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Biochimica et Biophysica Acta 1559 (2002) 135–144

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Role of the choline exchanger in Na^+-independent Mg^{2+} efflux from rat erythrocytes

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Received 22 August 2001; received in revised form 7 November 2001; accepted 22 November 2001

Abstract

Two types of Na^+-independent Mg^{2+} efflux exist in erythrocytes: (1) Mg^{2+} efflux in sucrose medium and (2) Mg^{2+} efflux in high Cl^- media such as KCl-, LiCl- or choline Cl-medium. The mechanism of Na^+-independent Mg^{2+} efflux in choline Cl medium was investigated in this study. Non-selective transport by the following transport mechanisms has been excluded: K^+,Cl^-- and Na^+,K^+,Cl^--symport, Na^+/H^+-, Na^+/Mg^{2+}-, Na^+/Ca^{2+}- and $K^+(Na^+)/H^+$ antiport, Ca^{2+}-activated K^+ channel and Mg^{2+} leak flux. We suggest that, in choline Cl medium, Na^+-independent Mg^{2+} efflux can be performed by non-selective transport via the choline exchanger. This was supported through inhibition of Mg^{2+} efflux by hemicholinum-3 (HC-3), dodecyltrimethylammonium bromide (DoTMA) and cinchona alkaloids, which are inhibitors of the choline exchanger. Increasing concentrations of HC-3 inhibited the efflux of choline and efflux of Mg^{2+} to the same degree. The K_d value for inhibition of [^{14}C]choline efflux and for inhibition of Mg^{2+} efflux by HC-3 were the same within the experimental error. Inhibition of choline efflux and of Mg^{2+} efflux in choline medium occurred as follows: quinine > cinchonine > HC-3 > DoTMA. Mg^{2+} efflux was reduced to the same degree by these inhibitors as was the [^{14}C]choline efflux.

Keywords: Na^+-independent Mg^{2+} efflux; Choline exchanger; Hemicholinum-3; Cinchona alkaloids; Rat erythrocytes

1. Introduction

Previously we have shown that Mg^{2+}-loaded and non-Mg^{2+}-loaded erythrocytes incubated in sucrose medium showed a considerable rate of Na^+-independent Mg^{2+} efflux [1–3]. This Na^+-independent Mg^{2+} efflux could be inhibited by DIDS, SITS or by isosmotic substitution of sucrose with NaCl, KCl, LiCl or choline Cl [1,3], indicating that extracellular Cl^- was responsible for inhibition. Inhibition through extracellular Cl^- and DIDS was abolished by the lipid soluble cation TPP^+ [2]. To explain the effects of DIDS, SITS and TPP^+, it was concluded that in sucrose medium the Na^+-independent efflux of Mg^{2+} was accompanied by net efflux of intracellular Cl^- via capnophorine (band 3 protein) for charge compensation. Inhibition through extracellular Cl^- has been assumed to be produced by reduction of net Cl^- efflux due to the dissipation of the

Abbreviations: DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DoTMA, dodecyltrimethylammonium bromide; HC-3, hemicholinum-3; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; TCA, trichloroacetic acid; TPP^+, tetraphenylphosphonium cation

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[1] This study constitutes part of his thesis.

0005-2736/02/$ – see front matter
PII: S0005-2736(01)00445-X

intracellular–extracellular Cl^- gradient and/or membrane potential [2,3].

However, when erythrocytes were suspended in KCl- or choline Cl-medium, there was a residual Mg^{2+} efflux that was not inhibited by DIDS and SITS [1–3]. To explain this residual second type of Na^+-independent Mg^{2+} efflux, non-selective transport by the following mechanisms may be considered: K^+,Cl^- symport, Na^+,K^+,Cl^- symport, Na^+/Mg^{2+} antiport, Na^+/H^+ antiport, Na^+/Ca^{2+} antiport, Ca^{2+}-activated K^+ channel, K^+(Na^+)/H^+ antiport, Mg^{2+} leak efflux and finally choline/Mg^{2+} antiport. In the present study we investigated whether one of these mechanisms may mediate Mg^{2+} efflux in Na^+-free high Cl^- medium.

Erythrocytes are known to contain a choline exchanger, which, in addition to transporting choline, is also able to transport other cations such as Cs^+, Rb^+, K^+, Li^+, Na^+ and is characterized by a unique affinity to Mg^{2+} [4]. Thus, the choline exchanger of erythrocytes is a rather unspecific transporter, also mediating the uptake of catecholamines [5]. We therefore also investigated whether the choline exchanger may perform choline/Mg^{2+} antiport.

The experiments were conducted with non Mg^{2+}-loaded rat erythrocytes, which have been shown by us to exhibit a significant Na^+-dependent and Na^+-independent Mg^{2+} efflux [3] so that Mg^{2+}-loading could be avoided; this technique has been used to obtain a measurable rate of Mg^{2+} efflux in other cell types [6].

2. Materials and methods

2.1. Materials

Nembutal (pentobarbital-sodium), 50 mg ml^{-1}, was from Abott, North Chicago, IL, USA; amiloride hydrochloride, bumetanide, choline chloride, clotrimazole, cinchonine, cinchonidine, DoTMA, furosemide, HC-3, quinine and quinidine were from Sigma, Deisenhofen, Germany. Nitrendipine was from Tocris, Biotrend Chemikalien, Cologne, Germany. [*methyl*-^{14}C]Choline-HCl, specific activity 2.04 GBq/mmol, was from Amersham Pharmacia Biotech, Little Chalfont, UK. All other chemicals were purchased at the highest grade of purity available from Merck, Darmstadt, Germany. Filtered, deionized and virtually Mg^{2+}-free water with a resistance of 15–18 MΩ/cm was used for solutions.

2.2. Red cell preparation and incubation

Red cells were prepared as described earlier [3]. Blood (6–8 ml) was always obtained from one anesthetized (50 mg kg^{-1} Nembutal i.p.) male Wistar rat weighing 350–450 g, whereby the abdominal vein was catheterized with a heparinized syringe. The blood was transferred to heparinized tubes, diluted 1:3–1:5 with 150 mmol l^{-1} NaCl containing 5 mmol l^{-1} D-glucose and 10 mmol l^{-1} HEPES–Tris, pH 7.4 (NaCl medium). The cell suspension was centrifuged at $1000\times g$ for 10 min at 20°C. The plasma and buffy coat containing the white cells were aspirated and discarded. The red cell pellets were washed twice at 20°C in 10 ml NaCl medium. The pellets were resuspended and incubated with gentle shaking as a 10% (v/v) suspension in NaCl medium or choline Cl medium containing 150 mmol l^{-1} choline Cl, 5 mmol l^{-1} D-glucose and 10 mmol l^{-1} HEPES–Tris, pH 7.4.

To prevent hemolysis, the cells were handled with utmost caution. Normally, hemolysis ranged between 0.5% and 1.5%. Where hemolysis was greater than 2%, the red cells were not used for the experiment. While investigating the effect of various substances, paired experiments were always performed in the different media.

2.3. Mg^{2+} efflux

At the beginning of incubation and at various intervals, 1-ml aliquots of the cell suspensions were centrifuged at $1000\times g$ for 10 min. This relatively low speed was used to minimize hemolysis. To determine Mg^{2+}, the supernatant was diluted with 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 atomic absorption spectrometry (Perkin Elmer, 2380). Mg^{2+} efflux was calculated from the difference in the increase of extracellular Mg^{2+} concentration during the time intervals and was related to the original cell volume measured by hematocrit. Hematocrit and hemolysis were measured in each sample.

2.4. [^{14}C]Choline fluxes

2.4.1. [^{14}C]Choline influx

The unidirectional influx of [^{14}C]choline was determined as described previously [7]. Briefly, the red cells were washed (three times) in NaCl medium. Then, the cells were resuspended (hematocrit 10%) in NaCl medium containing [^{14}C]choline. Final activity was 18.5 kBq ml^{-1} (0.5 μCi ml^{-1}) in the concentration range of 5–100 μmol l^{-1} choline and 37 kBq ml^{-1} (1 μCi ml^{-1}) for the higher choline concentrations up to 5 mmol l^{-1}. Furthermore, at choline Cl concentrations greater than 50 μmol l^{-1}, the NaCl concentration was adequately lowered so that total NaCl and choline Cl was 150 mmol l^{-1}.

Duplicate experiments were carried out in a final sample volume of 1.2 ml at room temperature. After 3 h, choline influx was terminated by transferring 0.4-ml aliquots of the suspensions to microcentrifuge tubes containing 0.8 ml dibutylphthalate. The cells were sedimented beneath the oil by centrifugation (15 000×g for 1 min). The aqueous supernatant was removed by aspiration and the radioactivity remaining on the walls was removed by washing (four times) with water. Dibutylphthalate was aspirated. The cell pellet was lysed for at least 15 min in 600 μl Triton X-100 (0.1% v/v) and precipitated through the addition of 600 μl TCA (5% w/v %) and subsequent centrifugation (15 000×g for min). The ^{14}C-activity of 1.1 ml of the lysate was measured in a β-scintillation counter. The influx rate was expressed in μmol choline l cells^{-1} h^{-1} and corrected for extracellular space in the cell pellet, the latter being estimated from the amount of [^{14}C]choline in pellet samples taken within a few seconds after addition of [^{14}C]choline.

In preliminary experiments (not shown) it was ensured that the time course of choline uptake was linear over a period of up to 4 h. This was established for 0.1 mmol l^{-1} and 5 mmol l^{-1} choline. Therefore, the influx rates determined in later experiments fell within the linear period.

2.4.2. [^{14}C]Choline efflux

[^{14}C]Choline efflux was measured after loading the erythrocytes at room temperature with 10 mmol l^{-1} ^{14}C-labeled choline Cl (final activity 74 kBq ml^{-1} = 2 μCi ml^{-1}) for 20–24 h. Also glucose was added, final concentration 20 mmol l^{-1}. Next day, when a steady state distribution of choline could be expected [4], the loaded erythrocytes were washed (three times) at 1000×g in 5 ml of a solution of 10 mmol l^{-1} unlabeled choline Cl, 140 mmol l^{-1} NaCl and 20 mmol l^{-1} HEPES–Tris, pH 7.4. Finally, the red cells were suspended (hematocrit 10% v/v) in choline Cl medium. After 2 h, efflux was terminated by transferring 0.5-ml aliquots to microcentrifuge tubes containing 700 μl dibutylphthalate and centrifuging at 15 000×g for 1 min. The subsequent procedure was the same as that described for [^{14}C]choline influx.

2.5. Statistical analysis

Data were expressed as mean values ± S.E.M. and statistical differences were determined by Student's paired and two-tailed t-test. A value of $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Exclusion of K^+,Cl^-- and Na^+,K^+,Cl^--symport

Erythrocytes can perform K^+,Cl^-- and Na^+,K^+,Cl^--symport. A role played by these transporters in Mg^{2+} efflux could be excluded for the following reasons:

K^+,Cl^- symport is activated by hypoosmotic swelling and Na^+,K^+,Cl^--symport is activated by hypertonic shrinkage [8,9]. Our experiments were performed under isoosmotic conditions. Therefore, these transporters would not be active.

K^+,Cl^-- and Na^+,K^+,Cl^--symport are inhibited by furosemide and bumetanide [8,10]. In preceding experiments with non Mg^{2+}-loaded rat erythrocytes, 0.1 mmol l^{-1} furosemide had no effect on Mg^{2+} efflux in NaCl and choline Cl medium [3]. In this study (Table 1), we found that even the high concentration of 1 mmol l^{-1} furosemide did not affect Mg^{2+} efflux in choline Cl medium. Moreover, 0.01 mmol l^{-1} and 0.1 mmol l^{-1} bumetanide, which is a more potent inhibitor of K^+,Cl^-- and Na^+,K^+,Cl^--symport [8,10], also had no effect on Mg^{2+} efflux in choline medium (Table 1).

K^+,Cl^-- and Na^+,K^+,Cl^--symport are specifically dependent on Cl^- [8]. After substituting Cl^- with

Table 1
Effect of various ion transport inhibitors on Mg^{2+} efflux of rat erythrocytes

Inhibitor	Concentration (mmol l^{-1})	Mg^{2+} efflux (μmol l cells^{-1} 2 h^{-1})	% of control
Control		143.3 ± 8.7	100
Amiloride	0.5	54.5 ± 3.9*	38
Furosemide	1.0	149.3 ± 23.2	104
Bumetanide	0.01	153.6 ± 7.3	107
Bumetanide	0.1	140.3 ± 7.4	98
Niflumic acid	0.1	151.6 ± 10.3	106
Nitrendipine	0.01	157.8 ± 8.6	110
Clotrimazole	0.01	170.7 ± 12.9	119

A 10% (v/v) cell suspension was incubated in choline Cl^- medium for 2 h. Means ± S.E.M., $n = 4–6$. *$P < 0.05$.

NO_3^-, SCN^-, Mg^{2+} efflux was not abolished (not shown).

In agreement with the Cl^- independence of Na^+-independent Mg^{2+} efflux in high Cl^- medium, 0.1 mmol l^{-1} niflumic acid (an inhibitor of Ca^{2+}-activated Cl^- channel [11] and of Cl^- transport through band 3 protein [12]) did not affect Mg^{2+} efflux in choline Cl medium (Table 1).

3.2. Exclusion of Na^+/H^+ antiport

Next, we considered whether Mg^{2+} efflux in choline Cl^- medium might operate via non-selective transport by the Na^+/H^+ antiporter. Na^+/H^+ antiport is inhibited by amiloride [13]. In non-Mg^{2+}-loaded rat erythrocytes, 0.5 mmol l^{-1} amiloride inhibited Mg^{2+} efflux in NaCl medium by 25% and in choline medium by 62% ([3] and Table 1). Since inhibition of Na^+/H^+ antiport occurs at 10 times lower amiloride concentrations, which do not effect Mg^{2+} efflux [14], Na^+/H^+ antiport cannot be involved in Mg^{2+} efflux. Moreover, at physiological intracellular pH, Na^+/H^+ antiport is practically quiescent and is activated by intracellular acidification due to the binding of H^+ to a modifier site [15]. Finally, it was reported that Na+/H+ antiport is absent in rat erythrocytes [16].

3.3. Exclusion of Na^+/Mg^{2+} antiport

Na^+/Mg^{2+} antiport is inhibited by amiloride and quinidine [3,17]. As mentioned in Section 3.2, in non-Mg^{2+}-loaded rat erythrocytes, amiloride inhibited Mg^{2+} efflux in NaCl medium and in choline Cl medium ([3] and Table 1). In addition, we found that the cinchona alkaloids quinine and quinidine inhibited Mg^{2+} efflux both in NaCl medium and in choline Cl medium (Table 2). From these results it cannot be excluded that Na^+-independent Mg^{2+} efflux may operate via Na^+/Mg^{2+} antiport. However, in preceding experiments we found that Na^+/Mg^{2+} antiport is specific for Na^+, not operating for example with K^+ or Li^+ instead of Na^+ [18,19]. As also shown in Table 2, at 1.2 mmol l^{-1} other cinchona alkaloids such as cinchonine and cinchonidine inhibited Mg^{2+} efflux only in choline Cl medium but not in NaCl medium. Taken together, both findings are strong arguments that Mg^{2+} efflux in choline Cl medium is not performed by non-selective transport via Na^+/Mg^{2+} antiport.

3.4. Exclusion of Na^+/Ca^{2+} antiport

In both this study (Table 1) as well as a previous one [3] we demonstrated inhibition of Mg^{2+} efflux in choline medium by amiloride. At the same concentration, amiloride can inhibit Na^+/Ca^{2+} antiport [14]. This may indicate Mg^{2+} efflux by Na^+/Ca^{2+} antiport. However, it was found that Na^+/Ca^{2+} antiport was competitively inhibited by Mg^{2+} that was not transported by the Na^+/Ca^{2+} antiporter [20]. This finding is in contrast to a recent study showing that the isoforms NCX1 and NCX3 of the Na^+/Ca^{2+} antiporter may perform Mg^{2+} influx [21].

In experiments with Mg^{2+}-loaded chicken and human erythrocytes, additional loading with Ca^{2+} did not inhibit Mg^{2+} efflux via Na^+/Mg^{2+} antiport [18,22], indicating that Ca^{2+} did not interfere with the Na^+/Mg^{2+} antiporter in these types of erythrocytes.

To date, Na^+/Ca^{2+} antiport in erythrocytes has only been described in dog and ferret. In all other erythrocytes, Na^+/Ca^{2+} antiport appears to be absent [23–26]. Thus, in rat erythrocytes the exchange of intracellular Mg^{2+} for extracellular Ca^{2+} or Na^+ by Na^+/Ca^{2+} antiport cannot be expected. Moreover, our experiments were performed in nominally Na^+- and Ca^{2+}-free medium. Also, Na^+/Ca^{2+} antiport is Na^+-specific and not active with Li^+,K^+ and choline [20]. Therefore, Na^+/Ca^{2+} antiporter could not operate as an Mg^{2+} transporter in rat erythrocytes under our experimental conditions.

3.5. Exclusion of Ca^{2+}-activated K^+ channel

Another candidate for Mg^{2+} transport might be the Ca^{2+}-activated K^+ channel (Gardos channel). We tested the effect of 10 μmol l^{-1} nitrendipine and clotrimazole, which are inhibitors of the Ca^{2+}-activated K^+ channel [27–29]. Both substances were without effect on Mg^{2+} efflux in choline Cl medium (Table 1). We therefore exclude the Ca^{2+}-activated K^+ channel as a Mg^{2+} transport mechanism. This assumption is supported by the fact that this channel is only operating when the intracellular Ca^{2+} concentration is elevated, which was not the case under our conditions.

3.6. Exclusion of $K^+(Na^+)/H^+$ exchanger

Finally it should be noted that a $K^+(Na^+)/H^+$ exchanger as discussed by Kummerow et al. [30] may also be excluded, since this exchanger is only operating in erythrocytes incubated in low-ionic-strength medium, e.g., sucrose medium.

3.7. Exclusion of Mg^{2+} leak flux

As shown below (Tables 2 and 3, Figs. 1–3), leak efflux as the main mechanism for Na^+-independent Mg^{2+} efflux in high Cl^- medium can also be excluded, since Mg^{2+} efflux in choline Cl^- medium could be inhibited by various inhibitors such as HC-3, DoTMA and some cinchona alkaloids. Since Mg^+ efflux was not completely abolished by these inhibitors under the conditions used, some residual Mg^{2+} leak efflux may be possible (see below, Section 3.8.4).

3.8. Choline/Mg^{2+} antiport

3.8.1. Effect of choline transport inhibitors on Mg^{2+} efflux

Since amiloride and quinidine are not specific inhibitors of Na^+/Mg^{2+} antiport, and since the Na^+-independent Mg^{2+} efflux occurred in choline Cl medium, we investigated whether the choline antiporter can mediate Mg^{2+} efflux.

Erythrocytes (and other cell types) possess (a) transport system(s) for choline. Human erythrocytes have been shown to exhibit a high-affinity, low-capacity choline transporter, which can exchange intracellular or extracellular choline for Cs^+, Rb^+, K^+, Li^+, Na^+ [4] and a low-affinity, high-capacity choline transporter, which is induced by malaria infection [7,31]. To our knowledge, choline transport in rat erythrocytes has not as yet been investigated. We therefore investigated whether these cells also express a choline exchanger.

First, the effect of choline transport inhibitors on Mg^{2+} efflux was tested. Fig. 1, which is analogous in form with the Lineweaver–Burk curve, shows the dose-dependent inhibition of Mg^{2+} efflux in choline Cl medium by HC-3, which is a specific competitive inhibitor of choline transport [32]. Calculation of the K_d value (according to [33]) delivered a value of 4.6 ± 0.4 mmol l^{-1} (mean ± S.E.M., $n = 5$).

Another inhibitor of choline transport in erythrocytes of different species is DoTMA [32,34]. In analogy to Fig. 1, again a dose-dependent inhibition of Mg^{2+} efflux in choline Cl medium could be produced. The K_d value of DoTMA was 0.19 ± 0.03 mmol l^{-1} (mean ± S.E.M., $n = 6$). At concentrations equal to or greater than 0.5 mmol l^{-1} DoTMA, hemolysis was larger than 5% so that the dose–effect relationship could be only investigated up to 0.35 mmol l^{-1} of DoTMA. In rat erythrocytes, DoTMA was a much less potent inhibitor of Mg^{2+} efflux than HC-3. The weaker effect of DoTMA in comparison with HC-3 has also been found for choline transport in erythrocytes of other species [32,34].

Several cinchona alkaloids have been described as other potent inhibitors of choline transport in human erythrocyte [31]. We therefore tested the effect of cinchonine on Mg^{2+} efflux. Cinchonine also produced a dose-dependent inhibition of Mg^{2+} efflux in choline Cl medium. The K_d value for inhibition

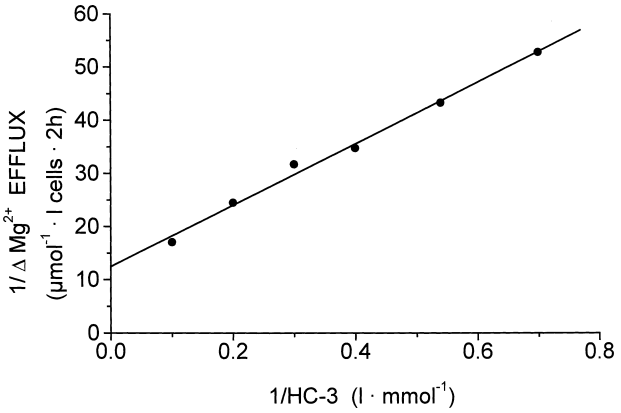

Fig. 1. Double reciprocal plot of the inhibition of Mg^{2+} efflux of rat erythrocytes in choline Cl medium at various concentrations of HC-3. The regression line was calculated using the least-square method. The K_d value was calculated according to [33]. Mean values of five experiments.

of Mg^{2+} efflux in choline Cl medium has been calculated to be 1.28 ± 0.07 mmol l^{-1} (mean ± S.E.M., $n = 4$). It should be mentioned that quinine, quinidine and cinchonidine also proved to be effective inhibitors of Mg^{2+} efflux in choline Cl medium (Table 2).

To provide evidence for the specificity of the inhibitory effects of HC-3, DoTMA and cinchonine on Mg^{2+} efflux in choline Cl medium, the effect of these inhibitors at the concentration of the K_d values has also been measured in NaCl medium. Table 2 shows that HC-3 and DoTMA did not inhibit but rather stimulated Mg^{2+} efflux in NaCl medium. Quinine and quinidine inhibited Mg^{2+} efflux both in choline Cl and NaCl medium. Most interestingly, at a concentration of 1.2 mmol l^{-1} the related drugs cinchonine and cinchonidine inhibited Mg^{2+} efflux in choline Cl medium by approximately 50% but had no effect in NaCl medium. Higher doses of cinchonine stimulated Mg^{2+} efflux in NaCl medium but inhibited Mg^{2+} efflux in choline Cl medium to a higher degree (data not shown).

Since the choline transporter also has an affinity to K^+ [4], the K_d values for the inhibition of Mg^{2+} efflux by the choline transport inhibitors have been determined in KCl medium. We found that the K_d values for HC-3, DoTMA and cinchonine were almost the same as in choline Cl medium ($n = 4$, results not shown). This fits well with the properties of the choline transporter and lends further support for a function of the choline transporter in Na^+-independent Mg^{2+} efflux.

Through the help of inhibitors we have thus shown that cinchonine and cinchonidine, which are inhibitors of choline transport inhibit Mg^{2+} efflux only in choline Cl medium but not in NaCl medium. This finding gives support to a Mg^{2+}/choline antiport mechanism and encouraged us to more directly investigate choline fluxes in rat erythrocytes for comparison and correlation with Mg^{2+} fluxes under the same conditions.

3.8.2. *[^{14}C]Choline efflux*

To measure choline efflux, rat erythrocytes were loaded overnight with 10 mmol l^{-1} choline Cl labeled with [^{14}C]choline. Under these conditions, the intracellular choline concentration was 293 ± 21 μmol l cells^{-1} (mean ± S.E.M., $n = 4$). This concentration is well above the physiological intracellular choline concentration, which in human erythrocytes lies within the range of 18–21 μmol l cells^{-1} [35].

Fig. 2 shows the time course of [^{14}C]choline efflux,

Table 2
Effect of various choline transport inhibitors on Mg^{2+} efflux of rat erythrocytes

Mg^{2+} efflux (μmol l cells^{-1} 2 h^{-1})					
Substance	Inhibitor (mmol l^{-1})	NaCl medium	% of control	Choline Cl medium	% of control
Control		223.9 ± 7.9	100	140.3 ± 6.3	100
HC-3	4.6	282.4 ± 7.0*	126	95.7 ± 8.2*	68
DoTMA	0.2	247.7 ± 8.3*	111	128.5 ± 3.7*	92
Quinine	1.2	169.5 ± 6.7*	76	53.8 ± 3.6*	38
Quinidine	1.2	101.3 ± 5.5*	45	32.6 ± 1.1*	23
Cinchonine	1.2	221.0 ± 14.0	99	75.1 ± 12.4*	54
Cinchonidine	1.2	209.2 ± 8.9	93	74.1 ± 6.0*	53

A 10% (v/v) cell suspension was incubated in (a) NaCl medium or (b) choline Cl medium for 2 h. Means ± S.E.M., $n = 4$–6. *$P < 0.05$.

which was slightly more curvilinear over 2 h than it was in the investigations by others in human erythrocytes [4]. To compare [^{14}C]choline efflux with Mg^{2+} efflux, [^{14}C]choline efflux was measured over 2 h, which is the same time used to determine Mg^{2+} efflux. [^{14}C]Choline efflux ranged from 103 to 147 μmol l cells^{-1} 2 h^{-1} (Fig. 2 and Tables 1–3). This was identical to the magnitude of Mg^{2+} efflux in choline Cl medium, which at 2 h ranged from 110 to 140 μmol l cells^{-1} 2 h^{-1} (Tables 1–3). Thus the capacity of the choline exchanger would be sufficient to mediate Mg^{2+} efflux in choline Cl medium.

Next, the effect of some choline transport inhibitors on [^{14}C]choline efflux was investigated. To better compare the effect of these drugs on [^{14}C]choline efflux with their effect on Mg^{2+} efflux of rat erythrocytes in choline Cl medium, the substances were tested at their K_d concentration for Mg^{2+} efflux in choline Cl medium. The results are shown in Table 3. It can be seen that the inhibitors reduced choline efflux as follows: quinine > cinchonine > HC-3 > DoTMA. This is the same series that was obtained for the inhibition of Mg^{2+} efflux in choline medium (Table 2).

To compare the magnitude of the inhibition of [^{14}C]choline efflux by HC-3 with the magnitude of inhibition of Mg^{2+} efflux by HC-3, the K_d value for the inhibition of [^{14}C]choline efflux by HC-3 was also evaluated. As can be derived from Fig. 3, a K_d value of 5.9 ± 0.9 mmol l^{-1} was obtained.

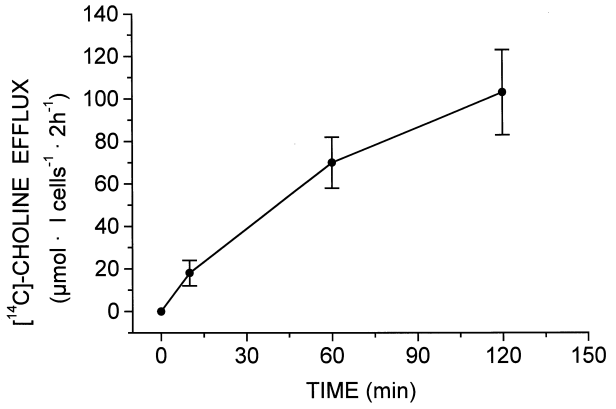

Fig. 2. Time course of the [^{14}C]choline efflux of rat erythrocytes loaded with [^{14}C]choline for 20 h. Efflux was measured in choline Cl medium. Means ± S.E.M., $n = 3$.

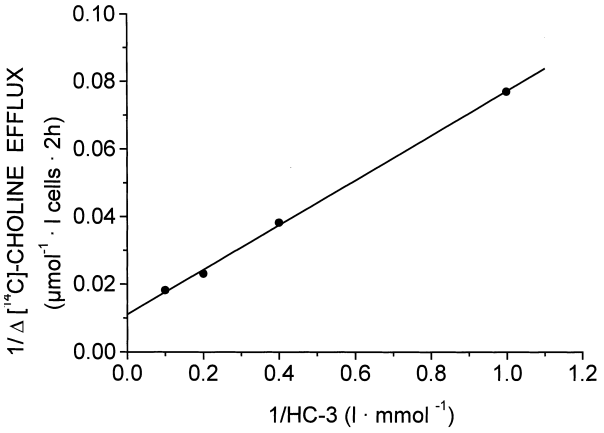

Fig. 3. Double reciprocal plot of the inhibition of [^{14}C]choline efflux of rat erythrocytes in choline Cl medium at various concentrations of HC-3. The regression line was calculated using the least-square method. The K_d value was calculated according to [33]. Mean values of five experiments.

3.8.3. [^{14}C]Choline influx

To obtain more clarity on the role of the choline transporter in Mg^{2+} efflux, we investigated [^{14}C]choline influx. NaCl medium was used for the following reasons: (1) for comparison with choline influx measured by other authors in human erythrocytes and (2) because measurement of choline influx in choline medium would need too high a [^{14}C]-activity. No attempts have been made to completely deprive the red cells of choline to really reach true zero trans conditions for choline. In pilot experiments we found a complete linear time dependence of [^{14}C]choline influx over 4 h at the two concentrations of 100 μmol l^{-1} and 1 mmol l^{-1} of choline ($n = 2$, not shown). As demonstrated later, the used concentration of 100 μmol l^{-1} external choline is near to the K_m value of choline influx. The physiological serum concentrations were reported to be in the range of 10–20 μmol l^{-1} in man, dog and rabbit [36,37].

The linearity with time allowed us to determine the apparent K_m value for [^{14}C]choline influx. Fig. 4A shows a typical Michaelis–Menten kinetic with increasing [^{14}C]choline influx at increasing extracellular choline concentration, approaching saturation at values above 5 mmol l^{-1} choline Cl. The Hofstee plot (see Fig. 4B) showed that there was only a single saturable choline transporter (between 5 μmol l^{-1} and 5 mmol l^{-1} of choline Cl). Calculation of the apparent K_m yielded 59.3 μmol l^{-1} (between 5 and

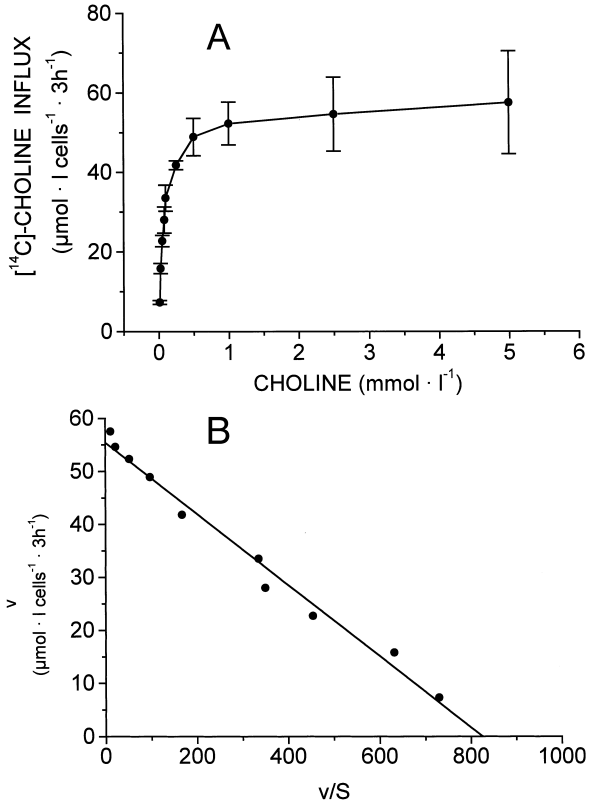

Fig. 4. (A) Kinetic of [^{14}C]choline influx in rat erythrocytes incubated in NaCl medium. Michaelis–Menten plot of [^{14}C]choline influx with increasing choline concentrations. Means ± S.E.M., $n = 4$–6. (B) Hofstee plot of values from A. The regression line was calculated using the least-square method. Mean values, $n = 4$–6.

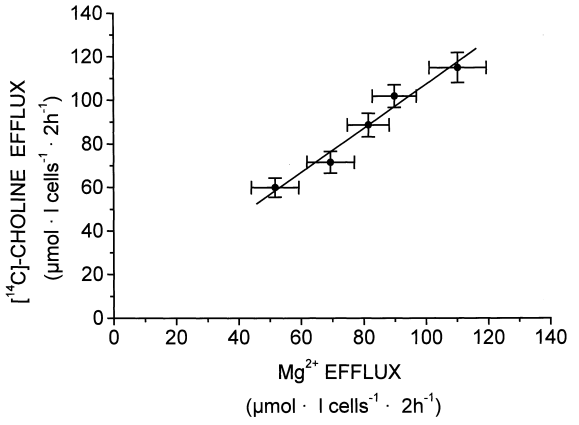

Fig. 5. Correlation of [^{14}C]choline efflux with Mg^{2+} efflux from rat erythrocytes inhibited by various concentrations of HC-3 (0, 1, 2.5, 5 and 10 mmol l^{-1}). The regression line was calculated using the least-square method. Means ± S.E.M., $n = 4$–5.

250 µmol l^{-1} choline Cl), indicating the existence of only one type of choline exchanger in rat erythrocytes as in non-malaria-infected human erythrocytes [38]. Our K_m value for choline influx in rat erythrocytes is similar to that found in eel erythrocytes for isoosmotic choline transport [34] but somewhat higher than in human erythrocytes where 25–56 µmol l^{-1} have been found, dependent on the used medium [4].

3.8.4. Relationship between [^{14}C]choline efflux and Mg^{2+} efflux

To estimate the relationship between [^{14}C]choline efflux and Mg^{2+} efflux we compared the effect of different doses of HC-3 on Mg^{2+} efflux and [^{14}C]choline efflux. As is shown in Fig. 5, the efflux rate of Mg^{2+} and choline was linearly correlated. This shows that the efflux of Mg^{2+} and choline are identically inhibited by HC-3. This could mean that one choline or one Mg^{2+} are transported by the same mechanism. In agreement with the identical inhibition of [^{14}C]choline efflux and Mg^{2+} efflux by HC-3, the K_d values of HC-3 for inhibition of [^{14}C]choline efflux (5.9 mmol l^{-1}, see Section 3.8.2) and of Mg^{2+} efflux (4.6 mmol l^{-1}, see Section 3.8.1) were, within the experimental error, the same. Moreover, when the inhibition of choline efflux in choline medium

Table 3
Effect of various choline transport inhibitors on [^{14}C]choline efflux of rat erythrocytes

Inhibitor	Concentration (mmol l^{-1})	[^{14}C]Choline efflux (µmol l cells^{-1} 2 h^{-1})	% of control
Control		147.8 ± 8.5	100
HC-3	4.6	68.9 ± 6.8*	47
DoTMA	0.2	105.5 ± 12.7*	71
Quinine	1.2	38.6 ± 8.5*	26
Cinchonine	1.2	50.3 ± 6.6*	34

The cells were loaded with 10 mmol l^{-1} of [^{14}C]-labeled choline for 20–24 h. After washing, the cells were suspended (hematocrit 10% v/v) in choline Cl medium. Efflux was measured for 2 h. For details see Section 2.4. Means ± S.E.M., $n = 5$. *$P < 0.05$.

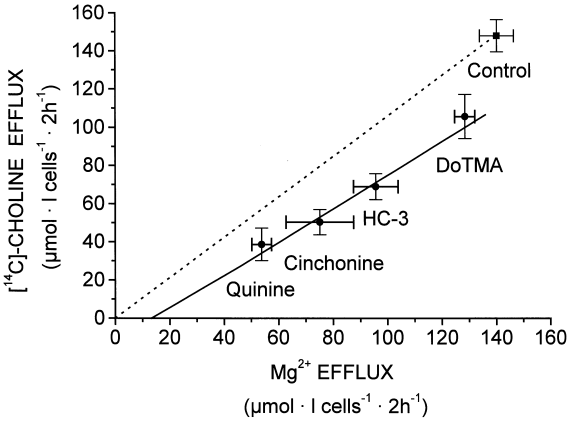

Fig. 6. Correlation of [^{14}C]choline efflux with Mg^{2+} efflux of rat erythrocytes inhibited by various inhibitors in choline Cl medium. Inhibitor concentrations corresponded to the K_d value for Mg^{2+} efflux in choline medium. Values taken from Tables 2 and 3. The regression line was calculated using the least-square method. Means ± S.E.M., $n = 4–6$.

(Table 3) and the inhibition of Mg^{2+} efflux by quinine, cinchonine, HC-3 and DoTMA (Table 2) were correlated, a linear relationship was obtained (Fig. 6). Again, this result may be taken as evidence that the choline exchanger can perform Mg^{2+} efflux via choline/Mg^{2+} exchange. However, in these experiments with inhibitor concentrations at their K_d values, residual Mg^{2+} efflux exceeded choline efflux. This may result from some Mg^{2+} leakage induced by the inhibitors at the used concentrations.

3.9. Concluding remarks

All inorganic cation transporters so far known in erythrocytes as a possible non-selective transport mechanism for Mg^{2+} efflux in choline Cl medium were excluded. The cumulative evidence for Mg^{2+}/choline antiport in rat erythrocytes is as follows:

The choline exchanger of erythrocytes is unspecific, also transporting Cs^+, Rb^+, K^+, Li^+, Na^+ and catecholamines [4,5].

[^{14}C]Choline efflux in choline Cl medium was the same as Mg^{2+} efflux in choline Cl medium. Thus, the capacity of the choline exchanger would be sufficient to mediate Mg^{2+} efflux in choline medium.

HC-3, a specific inhibitor of choline transport [32], and cinchonine did not inhibit Mg^{2+} efflux in NaCl medium but only in choline Cl medium, when used at the K_d value for Mg^{2+} efflux in choline Cl medium.

Mg^{2+} efflux and [^{14}C]choline efflux were inhibited by several specific (HC-3, DoTMA) and less specific inhibitors (quinine, quinidine, cinchonine, cinchonidine). The K_d values for the inhibition of Mg^{2+} efflux and of [^{14}C]choline efflux by HC-3 were the same within the experimental error.

Reduction of choline efflux by various inhibitors (quinine, cinchonine, HC-3 and DoTMA) was linearly correlated to the reduction of Mg^{2+} efflux in choline Cl medium.

These results indicate the participation of the choline exchanger in Mg^{2+} efflux. The physiological significance of Mg^{2+} transport through non-selective transport via the choline exchanger (e.g., by exchange of Mg^{2+} for choline or other monovalent cations) remains to be established. Studies on Na^+-dependent Mg^{2+} efflux relying on the incubation in choline Cl medium as a control should consider the interaction of choline/Mg^{2+} exchange.

Acknowledgements

The skilful technical assistance of Brigitte Papanis and Petra Lommes is greatly appreciated.

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