ethanol) where a continuous increase of the pH_i was observed, a slight decrease of the pH_i in the presence of amiloride occurred over the 30 min measuring period independent on the absence or presence of aldosterone.

Li⁺ efflux measurements

To investigate the effect of aldosterone (10 μM) on the Na^+/Li^+ exchanger, the Li^+ efflux from human RBCs in physiological solution was measured in the presence and absence of phloretin (100 μM). The obtained phloretin-sensitive Li^+ flux values are $0.25 \pm 0.01 \text{ mmol}/(\text{l}_{\text{cells}} \times \text{h})$ and $0.27 \pm 0.02 \text{ mmol}/(\text{l}_{\text{cells}} \times \text{h})$ in the absence (control) and presence of aldosterone, respectively. These values are not significantly different. Also for the phloretin-resistant Li^+ fluxes no significant differences could be detected ($1.00 \pm 0.25 \text{ mmol}/(\text{l}_{\text{cells}} \times \text{h})$ vs. $0.98 \pm 0.15 \text{ mmol}/(\text{l}_{\text{cells}} \times \text{h})$ for the fluxes in the absence and presence of aldosterone, respectively).

Discussion

In the present work the effect of the mineralocorticoid aldosterone on different ion transport systems in RBCs was examined. The effect of aldosterone was associated for a long time exclusively with the regulation of Na^+ and K^+ homeostasis by MR activation in epithelial cells [21]. In the last 15 years the nongenomic effect of aldosterone has been investigated in non-epithelial cells. It has been shown that aldosterone has physiological as well as patho-physiological effects on the heart, vascular and cerebral cells [22, 23].

The rapid effect of aldosterone, which is related to the nongenomic effect, activates the ion transport via channels and pumps. In some cells a transcellular reabsorption of Na^+ , in other cells an increase of the intracellular Ca^{2+} concentration was found [24, 25]. In the present

Fig. 6. Fluorescence measurements of the intracellular pH (pH_i) of human RBCs in physiological solution in the presence or absence of aldosterone (10 μM) and amiloride (100 μM). Aldosterone, or ethanol (control) was added before the start of the fluorescence measurements, amiloride was added 10 min before the start of the measurements. Each curve represents the average of measurement of 35 - 45 cells (taken from 3 different blood donors). Line without symbol: in the presence of 100 μM amiloride, ■ in the presence of 10 μM aldosterone (cp. Fig. 5), □ in the presence of 10 μM aldosterone and 100 μM amiloride. Representative error bars (S.D.) for the curves in the presence of amiloride plus aldosterone and amiloride are presented for selected times (5, 10, 15, 20, 25, and 30 min) and in an alternating procedure (to avoid overlapping). Representative error bars for the curve in the presence of aldosterone are shown in Fig. 5.

work the human RBC was taken as a simple model cell without a nucleus for testing the effect of aldosterone on different ion transport systems. The duration of the cell exposure to aldosterone did not exceed 30 - 40 min. For both reasons (no nucleus, short exposure time) a genomic effect can be excluded. Because of the short exposure time, this seems also to be the case for the experiments with young RBCs containing reticulocytes. To observe a possible effect of aldosterone, concentrations in the range

100 nM – 10 μ M, higher than the physiological relevant ones, were chosen. Aldosterone concentrations in this range were chosen also in other works. In an early report of aldosterone action on dog RBCs a concentration of 0.5 μ M was used [13]. Aldosterone levels up to 1 μ M were used analysing the nongenomic effect of this steroid in A6 cells [26]. Only aldosterone concentrations of more than 10 μ M were described to be unspecific (no receptor involved), i.e. could include possible effects of steroids on membrane fluidity [27].

Several studies showed that some of rapid nongenomic effects can be mediated by the classical membrane receptors and by receptors, which are distinct from the classical steroid receptor (Mannheim classification) [27, 28]. However, on RBCs such effects have not been described. It is evident that aldosterone as a lipophilic hormone can cross the RBC membrane and therefore might affect transport systems from both sides of the membrane.

The nongenomic effect of aldosterone on three transport systems was studied in the present work with tracer kinetics. For the Na^+/K^+ pump, the $\text{Na}^+,\text{K}^+,2\text{Cl}^-$ symporter, and the (ouabain + bumetanide + EGTA)-resistant K^+ transport pathway no significant aldosterone effect could be found (Fig. 1). No aldosterone effect could be found for these transport pathways also in chicken RBCs. These findings are in agreement with an earlier report showing that the total $\text{K}^+ (^{86}\text{Rb}^+)$ influx of human RBCs (no transport inhibitors were used) is not affected by aldosterone [29]. However, for some nucleus containing cell types it was reported that aldosterone activates the Na^+/K^+ pump quickly [30].

Since the (ouabain + bumetanide + EGTA)-resistant K^+ influx accounts only for 5% of the total K^+ influx and involves the transport via the $\text{K}^+(\text{Na}^+)/\text{H}^+$ exchanger, measurements in LIS solution were performed. It is known that the $\text{K}^+(\text{Na}^+)/\text{H}^+$ exchanger is activated under LIS conditions [16, 31, 32]. However, also in LIS medium no aldosterone effect was observed.

The K^+ influx investigations of young RBCs (which were separated by density centrifugation) containing a higher fraction of reticulocytes than normal blood showed that aldosterone has a significant effect on the $\text{Na}^+,\text{K}^+,2\text{Cl}^-$ symporter (Fig. 2). The addition of aldosterone leads to an inhibition of this transport pathway. One has to take into consideration that the K^+,Cl^- symporter, which is silent in mature human RBCs, is activated in reticulocytes and young RBCs. The presence of 100 μ M bumetanide only partly inhibits this transporter. Therefore, it cannot be excluded that aldosterone affects the K^+,Cl^- symporter.

That the K^+ influx mediated by the $\text{Na}^+,\text{K}^+,2\text{Cl}^-$ symporter in mature RBCs (Fig. 1) is lower than in young RBCs (Fig. 2) can be also explained by the possibility of an involvement of the K^+,Cl^- symporter. However, the mechanism of this aldosterone effect remains unclear, the $\text{Na}^+,\text{K}^+,2\text{Cl}^-$ symporter and/or the K^+,Cl^- symporter could be involved. To clarify this problem further detailed investigations are required. For several nucleus containing cell types (e.g., rat arterial vessel cells, myocytes) it was described that aldosterone activates the $\text{Na}^+,\text{K}^+,2\text{Cl}^-$ symporter [23, 30, 33]. For vascular smooth muscle cells (VSMC) no rapid effect of aldosterone on the $\text{Na}^+,\text{K}^+,2\text{Cl}^-$ symporter was found [30].

The influence of aldosterone on the Na^+/Li^+ exchanger of human RBCs was investigated by measuring the Li^+ efflux using an atom absorption spectrometer. For this transport pathway no significant effect of aldosterone could be found.

Investigations of the intracellular Ca^{2+} content and pH_i were carried out on a single cell level. To our knowledge, this is the first time such measurements have been applied to study the nongenomic aldosterone effect.

Ca^{2+} uptake in human and chicken RBCs was measured in the presence of o-vanadate to inhibit the Ca^{2+} pump. With an intact Ca^{2+} pump the fluo-4 fluorescence intensity was too low to detect a significant fluorescence signal and hence not applicable for the investigations of an aldosterone effect under such conditions (Fig. 3B, control without o-vanadate). No increase of the intracellular Ca^{2+} content of the RBCs could be registered in physiological solution when the Ca^{2+} pump was not inhibited.

Fluo-4 allows only a qualitative determination of the Ca^{2+} content. However, as we published recently, this dye can be used for Ca^{2+} measurements in intact RBCs, whereas fura-2 is not applicable [17].

The investigations on single human RBCs with an inhibited Ca^{2+} pump demonstrate a clear aldosterone effect. This effect is dependent on time and occurs at different aldosterone concentrations (1 μ M and 10 μ M) (Fig. 3B). One should note that each curve in Fig. 3B represents the average of measurements of 35 individual RBCs. Although the differences in the curves at the beginning of the experiments remain unclear, it seems evident that aldosterone over the 30 min measuring period leads to reduction of the Ca^{2+} content. This would mean that it reduces a Ca^{2+} uptake pathway which might be of importance for hypertension and/or heart failure. Since the addition of the Ca^{2+} channel blocker nifedipine prevents the increase of the intracellular Ca^{2+} content

(both in the absence and presence of aldosterone), it can be concluded that aldosterone affects Ca^{2+} channels. However, as long as the Ca^{2+} pump is not affected by aldosterone, it is difficult to imagine the physiological relevance of such an effect. Also for chicken RBCs a significant effect of aldosterone was found showing a decrease of the intracellular Ca^{2+} content under conditions where the Ca^{2+} pump is blocked (Fig. 4B). This result is even more complicated to interpret because most of the intracellular Ca^{2+} is located in the nucleus (Fig. 4A). Therefore, the transport of Ca^{2+} across the nucleus membrane has to be considered. For many cell types with a nucleus it was observed that an increase of the intracellular Ca^{2+} concentration occurs some minutes after aldosterone addition [34, 35]. This increase is due to the fact that Ca^{2+} is released from intracellular stores.

Single cell fluorescence measurements of the pH_i reveal a permanent alkalinisation of the control, i.e. in the presence of ethanol (Fig. 5). This curve represents the average of 45 cells from 3 different donors. For all investigated single cells an increase was found, which in average is about 0.1 pH units in 30 min. The pH_i at the beginning of the fluorescence measurements is about 7.3 and already 0.1 pH units higher than under conditions where no ethanol was added. It should be noticed that ethanol was added some minutes before the fluorescence measurements were started. This time was necessary to let the cells settle down to the cover slip surface (see Material and Methods). In the absence of ethanol the pH_i is about 7.2 and stable over 30 min. In the presence of aldosterone the progression of the curves changes completely. After a maximum pH_i is reached an acidification of about 0.1 pH units occurs for all 3 curves where aldosterone is present. The effect of acidification can be seen also in the presence of ethanol and amiloride, an inhibitor of the Na^+/H^+ exchanger, independent of the presence or absence of aldosterone (Fig. 6). Therefore, it seems that aldosterone inhibits the Na^+/H^+ exchanger in RBCs. The acidification of the cells should have an effect on other cellular parameters like cell volume and transmembrane potential. Such effects were not

investigated in the present publication. In addition, an acidification of the cells activates the K^+, Cl^- symporter (e.g. [36]) and inactivates the $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ symporter [37] in mammalian RBCs. Therefore, the aldosterone-induced reduction of the $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ symporter shown in Fig. 2 could be at least partly a result of such an effect.

For different cell types (vascular smooth muscle cells, human mono leucocytes, arterial cells, collecting duct cells and primary renal proximal tubule epithelial cells) an effect of aldosterone on the Na^+/H^+ exchanger was described [38–42]. In these cell types a fast activation of this transporter results in an increase of the intracellular Na^+ concentration and the pH_i (due to H^+ efflux). However, based on our results an effect of ethanol has to be taken into consideration.

The described effects of aldosterone on the Ca^{2+} content and the Na^+/H^+ exchanger (pH_i) might be inter-related. Our results fit to a finding demonstrating that intracellular Ca^{2+} activates the Na^+/H^+ exchanger in human RBCs [43]. Extracellular acidification inhibits this transport pathway [43]. This would explain the aldosterone-induced reduction of the Ca^{2+} content together with an acidification of the cells (Figs. 3B and 5). The extracellular pH can be assumed as constant during our measurements. The experiments were performed with single cells in a buffered external medium at a very low haematocrit (0.015 %). However, the pH_i measurements were carried out in the absence of extracellular Ca^{2+} . Therefore, a direct effect of aldosterone on the Na^+/H^+ exchanger seems more likely.

Summarising, significant effects of aldosterone on transport pathways in the RBC membrane or on the Ca^{2+} content and the pH_i , the following results were found: (i) Aldosterone inhibits the $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ symporter in young RBCs containing a higher fraction of reticulocytes than normal cells. (ii) Aldosterone affects the Ca^{2+} uptake in human and chicken RBCs under conditions where the Ca^{2+} pump is blocked by o-vanadate. The effect is nifedipine-sensitive. (iii) Aldosterone influences the pH_i of human RBCs resulting in an acidification compared to the control. It is due to an effect on the Na^+/H^+ exchanger.

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