Stimulation of Na^+/Mg^{2+} antiport in rat erythrocytes by intracellular Cl^-

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Abstract **Mg^{2+} efflux from rat erythrocytes was measured in NaCl, $NaNO_3$, NaSCN and Na gluconate medium. Substitution of extracellular and intracellular Cl^- with the permeant anions NO_3^- and SCN^- reduced Mg^{2+} efflux via Na^+/Mg^{2+} antiport. After substitution of extracellular Cl^- with the non-permeant anion gluconate, Mg^{2+} efflux was not significantly reduced. In Na gluconate medium, an influence of the changed membrane potential and intracellular pH on Mg^{2+} efflux could be excluded. The results indicate the existence of Cl^--independent Na^+/Mg^{2+} antiport and of Na^+/Mg^{2+} antiport stimulated by intracellular Cl^-. Intracellular Cl^-, as determined by means of $^{36}Cl^-$, was found to stimulate Na^+/Mg^{2+} antiport through a cooperative effect according to a sigmoidal kinetics. The Hill coefficient for intracellular Cl^- amounted to 1.4–1.8, indicating that two intracellular Cl^- may be simultaneously active. With respect to specificity, Cl^- was most effective, followed by Br^-, J^-, and F^-. Stimulation of Na^+/Mg^{2+} antiport by intracellular Cl^- together with intracellular Mg^{2+} may play a role during deoxygenation of erythrocytes and in essential hypertension.**

Key words: Na^+/Mg^{2+} antiport; $[Cl^-]_i$ stimulation; Deoxygenation; Essential hypertension; Rat erythrocyte

1. Introduction

In erythrocytes and other cell types, the concentration of intracellular free Mg^{2+} ($[Mg^{2+}]_i$) is about the same as the concentration of extracellular free Mg^{2+}. Taking into account the intracellular negative membrane potential, there must be a permanent transport of Mg^{2+} out of the cells. This Mg^{2+} transport is performed by Na^+/Mg^{2+} antiport. In erythrocytes, Na^+/Mg^{2+} antiport is electroneutral, driven by the extracellular–intracellular Na^+ gradient, allosterically activated by an increase of $[Mg^{2+}]_i$, and inhibited e.g. by amiloride [1,2]. Thus, Na^+/Mg^{2+} antiport has properties similar to Na^+/H^+ antiport [3,4].

Under isoosmotic conditions, the activity of the Na^+/H^+ antiporter of apical membranes of colonic crypt cells was dependent on extracellular Cl^- [5]. Under hyperosmotic conditions, the activation of Na^+/H^+ antiport was inhibited by removing extracellular Cl^-, as observed in dog erythrocytes [6,7], rabbit erythrocytes [8], mesangial cells [9] and barnacle muscle fibers [10]. Expression of the different isoforms of the Na^+/H^+ exchanger in Chinese hamster ovary cells deficient in the Na^+/H^+ exchanger revealed that, under isoosmotic conditions, intracellular Cl^- was critical for the activity of the major isoforms NHE1, NHE2 and NHE3 [11]. Also, the Na^+,K^+,Cl^- cotransporter in various cell types [12,13] and non-selective cation channels in human erythrocytes [14] and in fetal pneumocytes [15] were supposed to be regulated by intracellular Cl^-. These results indicate a more general role of intracellular Cl^- in ion transport processes. Therefore, we investigated whether Na^+/Mg^{2+} antiport could also be affected by intracellular Cl^-.

2. Materials and methods

2.1. Materials

Nembutal® (pentobarbital sodium) was obtained from Abbott (North Chicago, IL, USA), tetraphenylphosphonium chloride (TPP^+, as cation) and tetraphenylboron sodium (TPB^-, as anion) from Aldrich-Sigma (Deisenhofen, Germany). All other chemicals were purchased at the highest grade of purity available from Merck (Darmstadt, Germany). Filtered, de-ionized and virtually Mg^{2+}-free water with a resistance of 15–18 MΩ/cm was used for solutions.

2.2. Red blood cell preparation and incubation

Rat erythrocytes were prepared as described earlier [16]. Blood (6–8 ml) was always obtained from only one anesthetized (50 mg/kg Nembutal® i.p.) male Wistar rat weighing 350–450 g. The abdominal vein was catheterized with a heparinized syringe. The blood was transferred to several heparinized tubes, diluted 1:3–1:5 with NaCl medium, consisting of 150 mmol/l NaCl, 5 mmol/l D-glucose and 10 mmol/l HEPES–Tris, pH 7.4. The cell suspension was centrifuged at $1000\times g$ for 10 min. The plasma and buffy coat containing the white cells were aspirated and discarded. The sedimented red cells were washed twice in 10 ml NaCl medium. Finally, the red cells were resuspended and incubated with gentle shaking as a 10% (v/v) suspension in NaCl, $NaNO_3$, NaSCN or Na gluconate medium containing 150 mmol/l of each sodium salt, 5 mmol/l D-glucose and 10 mmol/l HEPES–Tris, pH 7.4.

To minimize hemolysis, the cells were handled with utmost caution at a temperature of 24°C. Hemolysis ranged between 0.5 and 1.5%, and when greater than 2%, the red cells were not used for the experiment. Paired experiments were always performed in the different media.

2.3. Mg^{2+} efflux

At the beginning and after 2 h of incubation, 1 ml aliquots of the cell suspensions were centrifuged at $1000\times g$ for 10 min. This relatively low speed was necessary to minimize hemolysis. To determine Mg^{2+}, the supernatant was diluted with trichloroacetic acid (TCA). The final concentration of TCA was 5% (w/v), containing 0.1% (w/v) La_2O_3 and 0.16% (v/v) HCl. Mg^{2+} was measured in triplicate by means of atomic absorption spectrometry (Perkin Elmer, 2380). Mg^{2+} efflux was calculated from the difference in the increase in extracellular Mg^{2+} concentration during the time interval, and was related

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to the original cell volume measured with hematocrit. Hematocrit and hemolysis were measured in each sample. Mg^{2+} efflux was corrected for hemolysis. For this purpose, TCA was added to the sedimented erythrocytes and Mg^{2+} in the TCA extract was measured as described above.

2.4. Determination of intracellular Cl^- concentration

The intracellular Cl^- concentration ($[Cl^-]_i$) was determined by equilibration of $^{36}Cl^-$ between incubation media and cells. $[Cl^-]_i$ was calculated from the specific activity of extracellular Cl^- and the measured intracellular $^{36}Cl^-$ activity.

For determination of $[Cl^-]_i$, rat erythrocytes were incubated in NaSCN medium in which NaSCN was gradually isoosmotically substituted with NaCl. The medium contained $^{36}Cl^-$ (Amersham Pharmacia Biotech, Amersham, UK, specific activity >110 MBq/g Cl^-). After incubation for 30 min at 24°C, 2×500 μl cell suspension was centrifuged at 15 800×*g* for 2 min. The supernatant was carefully removed. 600 μl of 0.1% Triton X-100 and 600 μl of 5% (w/v) TCA were added to the cell sediments or to 50 μl supernatants. After centrifugation, 1000 μl of the Triton–TCA extracts was added to 10 ml Ultima Gold cocktail, and after disappearance of chemoluminescence, ^{14}C activity was measured in a β counter.

To determine intracellular $^{36}Cl^-$ activity, $^{36}Cl^-$ activity trapped between the sedimented erythrocytes was subtracted. Therefore, in separate experiments, the volume of the trapped medium in the cell sediments was estimated under the same conditions by means of ^{14}C sucrose ([U-^{14}C]sucrose, Amersham Pharmacia Biotech, 23.8 GBq/mmol).

3. Results and discussion

3.1. Stimulation of Na^+/Mg^{2+} antiport by intracellular Cl^-

When NaCl of the incubation medium was substituted isoosmotically with $NaNO_3$ or NaSCN, Mg^{2+} efflux was drastically reduced. However, substitution of NaCl with Na gluconate led to no significant reduction of Mg^{2+} efflux (Fig. 1). After NaCl of the incubation medium was substituted with $NaNO_3$ and NaSCN, NO_3^- and SCN^- were rapidly transferred into the erythrocytes and replaced intracellular Cl^-. On the other hand, gluconate is a non-permeating anion [5,17,18] that does not replace intracellular Cl^-. Therefore, it can be concluded that intracellular Cl^- is required for Mg^{2+} efflux via Na^+/Mg^{2+} antiport.

It should be considered that substituting NaCl with Na gluconate will change the membrane potential from −8.5 mV to +50 mV and intracellular pH (pH_i) from pH_i 7.3 to 8.3, as measured earlier in human erythrocytes [17].

To exclude any effect of the membrane potential, this was changed by addition of the lipophilic cation TPP^+ and by the lipophilic anion TPB^-, which may alter the membrane potential to more positive (TPP^+) or more negative (TPB^-) values. The results are documented in Table 1. TPB^- increased Mg^{2+} efflux in both the NaCl medium and the Na gluconate medium. TPP^+ increased Mg^{2+} efflux only to a small degree in both media.

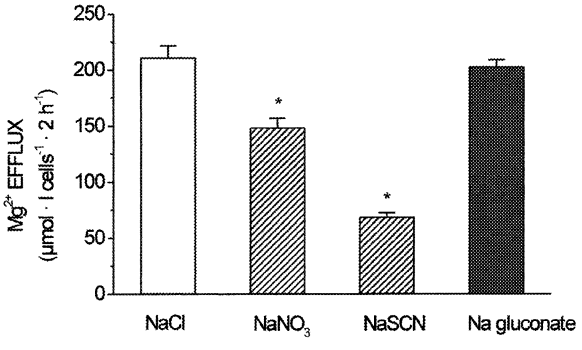

Fig. 1. Mg^{2+} efflux from rat erythrocytes incubated as a 10% (v/v) suspension in NaCl, $NaNO_3$, NaSCN or Na gluconate medium containing 150 mmol/l of each sodium salt, 5 mmol/l D-glucose and 10 mmol/l HEPES–Tris, pH 7.4. Means ± S.E.M., $n=6$, $^*P<0.05$ compared to incubation in NaCl medium.

Table 1
Effect of the charged lipophilic ions TPP^+ and TPB^-, each at 50 μmol/l, on Mg^{2+} efflux[a] from rat erythrocytes in NaCl or Na gluconate medium

Medium	Control	TPP^+	TPB^-
NaCl	229.9 ± 14.0[b]	267.0 ± 15.5*	326.4 ± 18.2*
Na gluconate	204.4 ± 17.6	221.1 ± 11.9	369.1 ± 29.8*

*$^P<0.05$ when compared to controls.
[a]Mg^{2+} efflux expressed as μmol/l cells/2 h.
[b]Values as means ± S.E.M. of six paired experiments.

Despite a completely different membrane potential of erythrocytes in NaCl medium and Na gluconate medium, TPP^+ and TPB^- increased Mg^{2+} efflux in both media. Thus, the different membrane potentials of erythrocytes in NaCl medium and Na gluconate medium had no significant effect on Mg^{2+} efflux through Na^+/Mg^{2+} antiport. This conclusion agrees with previous results, showing that Na^+/Mg^{2+} antiport in erythrocytes was electroneutral and thus independent of the membrane potential [19]. Also, in sublingual acini of rats electroneutral Na^+/Mg^{2+} antiport was independent of membrane potential [20].

The increase in Mg^{2+} efflux by TPB^- in NaCl and Na gluconate media may be explained by an interaction of TPB^- with Mg^{2+} and/or positively charged groups at the inner site of the cell membrane and/or at the inner site of the Na^+/Mg^{2+} antiporter. Another explanation is that the lipophilic TPB^- may act as an ionophore for Mg^{2+}, mediating Mg^{2+} efflux under our experimental conditions.

An effect of the increased pH_i in Na gluconate medium on Mg^{2+} efflux seems to be improbable because Mg^{2+} efflux in NaCl and in Na gluconate media were almost identical (Fig. 1, Table 1). Since an effect of the membrane potential on Mg^{2+} efflux has been excluded, it might also be expected that a different pH_i in Na gluconate medium cannot play a significant role in Na^+/Mg^{2+} antiport. Also, in hamster erythrocytes Na^+/Mg^{2+} antiport was not significantly reduced when at an extracellular pH of 7.4 in NaCl medium, the pH_i was increased from 7.0 to 7.7 [21].

Taken together, our experiments may be interpreted as indicating that intracellular Cl^- is required for Mg^{2+} efflux through Na^+/Mg^{2+} antiport.

In order to obtain a quantitative relationship between Cl^- and the activity of Na^+/Mg^{2+} antiport, we investigated the effect of increasing extracellular Cl^- concentrations ($[Cl^-]_o$) on Mg^{2+} efflux with graded isoosmotic substitution of NaSCN with NaCl. Substitution of NaSCN with NaCl was used because Mg^{2+} efflux in NaSCN medium was slower than Mg^{2+} efflux in media containing other tested anions (see Fig. 1).

As shown in Fig. 2, an increase in $[Cl^-]_o$ stimulated Mg^{2+} efflux due to a sigmoidal kinetics, indicating a cooperative or allosteric effect and thus a regulatory function of Cl^-. There was a remaining Mg^{2+} efflux at zero $[Cl^-]_o$. This may represent a Cl^--independent activity of Na^+/Mg^{2+} antiport or Mg^{2+} efflux through another pathway. Plotting the data of

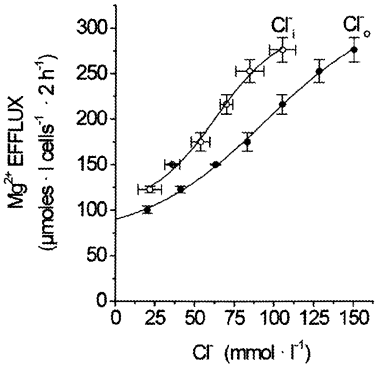

Fig. 2. Mg^{2+} efflux from rat erythrocytes as a function of $[Cl^-]_o$ and $[Cl^-]_i$. The cells were incubated in NaSCN medium in which NaSCN was gradually isoosmotically substituted with NaCl as indicated. Means ± S.E.M. of five experiments.

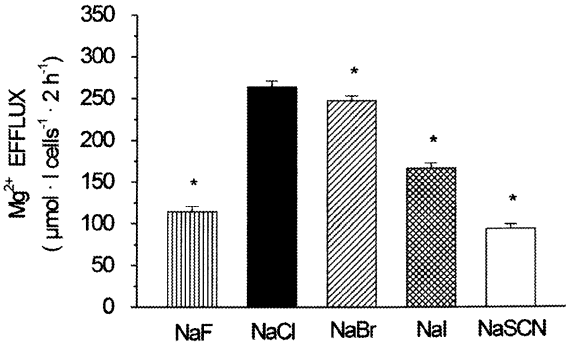

Fig. 4. Mg^{2+} efflux from rat erythrocytes incubated in NaF, NaCl, NaBr, NaJ, and NaSCN media, containing 150 mmol/l of each salt, 5 mmol/l D-glucose and 10 mmol/l HEPES–Tris, pH 7.4. Means ± S.E.M., $n = 4$, $^*P < 0.05$ compared to incubation in NaCl medium.

Fig. 2 for $[Cl^-]_o$ according to Lineweaver–Burk or Eadie–Hofstee did not yield linear curves (not shown), thus excluding Michaelis–Menten kinetics. These results agree with the sigmoidal curve in Fig. 2. Also, in rat mesangial cells, shrinkage-induced Na^+/H^+ exchange depended in a sigmoidal fashion on $[Cl^-]_o$ [9] and thus probably on $[Cl^-]_i$, which was not measured by the authors [9]. In Fig. 2, therefore, Mg^{2+} efflux was also plotted as a function of $[Cl^-]_i$. A sigmoidal kinetics of Mg^{2+} efflux was found again for $[Cl^-]_i$.

Fig. 2 also contains the $[Cl^-]_i$ values at the corresponding $[Cl^-]_o$ values. The curve and the values for $[Cl^-]_i$ are shifted to lower values compared to $[Cl^-]_o$, which accords with the Donnan factor for Cl^- in erythrocytes [22,23].

The values for Mg^{2+} efflux at the various $[Cl^-]_i$ of Fig. 2 were further plotted according to Hill ($\log v/V_{max} - v = n \log S - \log K$). To analyze Cl^- stimulation of Na^+/Mg^{2+} antiport, Mg^{2+} efflux at zero $[Cl^-]_o$ amounting to 90 µmol/l cells/2 h (see Fig. 2) was subtracted from V_{max} and the v values of Fig. 2. V_{max} cannot be measured directly and must be obtained by extrapolation. Obviously, there is uncertainty in extrapolating V_{max}. Therefore, the Hill plot was constructed with a V_{max} value of 450 and also of 600 µmol/l cells/2 h. The resulting curves are shown in Fig. 3. From the slopes in Fig. 3, Hill coefficients of $n = 1.5$ and of $n = 1.4$ were obtained (curve A and curve B in Fig. 3). Thus, using the higher V_{max} value resulted in a somewhat lower value for n. When, for Mg^{2+} efflux at zero $[Cl^-]_i$, 100 µmol/l cells/2 h instead of 90 µmol/l cells/2 h was set in calculation of n, higher values with $n = 1.8$ and $n = 1.6$ (not shown) were obtained. Taken together, Hill coefficients between 1.4 and 1.8 indicate that two intracellular Cl^- may be involved in the stimulation of Mg^{2+} efflux.

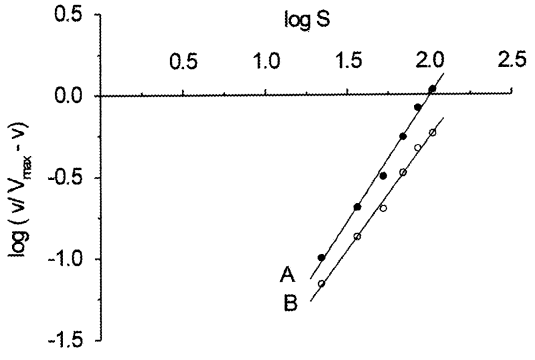

Fig. 3. Hill plot ($\log v/V_{max} - v = n \log S - \log K$) of Mg^{2+} efflux from rat erythrocytes at various Cl^- concentrations (S). Values for v were taken from Fig. 2, V_{max} was extrapolated from Fig. 2. Because of the uncertainty of this extrapolation, V_{max} values of 450 µmol/l cells/2 h and 600 µmol/l cells/2 h were used. Mg^{2+} efflux at zero $[Cl^-]_o$ was subtracted from V_{max} and the v values. Curve A: Hill plot as a function of $[Cl^-]_i$ with $V_{max} = 450$ µmol/l cells/2 h. Curve B: Hill plot as a function of $[Cl^-]_i$ with $V_{max} = 600$ µmol/l cells/2 h.

Next, we investigated whether the activation of Na^+/Mg^{2+} antiport by intracellular Cl^- is specific for Cl^-. As shown in Fig. 4, among the halides activation of Na^+/Mg^{2+} antiport by Cl^- was most effective, followed by Br^-, J^-, and F^-, indicating a physiological role of Cl^- in stimulating Mg^{2+} efflux. Also, shrinkage-induced Na^+/H^+ exchange in rat mesangial cells expressed a similar sequence: $Cl^- > Br^- > J^- > SCN^- > F^-$ [9]. The rather small effect of F^- compared to SCN^- may be due to the formation of poorly water-soluble MgF_2 (solubility product $K_{sp} = [Mg^{2+}] \times [F^-]^2 = 5 \times 10^{-8}$ $(mol/l)^3$ [24]. Moreover, F^- inhibits glycolysis (enolase) and thus reduces the ATP concentration in anuclear erythrocytes. Also, an effect of F^- on GTP binding proteins may be involved.

Whether the stimulation of Na^+/Mg^{2+} antiport by intracellular Cl^- is restricted to erythrocytes remains open. Also, in some other cell types in which Na^+/Mg^{2+} antiport has so far not been measured, $[Cl^-]_i$ is rather high and could be changed. In fetal pneumocytes, $[Cl^-]_i$ was experimentally reduced from 50 mmol/l to 20 mmol/l [15]. In endothelial cells, $[Cl^-]_i$ was changed by 25% [12]. In neurons of the suprachiasmatic nucleus, $[Cl^-]_i$ was shown to vary in a circadian rhythm from 20 mmol/l to 6 mmol/l [25]. Also, in erythrocytes and other cell types, activation of protein kinase A and of protein kinase C could increase Cl^- efflux and reduce $[Cl^-]_i$ [26]. Furthermore, in cell types other than erythrocytes there may exist isoforms of the Na^+/Mg^{2+} antiporter which may express a different $[Cl^-]_i$ dependence. This latter aspect has not been investigated to date.

3.2. Possible mechanism of action of Cl^- on Na^+/Mg^{2+} antiport

The mechanism of action of intracellular Cl^- remains undefined. An effect of an altered membrane potential can be excluded, as discussed above. Since the effect of Cl^- was studied by substitution with SCN^-, one may think of a non-specific effect of the two anions on Na^+/Mg^{2+} antiport

according to their position in the lyotropic Hofmeister series: $SO_4^{2-} > Cl^- > Br^- > NO_3^- > J^- > SCN^-$. This effect may contribute to the reduction of Mg^{2+} efflux by Br^-, NO_3^-, J^- and SCN^-. However, the sigmoidal kinetics and the Hill coefficient of 1.4–1.8 may indicate that two intracellular Cl^- may affect the Na^+/Mg^{2+} antiporter. According to the proposed mechanism of Na^+/Mg^{2+} antiport [27], intracellular Cl^- may alter the affinity of the Na^+/Mg^{2+} antiporter to intracellular Mg^{2+} either at the transport site or at an allosteric modifier site. Similarly, in experiments with truncated mutants of NHE1 [11], the action of Cl^- was localized at the cytoplasmic COOH-terminal domain of the antiporter and it has been discussed that intracellular Cl^- may change the sensitivity of the Na^+/H^+ antiporter to H^+ by directly binding of Cl^- to the COOH-terminal region of the antiporter or to an ancillary protein that in turn interacts with the cytosolic aspect of the antiporter [11].

It may be at least possible that a Cl^--dependent protein kinase [28] is involved in the stimulation of Na^+/Mg^{2+} antiport by intracellular Cl^-. The mechanism of action of Cl^- on Na^+/Mg^{2+} antiport must be defined in future experiments.

3.3. Possible biological implications

Modulation of Na^+/Mg^{2+} antiport by an alteration of $[Cl^-]_i$ may play a role in the gas exchange of erythrocytes. During deoxygenation, $[Cl^-]_i$ increases through exchange with HCO_3^- (chloride shift or Hamburger shift), which may amount to 10% of the total Cl^- concentration of erythrocytes [22]. However, intracellular Cl^- and other ions are compartmentalized [29,30]. Therefore, the Cl^- concentration at the Cl^- binding sites of the Na^+/Mg^{2+} antiporter and the alterations of $[Cl^-]_i$ at these sites during deoxygenation are not exactly known.

In addition, there may be a synergistic effect of intracellular Cl^- and intracellular Mg^{2+} on the activity of Na^+/Mg^{2+} antiport. During deoxygenation, the concentration of intracellular free Mg^{2+} in erythrocytes was reported to increase from 0.39 mmol/l to 0.54–0.62 mmol/l [31], or by other methods, from 0.25 mmol/l to 0.67 mmol/l [32], and from 0.38 mmol/l to 0.69 mmol/l [33]. This increase in intracellular free Mg^{2+} concentration is mainly due to the increased binding of 2,3-bisphosphoglycerate (2,3-BPG) to deoxyhemoglobin combined with the liberation of Mg^{2+} that was bound in the Mg–2,3-BPG complex [34]. The increase in intracellular free Mg^{2+} incites tyrosine phosphorylation of band 3 protein [33], which leads to a strengthening of its association with the spectrin network and to a decrease in cell membrane fluctuations [33]. The increase in intracellular Mg^{2+} concentration during deoxygenation may additionally increase the activity of Na^+/Mg^{2+} antiport.

In preceding experiments with erythrocytes, Na^+/Mg^{2+} antiport was drastically increased [3,16,27,35] by an allosteric effect [3] when intracellular Mg^{2+} was increased by Mg^{2+} loading. The increased concentration of intracellular Cl^- and intracellular free Mg^{2+} during deoxygenation may enhance the activity of Na^+/Mg^{2+} antiport and Mg^{2+} efflux, leading to less periodic alterations of the intracellular free Mg^{2+} concentration and thus to a smoothing of alterations in band 3 phosphorylation and cell membrane fluctuations.

Also, in essential hypertension there may be a relationship between intracellular Cl^- and Na^+/Mg^{2+} antiport. In essential hypertension, $[Cl^-]_i$ in erythrocytes was found to be reduced from 84.4 mmol/l to 70.6 mmol/l [36]. On the other hand, in 45% of patients with essential hypertension, Na^+/Mg^{2+} antiport was increased [37,38]. In these studies, Na^+/Mg^{2+} antiport was measured in Mg^{2+}-loaded erythrocytes, that is under nearly V_{max} conditions of Na^+/Mg^{2+} antiport. This may indicate an increased number of Na^+/Mg^{2+} antiporters in the erythrocytes of these patients. The reduced $[Cl^-]_i$ of erythrocytes in essential hypertension may represent a compensatory mechanism to reduce the activity of Na^+/Mg^{2+} antiport in order to preserve $[Mg^{2+}]_i$. This may explain that in erythrocytes of patients with essential hypertension, no significant alteration of $[Mg^{2+}]_i$ in erythrocytes was found [39]. However, a reduced $[Mg^{2+}]_i$ was found in erythrocytes of other patients with essential hypertension [40] and spontaneously hypertensive rats [41]. For review of the role of Mg^{2+} in hypertension see [42]. A different interaction of intracellular Cl^- and intracellular Mg^{2+} with the Na^+/Mg^{2+} antiporter due to different properties of the Na^+/Mg^{2+} antiporter may be responsible for these as yet not explained differences. The biological significance of the alterations in intracellular Cl^-, intracellular Mg^{2+} and Na^+/Mg^{2+} antiport in erythrocytes and other cell types in vivo remains to be defined in future experiments.

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