*Biochimica et Biophysica Acta*, 1149 (1993) 49–54



BBAMEM 75989

# $Na^+$- and anion-dependent $Mg^{2+}$ influx in isolated hepatocytes

Theodor Günther and Vera Höllriegl

*Institute of Molecular Biology and Biochemistry, Free University of Berlin, Berlin (Germany)*

(Received 22 February 1993)

Key words: Magnesium ion influx; Cotransport; Sodium ion; Chloride; Bicarbonate; Phosphate; (Rat hepatocyte)

Hepatocytes, which were $Mg^{2+}$-depleted during isolation, took up $Mg^{2+}$ during reincubation. $Mg^{2+}$ uptake was dependent on the concentration of extracellular $Mg^{2+}$, $Na^+$, $Cl^-$, bicarbonate and phosphate. $Li^+$ and choline$^+$ did not substitute for extracellular $Na^+$ in $Mg^{2+}$ influx. $Mg^{2+}$ influx was maximal when all three anion species were present, and did not occur when these anions were replaced by gluconate. Bicarbonate, phosphate and $Cl^-$ could substitute for each other. $Mg^{2+}$ uptake in hepatocytes was inhibited by *p*-chloromercuribenzene sulfonate, ouabain, gramicidin D, amiloride and verapamil. The results were explained by the assumption that net $Mg^{2+}$ influx in hepatocytes is operating via electroneutral $Na^+$, $Mg^{2+}$/anion cotransport driven by the $Na^+$ gradient. However, electrogenic $Mg^{2+}$ uptake gated by extracellular $Na^+$ and anions could not be excluded.

## Introduction

Preceding experiments revealed net $Mg^{2+}$ influx in $Mg^{2+}$-depleted Yoshida ascites tumor cells [1], cardiomyocytes [2], MDCK cells [3] and reticulocytes [4]. Net $Mg^{2+}$ influx stopped when the physiological intracellular $Mg^{2+}$ concentration was reached [1–3], indicating regulation of net $Mg^{2+}$ influx due to feed-back inhibition by intracellular $Mg^{2+}$. Net $Mg^{2+}$ influx into Yoshida cells was performed by electroneutral $Mg^{2+}$/$HCO_3^-$ cotransport [1]. For other cells, the mechanism of net $Mg^{2+}$ influx has not been defined so far. Therefore, we investigated the mechanism of net $Mg^{2+}$ uptake by isolated hepatocytes, which were $Mg^{2+}$-depleted during isolation.

## Materials and Methods

### *Preparation of hepatocytes*

Isolated perfused rat livers were dispersed by a two-step procedure of $Ca^{2+}$ removal, followed by collagenase (Sigma, type I) treatment according to Seglen [5]. During collagenase treatment, $Mg^{2+}$-free medium was used. The isolated hepatocytes were filtered through two layers of gauze, washed twice in prewarmed (37°C) $Mg^{2+}$-free and albumin-free $Na^+$ medium (see below) by centrifugation at $44 \times g$ for 1 min and purified from damaged cells and cell debris by Percoll centrifugation.

For Percoll centrifugation, 1 volume cells was suspended in 4 volumes $Mg^{2+}$-free and albumin-free $Na^+$ medium and mixed 1:1 (v/v) with 70% Percoll (Pharmacia) in $Mg^{2+}$-free and albumin-free $Na^+$ medium and centrifuged at $1500 \times g$ for 5 min.

### *$Mg^{2+}$ influx*

The sedimented hepatocytes (95% viable by Trypan blue exclusion) were resuspended in prewarmed (37°C) $Na^+$ medium. Cell concentration amounted to 2–3% (v/v) corresponding to $(1–2) \cdot 10^6$ cells/ml. Cell suspensions were gassed with 95% $O_2$/5% $CO_2$ or with 100% $O_2$ when bicarbonate-free medium was used.

At the beginning of incubation and after various times as indicated, 1-ml aliquots of the cell suspensions were centrifuged (1 min at $44 \times g$). The supernatant was sucked off and 1 ml 5% (w/v) TCA was added to the pellet. In the experiments with increasing $[Mg^{2+}]_o$, the cells were washed once with $Mg^{2+}$- and $Ca^{2+}$-free incubation medium containing 2 mM EDTA. After homogenisation and centrifugation, $Mg^{2+}$ concentration of the TCA extract was measured by atomic absorption spectrophotometry (Philips, SP 9). Protein content of the TCA precipitates was measured with the Pierce BCA Protein Assay [6]. $Mg^{2+}$ influx was calculated from the increase of cellular $Mg^{2+}$ content.

Correspondence to: T. Günther, Institute of Molecular Biology and Biochemistry, Free University of Berlin, Arnimallee 22, D-W-1000 Berlin 33, Germany.

Abbreviations: $[X]_o$, $[X]_i$, extracellular, intracellular concentrations of the corresponding ions or salts; $P_i$, inorganic phosphate; PCMBS, *p*-chloromercuribenzene sulfonate; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TCA, trichloroacetic acid.

*Cellular $Na^+$, $K^+$ and ATP content*

For determination of cellular $Na^+$ and $K^+$ contents, the cells were once washed in cold choline Cl medium and sedimented. The supernatant was sucked off and 1 ml 5% TCA was added to the pellet as described above. $Na^+$ and $K^+$ concentration of the TCA extract was measured by flame photometry (KLiNa-flame, Beckman). ATP content of liver and isolated hepatocytes was determined enzymatically in TCA extracts by an optical test according to the instructions of the manufacturer (Sigma, procedure No. 336-UV).

*Media*

Incubation was performed in $Na^+$ medium, containing (in mM): 125 NaCl, 15 $NaHCO_3$, 5 KCl, 1 $KH_2PO_4$, 5 glucose, 1 adenosine, 2.4 $CaCl_2$, 0.9 $MgCl_2$, 50 g/l bovine serum albumin (Serva, fraction V), 20 Hepes-NaOH (pH 7.4), and adjusted to a final pH of 7.4. For preparation of albumin-free $Na^+$ medium, albumin was omitted and $[CaCl_2]_o$ was reduced to 1.2 mM and $[MgCl_2]_o$ to 0.6 mM.

When bicarbonate-free medium or media with different $[HCO_3^-]_o$ were used, $NaHCO_3$ was substituted by NaCl. For media with different $[P_i]_o$, phosphate was added as $Na_2HPO_4$. For preparing $K^+$, choline$^+$ or $Li^+$ medium, $Na^+$ salts were substituted by $K^+$, choline$^+$ or $Li^+$ salts, and Hepes-Tris was used for buffering.

*Concentration of free $Mg^{2+}$ and free $Ca^{2+}$ in media*

Concentration of free $Mg^{2+}$ and $Ca^{2+}$ in the media was measured by means of an $Mg^{2+}$- and $Ca^{2+}$-sensitive electrode (Microlyte 6, Kone Instruments, Espoo, Finland).

## Results and Discussion

*Characterization of the isolated hepatocytes*

During isolation of hepatocytes $Mg^{2+}$ content was reduced from 64.5 ± 2.2 to 47.9 ± 1.5 nmol/mg protein (mean ± S.E. of eight experiments). $K^+$ and ATP contents were also reduced, whereas $Na^+$ content was increased (Table I).

The major part of $Mg^{2+}$ depletion during cell isolation might have been caused by reduction of ATP content, in the sense that $Mg^{2+}$ was released from ATP because of the lower $Mg^{2+}$ binding to ADP than to ATP. This might have led to a transient increase in free $[Mg^{2+}]_i$ and consequently to an efflux of $Mg^{2+}$. This mechanism would be in analogy with $Mg^{2+}$ efflux from erythrocytes and other cells, in which $Mg^{2+}$ efflux was induced by loading the cells with $Mg^{2+}$ [7,8].

After reincubation of isolated hepatocytes in $Na^+$ medium, intracellular $Na^+$, $K^+$ and ATP concentrations were rapidly restored (Table I). After reincubation in $Na^+$ medium with different anion contents, the same results were obtained (data not shown). Therefore, the different rate of $Mg^{2+}$ influx in the presence of different anions (Table II) was not caused by reduced ATP concentration and energy state of the cells.

$Mg^{2+}$ uptake by isolated $Mg^{2+}$-depleted hepatocytes occurred at a slower rate (Fig. 1) than restoration of intracellular $Na^+$, $K^+$ and ATP (Table I). Therefore, most of the $Mg^{2+}$ uptake took place when ATP content and energy state of the cells had already been restored. Control experiments, in which the cells were incubated in $Mg^{2+}$-free $Na^+$ medium for 15 min (to restore $[ATP]_i$, $[Na^+]_i$ and $[K^+]_i$) followed by addition of $Mg^{2+}$ to start $Mg^{2+}$ influx, did not yield a significantly different $Mg^{2+}$ influx (data not shown).

*$Na^+$ dependency of $Mg^{2+}$ influx*

Since $Mg^{2+}$ uptake in Yoshida ascites tumor cells was performed by electroneutral $Mg^{2+}/HCO_3^-$ cotransport [1], we tested whether $Mg^{2+}$ influx in hepatocytes is operating by an electroneutral mechanism or

TABLE I

*$K^+$, $Na^+$ and ATP content of rat liver cells before and after isolation and after reincubation in $Na^+$ medium*

Mean ± S.E. of eight experiments.

| | Content (nmol/mg protein) | | |
|---|---|---|---|
| | $K^+$ | $Na^+$ | ATP |
| Before isolation | 601 ± 26 | [a] | 29.9 ± 1.2 |
| After isolation | 303 ± 11 | 335 ± 13 | 13.3 ± 0.7 |
| 15 min after reincubation | 490 ± 9 | 124 ± 4 | 23.6 ± 1.1 |
| 30 min after reincubation | 521 ± 10 | 117 ± 5 | 26.4 ± 0.9 |
| 60 min after reincubation | 531 ± 12 | 102 ± 4 | 28.0 ± 1.3 |

[a] The major part of $Na^+$ in liver is extracellularly localized.

TABLE II

*Effect of extracellular $Cl^-$, $HCO_3^-$ and $P_i$ on $Mg^{2+}$ influx ($\Delta Mg^{2+}$) in isolated hepatocytes*

Addition of anions as indicated. When anion species were omitted, they were equimolarly substituted by gluconate. Mean ± S.E. of four experiments in duplicates.

| $[Cl^-]_o$ (133 mM) | $[HCO_3^-]_o$ (15 mM) | $[P_i]_o$ (1.0 mM) | $\Delta Mg^{2+}$ (nmol/mg protein per h) |
|---|---|---|---|
| + | + | + | 12.0 ± 0.4 |
| + | + | − | 5.4 ± 0.3 |
| − | + | + | 3.8 ± 0.5 |
| + | − | + | 6.0 ± 0.6 |
| − | + | − | 3.0 ± 0.5 |
| + | − | − | 2.5 ± 0.6 |
| − | − | + | 1.3 ± 0.2 |
| − | − | − | 0.0 ± 0.2 |

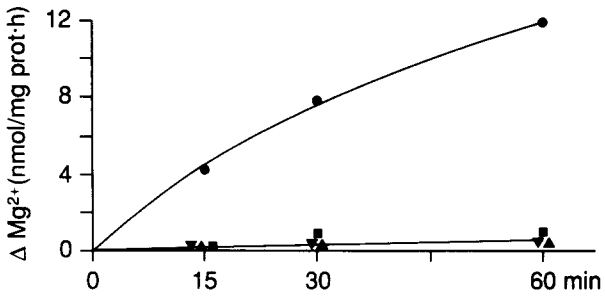


Fig. 1. $Mg^{2+}$ uptake by isolated hepatocytes. Hepatocytes were incubated in $Na^+$ medium (●), $K^+$ medium (▲), $Li^+$ medium (■) or choline$^+$ medium (▼). Mean of four experiments.

TABLE III

*Inhibition of $Mg^{2+}$ influx ($\Delta Mg^{2+}$) in hepatocytes*

Cells were incubated in $Na^+$ medium with the addition of inhibitors as indicated. Mean ± S.E. of three experiments.

| Addition | | $\Delta Mg^{2+}$ (nmol/mg protein per h) |
|---|---|---|
| Control | – | 11.5 ± 0.9 |
| Gramicidin D | 2.5 μg/ml | 1.0 ± 0.6 |
| Ouabain | 1 mM | 0.9 ± 0.3 |
| PCMBS | 0.5 mM | 0.3 ± 0.3 |

whether it is dependent on the membrane potential. When extracellular $Na^+$ was replaced by $K^+$ to depolarize the membrane potential, an electrogenic system should be affected. As shown in Fig. 1, $Mg^{2+}$ uptake by hepatocytes was prevented when $Na^+$ was substituted by $K^+$. This result shows that $Mg^{2+}$ influx may depend on the membrane potential or on $[Na^+]_o$.

Therefore, we measured $Mg^{2+}$ influx in media in which $Na^+$ was replaced by $Li^+$ or choline$^+$. As also shown in Fig. 1, in $Li^+$ and choline$^+$ medium a significant uptake of $Mg^{2+}$ did not occur, indicating that $Mg^{2+}$ influx is specifically dependent on $[Na^+]_o$.

The effect of $[Na^+]_o$ on $Mg^{2+}$ influx obeyed saturable, non-cooperative kinetics (Michaelis-Menten kinetics, $K_m = 50$ mM, $V_{max} = 15$ nmol $Mg^{2+}$/mg protein per h, Fig. 2).

From this result it can be suggested that 1 extracellular $Na^+$ is needed in $Mg^{2+}$ transport. It may be bound to the $Mg^{2+}$ transporter which thereafter can transport $Mg^{2+}$, or 1 $Na^+$ is cotransported together with $Mg^{2+}$. The driving force for this transport may be the membrane potential or the extracellular-intracellular $Na^+$ gradient.

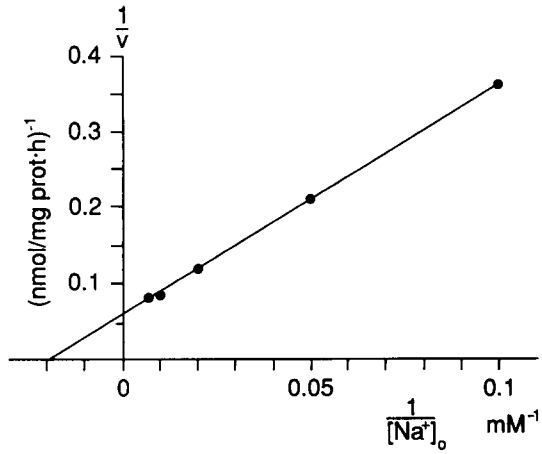


Fig. 2. $Na^+$ dependency of $Mg^{2+}$ influx in hepatocytes. Double-reciprocal plot. NaCl of $Na^+$ medium was replaced by choline Cl. Mean of four experiments.

To analyse this mechanism we tested the effect of PCMBS, ouabain and gramicidin D which disturbed or prevented the restoration of the $Na^+$ and $K^+$ gradient in the isolated hepatocytes. Ouabain concentration must be high because of the low ouabain sensitivity of rat liver [9]. At 1 mM ouabain the $Na^+$ gradient of hepatocytes was drastically reduced [10] (data not shown). A low concentration of gramicidin D disturbed the $Na^+$ gradient [10] (data not shown) and membrane potential [10] without affecting ATP [11]. We found that these substances prevented $Mg^{2+}$ uptake by hepatocytes (Table III). The inhibition of $Mg^{2+}$ uptake by gramicidin D cannot be caused by reduction of ATP and consequent increase of free $[Mg^{2+}]_i$ but must be caused by destruction of the $Na^+$ gradient or membrane potential. Moreover, these results show that the presence of extracellular $Na^+$ was not sufficient for $Mg^{2+}$ influx and that, additionally, there must exist an $Na^+$ gradient or a membrane potential.

Hence, these results are compatible with $Na^+$, $Mg^{2+}$ cotransport driven by the $Na^+$ gradient or the membrane potential. Alternatively, $Mg^{2+}$ influx may be performed by $Na^+$-gated $Mg^{2+}$ uptake driven by the membrane potential. However, the inhibition of $Mg^{2+}$ uptake by amiloride (see below), which inhibits $Na^+$-coupled cotransport of alanine [9] and hexose [12], may indicate that $Na^+$ is cotransported together with $Mg^{2+}$.

*Anion dependency of $Mg^{2+}$ influx*

Since $Mg^{2+}$ uptake by Yoshida cells was performed by electroneutral $Mg^{2+}/HCO_3^-$ cotransport, $Na^+$, $Mg^{2+}$ cotransport or $Na^+$-gated $Mg^{2+}$ influx in hepatocytes may be accompanied by anions either in an electroneutral or electrogenic way. Therefore, we tested the effect of extracellular anions.

Maximal $Mg^{2+}$ influx was achieved when $Cl^-$, $HCO_3^-$ and $P_i$ were present in the $Na^+$ medium. When two or one anion species were present, a lower rate of $Mg^{2+}$ influx was obtained (Table II).

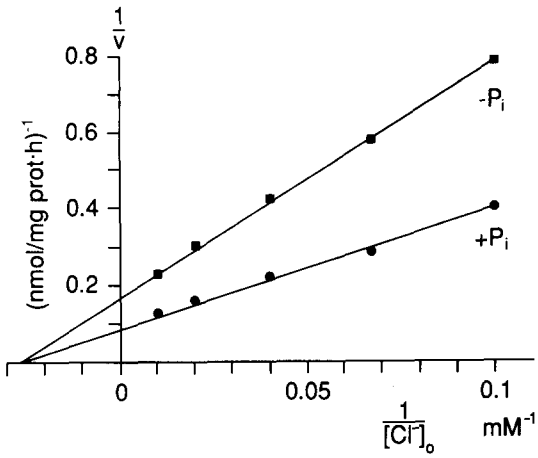


Fig. 3. Effect of $[Cl^-]_o$ on $Mg^{2+}$ influx in hepatocytes. Double-reciprocal plot. The cells were incubated in $P_i$-containing ($+P_i$) and $P_i$-free $Na^+$ medium ($-P_i$). $Mg^{2+}$ influx at $[Cl^-]_o = 0$ was subtracted. Mean of four experiments.

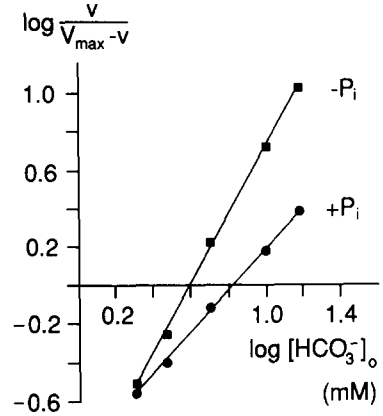


Fig. 5. Effect of $[HCO_3^-]_o$ on $Mg^{2+}$ influx in hepatocytes. Hill plot. The cells were incubated in $Na^+$ medium ($+P_i$) or in $P_i$-free $Na^+$ medium ($-P_i$). $Mg^{2+}$ influx at $[HCO_3^-]_o = 0$ was subtracted. Mean of four experiments.

When $Cl^-$, $HCO_3^-$ and $P_i$ were substituted by gluconate, $Mg^{2+}$ influx was prevented (Table II). This effect was not caused by reduction of free $[Mg^{2+}]_o$ of the medium (see below).

When the effect of one anion species on $Mg^{2+}$ influx was tested in the presence of the other two anion species, $Mg^{2+}$ influx revealed saturable, non-cooperative kinetics (Figs. 3 and 4) or a Hill coefficient of 1 (Fig. 5). According to the analogous Michaelis-Menten kinetics saturable, non-cooperative kinetics or a Hill coefficient of 1 indicate that each anion was limiting the rate of $Mg^{2+}$ uptake in the presence of the other two anion species.

When $P_i$ was omitted from the $Na^+$ medium, $Cl^-$ dependency of $Mg^{2+}$ influx obeyed saturable, non-cooperative kinetics (Fig. 3), indicating that also in the absence of $P_i$ only 1 $Cl^-$ was operating in $Mg^{2+}$ uptake. The affinity ($K_m = 38$ mM) was the same as in the presence of $P_i$ (Fig. 3). This result suggests that $H_2PO_4^-$ was substituted by $HCO_3^-$ and that now 2 $HCO_3^-$ were simultaneously operating in $Mg^{2+}$ influx together with 1 $Cl^-$. Indeed, when the effect of $HCO_3^-$ on $Mg^{2+}$ influx was investigated in the absence of $P_i$, the Hill plot revealed a Hill coefficient of 2 (Fig. 5), indicating that under this condition 2 $HCO_3^-$ were simultaneously operating in $Mg^{2+}$ influx together with 1 $Cl^-$.

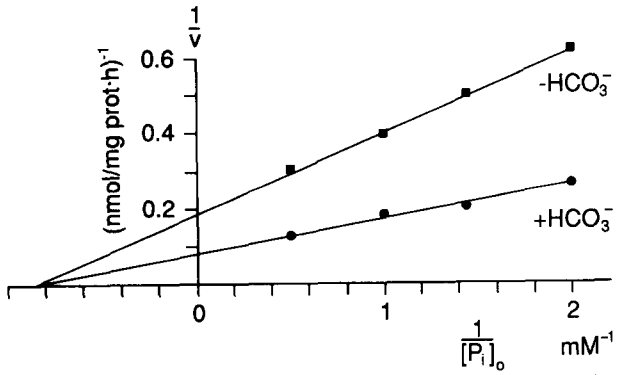


Fig. 4. Effect of $[P_i]_o$ on $Mg^{2+}$ influx in hepatocytes. Double-reciprocal plot. The cells were incubated in $Na^+$ medium ($+HCO_3^-$) or in $HCO_3^-$-free $Na^+$ medium ($-HCO_3^-$). $Mg^{2+}$ influx at $[P_i]_o = 0$ was subtracted. Mean of four experiments.

$P_i$ dependency of $Mg^{2+}$ influx in the absence of $HCO_3^-$ revealed saturable, non-cooperative kinetics (Fig. 4). Thus, in the presence and absence of $HCO_3^-$ only 1 $P_i$ was involved in $Mg^{2+}$ influx. Since the $pK_2$-value of phosphate amounts to 6.7 [13], $P_i$ in the media consists of $H_2PO_4^-$ and $HPO_4^{2-}$. Saturable, non-cooperative kinetics in the absence of $HCO_3^-$ might indicate that instead of 1 $H_2PO_4^-$ 1 $HPO_4^{2-}$ is operating in $Mg^{2+}$ influx together with 1 $Cl^-$. Thus, these kinetic experiments showed that three negative charges of anions were simultaneously involved in $Na^+$-dependent $Mg^{2+}$ uptake.

These results can be explained by the assumption that three anions are taken up by hepatocytes together with $Na^+$ and $Mg^{2+}$ in an electroneutral cotransport. The driving force may be the extra-intracellular $Na^+$ gradient. $Mg^{2+}$ influx would be maximal as $Na^+$, $Mg^{2+}/Cl^-$, $HCO_3^-$, $H_2PO_4^-$ cotransport and would be less active with other combinations of these anions (Table II). However, since the rate of $Mg^{2+}$ uptake is low and since there are other mechanisms by which anions and $Na^+$ are transported, simultaneous uptake of anions and $Na^+$ was not measured to define $Na^+$, $Mg^{2+}$/anion cotransport and its stoichiometry. Therefore, it cannot be excluded that there may be an electrogenic $Mg^{2+}$ uptake gated by extracellular $Na^+$ and anions.

*Ion interactions in media*

There may be interactions of ions in the media. When $[HCO_3^-]_o$ and $[P_i]_o$ were changed at constant

TABLE IV

*Effect of $[Mg^{2+}]_o$ on $Mg^{2+}$ influx ($\Delta Mg^{2+}$) in hepatocytes*

The cells were incubated in $Na^+$ medium or in gluconate medium. Mean of 2 experiments. Total $[Mg^{2+}]_o$ was measured by atomic absorption spectrophotometry, and free $[Mg^{2+}]_o$ was measured by an $Mg^{2+}$-sensitive electrode (Microlyte 6).

| | Total $[Mg^{2+}]_o$ (mM) | Free $[Mg^{2+}]_o$ (mM) | $\Delta Mg^{2+}$ (nmol/mg protein per h) |
|---|---|---|---|
| $Na^+$ medium | 0.9 | 0.7 | 10.3 |
| | 1.5 | 1.2 | 12.0 |
| | 3.0 | 2.3 | 15.1 |
| | 6.0 | 4.3 | 16.0 |
| Gluconate medium | 0.9 | 0.6 | 0.0 |
| | 1.5 | 0.7 | 0.2 |
| | 3.0 | 1.5 | 0.1 |
| | 6.0 | 3.0 | 0.3 |

total $[Mg^{2+}]_o$, the interaction and binding of $Mg^{2+}$ (and $Ca^{2+}$) to albumin and to $HCO_3^-$ and $P_i$ may be changed. At pH 7.4 and 5% albumin, approximately one third of total $Mg^{2+}$ in the media may be bound to albumin [14,15], representing an $Mg^{2+}$ buffer. Thus, the concentration (or more exactly the activity) of extracellular free $Mg^{2+}$ may be held constant at changed $[HCO_3^-]_o$ and $[P_i]_o$. $Mg^{2+}$ buffering by albumin may be effective since the interactions of $HCO_3^-$ and $P_i$ with $Mg^{2+}$ are weak at physiological concentrations [14].

In agreement with this conclusion, measuring free $[Mg^{2+}]_o$ in the absence or presence of 1 mM $P_i$ and in the absence or presence of 15 mM $HCO_3^-$ did not reveal significantly different values.

When $Cl^-$, $HCO_3^-$ and $P_i$ were substituted by gluconate, the interaction between $Mg^{2+}$ and anions may be changed either by affecting the activity coefficient of $Mg^{2+}$ or by weak binding of $Mg^{2+}$ to gluconate. The apparent association constant of $Mg^{2+}$ gluconate was determined to be log $K = 0.70$ [16]. In the medium a weak interaction between $Mg^{2+}$ and gluconate may be compensated by the $Mg^{2+}$ buffer (albumin). In order to analyse whether these effects play a significant role, $Mg^{2+}$ dependency of $Mg^{2+}$ uptake and free $[Mg^{2+}]_o$ were measured in $Na^+$- and gluconate medium.

As shown in Table IV, when $[Mg^{2+}]_o$ was increased, there was no significant $Mg^{2+}$ influx in gluconate medium, whereas at similar $[Mg^{2+}]_o$ in $Na^+$ medium, $Mg^{2+}$ was taken up, depending on $[Mg^{2+}]_o$. Therefore, interaction of $Mg^{2+}$ with gluconate was not the reason why $Mg^{2+}$ influx did not occur in gluconate medium (Table II). The values taken from Table IV revealed saturable, non-cooperative kinetics with $K_m$ for $Mg^{2+}$ uptake of 0.6 mM (Fig. 6). Hence, $Mg^{2+}$ uptake by the hepatocytes occurred at physiological free $[Mg^{2+}]_o$.

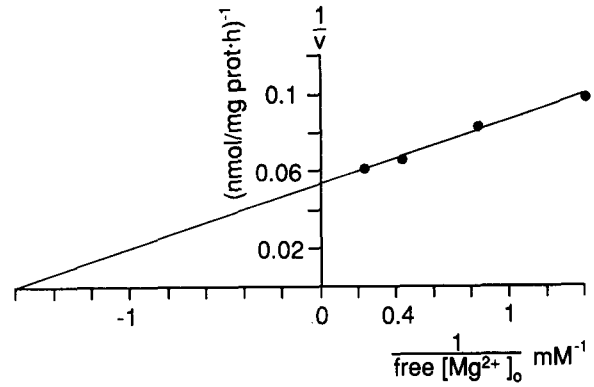


Fig. 6. Double-reciprocal plot of $Mg^{2+}$ influx in hepatocytes incubated in $Na^+$ medium. Values were taken from Table IV.

Accuracy of $[HCO_3^-]_o$ should also be considered. In the media, pH was adjusted to 7.4 and remained constant throughout gassing with 95% $O_2$/5% $CO_2$. In the presence of $NaHCO_3$, there will be $HCO_3^-$ from $NaHCO_3$ as well as from carbonic acid (dissolved and dissociated $CO_2$). However, with the pH and $[NaHCO_3]_o$ used the concentration of $CO_3^{2-}$ could be neglected and total $[HCO_3^-]_o$ was equivalent to that of the bicarbonate added [13].

*Effect of inhibitors*

As described above, $Mg^{2+}$ influx was inhibited by various substances (Table III), which disturbed the $Na^+$ gradient and thus the driving force.

An anion-coupled cotransport was concluded for the uptake of $Zn^{2+}$ by human erythrocytes, which may function as $(Zn, 2HCO_3, Cl)^-$ [17], $(Zn, CO_3, Cl)^-$ or as $(Zn, HCO_3, Cl, OH)^-$ cotransport [18] via band 3 protein (capnophorin).

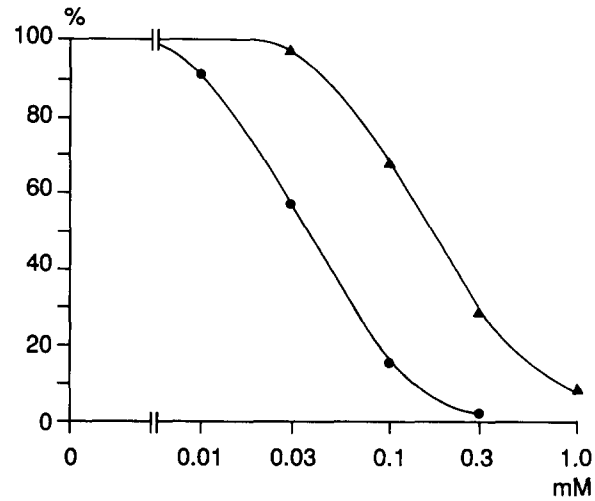


Fig. 7. Inhibition of $Mg^{2+}$ influx in hepatocytes by amiloride (▲) and verapamil (●). The cells were incubated in $Na^+$ medium. Mean of three experiments. 100% corresponds to an $Mg^{2+}$ uptake of 11.2 ± 1.0 (mean ± S.E.) nmol/mg protein per h.

TABLE V

*Effect of $Ca^{2+}$ and albumin in medium on $Mg^{2+}$ influx ($\Delta Mg^{2+}$) in hepatocytes*

Cells were incubated in $Na^+$ medium with different contents of $Ca^{2+}$ and albumin. In albumin-free medium $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ were reduced to 1.2 and 0.6 mM. Mean ± S.E. of four experiments.

| $[Ca^{2+}]_o$ (mM) | $[Mg^{2+}]_o$ (mM) | Albumin (g/l) | $\Delta Mg^{2+}$ (nmol/mg protein) | |
|---|---|---|---|---|
| | | | 60 min | 90 min |
| 2.4 | 0.9 | 50 | 11.3 ± 1.2 | 17.8 ± 1.1 |
| 5.0 | 0.9 | 50 | 10.2 ± 0.5 | 17.6 ± 1.0 |
| – | 0.9 | 50 | 4.0 ± 0.6 | 5.0 ± 0.5 |
| 1.2 | 0.6 | – | 4.2 ± 0.5 | 3.0 ± 1.0 |

$Mg^{2+}$ uptake was not significantly inhibited by 30 $\mu$M SITS and 100 $\mu$M bumetanide (data not shown), indicating that band 3 protein (capnophorin) and $Na^+$, $K^+$, 2 $Cl^-$ cotransport which are inhibited by these inhibitors [19] are not involved in $Mg^{2+}$ uptake by hepatocytes.

In addition, we tested the effect of verapamil and amiloride. $Mg^{2+}$ uptake in $Mg^{2+}$-depleted cardiac myocytes [2] and MDCK cells [3] was inhibited by 90% by 25 $\mu$M verapamil. $Mg^{2+}$ uptake in hepatocytes was also inhibited by verapamil ($IC_{50} = 35$ $\mu$M, Fig. 7). Possibly, $Mg^{2+}$ influx in hepatocytes is similar to $Mg^{2+}$ influx in cardiomyocytes and MDCK cells, in which the mechanism of $Mg^{2+}$ influx has not been characterized so far.

Although $Mg^{2+}$ uptake was inhibited by verapamil there seems to be no relationship between $Mg^{2+}$ uptake and $Ca^{2+}$ uptake. $Mg^{2+}$ uptake in MDCK cells was not significantly different at zero and 5 mM $[Ca^{2+}]_o$ [3]. Also, in hepatocytes $Mg^{2+}$ uptake was not significantly changed when $[Ca^{2+}]_o$ was increased from 2.4 to 5 mM (Table V) which corresponds to free $[Ca^{2+}]_o$ of 1.2 and 2.9 mM, as measured by Microlyte 6. In $Ca^{2+}$-free or albumin-free medium $Mg^{2+}$ influx was reduced (Table V) due to cell injury as was seen by reduced Trypan blue exclusion and loss of $[K^+]_i$ (data not shown). Elevation of $[Mg^{2+}]_o$ to 5 mM had no effect on $^{45}Ca^{2+}$ uptake by MDCK cells [3]. These results suggest that $Ca^{2+}$ and $Mg^{2+}$ may enter cells by separate pathways. Moreover, the inhibition of $Mg^{2+}$ uptake by verapamil shows that verapamil does not selectively block $Ca^{2+}$ influx but can also inhibit other influx systems, e.g. for $Mg^{2+}$ or $Na^+$ [20].

Amiloride inhibited $Na^+$-coupled transport of alanine [9] and hexose [12]. $Na^+$-dependent $Mg^{2+}$ influx in hepatocytes was also inhibited by amiloride ($IC_{50} =$ 0.18 mM, Fig. 7). The mechanism by which $Na^+$-coupled cotransport systems are inhibited by amiloride may be the interaction of amiloride at the extracellular $Na^+$-binding site. The analogous mechanism of amiloride, although with different affinities [21], may hold for the inhibition of $Na^+$ channels, $Na^+/H^+$, $Na^+/Ca^{2+}$ and $Na^+/Mg^{2+}$ exchange by amiloride.

## Conclusion

The experiments showed that $Mg^{2+}$ uptake in hepatocytes depended on $[Na^+]_o$ and that $Mg^{2+}$ influx was optimal in the presence of extracellular $Cl^-$, $HCO_3^-$ and $H_2PO_4^-$. $Mg^{2+}$ uptake was inhibited by amiloride as was found for $Na^+$-coupled uptake of alanine [9] and hexose [12]. The results can be explained by the assumption that $Mg^{2+}$ influx in hepatocytes is operating via a mechanism of its own, which may be electroneutral $Na^+$, $Mg^{2+}$/anion cotransport, driven by the $Na^+$ gradient. However, since cotransport of $Na^+$ and anions was not directly measured electrogenic $Mg^{2+}$ uptake gated by extracellular $Na^+$ and anions cannot be excluded.

## References


1 Günther, T., Vormann, J. and Averdunk, R. (1985) FEBS Lett. 197, 297–300.
2 Quamme, G.A. and Rabkin, S.W. (1990) Biochem. Biophys. Res. Commun. 167, 1406–1412.
3 Quamme, G.A. and Dai, L.J. (1990) Am. J. Physiol. 259, C521–C525.
4 Günther, T. and Vormann, J. (1991–1992) Magnesium Trace Elem. 10, 17–20.
5 Seglen, P.O. (1973) Expt. Cell Res. 82, 391–398.
6 Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
7 Günther, T., Vormann, J. and Höllriegl, V. (1990) Biochim. Biophys. Acta 1023, 455–461.
8 Günther, T. and Vormann, J. (1990) Magnesium Trace Elem. 9, 279–282.
9 Renner, E.L., Lake, J.R., Cragoe, E.J., Jr. and Scharschmidt, B.F. (1988) Biochim. Biophys. Acta 938, 386–394.
10 Kristensen, L.O. and Folke, M. (1986) Biochim. Biophys. Acta 855, 49–57.
11 Kristensen, L.O. (1980) J. Biol. Chem. 255, 5236–5243.
12 Cook, J.S, Shaffer, C. and Cragoe, E.J., Jr. (1987) Am. J. Physiol. 253, C199–C204.
13 Umbreit, W.W., Burris, R.H. and Stauffer, J.C. (1959) Manometric Techniques, pp. 1–338, Burgess, Minneapolis, MN.
14 Copeland, B.E. and Sunderman, F.W. (1952) J. Biol. Chem. 197, 331–341.
15 Willis, M.J. and Sunderman, F.W. (1952) J. Biol. Chem. 343–345.
16 Cannan, R.K. and Kibrick, A. (1938) J. Am. Chem. Soc. 60, 2314–2320.
17 Torrubia, J.O.A. and Garay, R. (1989) J. Cell. Physiol. 138, 316–322.
18 Kalfakakou, V. and Simons, T.J.B. (1990) J. Physiol. 421, 485–497.
19 Hoffmann, E.K. (1986) Biochim. Biophys. Acta 864, 1–31.
20 Nachshen, D.A. and Blaustein, M.P. (1979) Mol. Pharmacol. 16, 579–586.
21 Günther, T., Vormann, J., Cragoe, E.J., Jr. and Höllriegl, V. (1989) Magnesium-Bull. 11, 103–107.