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Activation of Na^+/Mg^{2+} antiport in thymocytes by cAMP

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Mg^{2+} efflux from Mg^{2+}-loaded rat thymocytes was stimulated by 0.1 mM dibutyryl cAMP (db cAMP). The activation of Mg^{2+} efflux by db cAMP was more expressed at lower Mg^{2+}-loading. cAMP induced only a very small increase in the concentration of intracellular free Mg^{2+} which cannot explain the activation of Na^+/Mg^{2+} antiport. From these results it was concluded that cAMP increases the affinity of the Na^+/Mg^{2+} antiporter for intracellular Mg^{2+}, probably by phosphorylation.

Magnesium; Efflux; cAMP; Intracellular free Mg^{2+}; Rat thymocyte

1. INTRODUCTION

Na^+-dependent Mg^{2+} efflux from Mg^{2+}-loaded rat thymocytes is operating via Na^+/Mg^{2+} antiport. Na^+-independent Mg^{2+} efflux was not detectable in these cells [1]. In perfused hearts and liver, a transient Mg^{2+} efflux can be induced by β-adrenergic substances and db cAMP [2–4]. Therefore, we investigated the effect of db cAMP on Na^+/Mg^{2+} antiport in thymocytes.

2. MATERIALS AND METHODS

Rat thymus was obtained from Wistar rats. Thymocytes were isolated by gently sieving the thymus into KCl medium (in mM: 150 KCl, 3 $MgCl_2$, 5 glucose, 50 sucrose, 30 HEPES/Tris, pH 7.4), washed once in Mg^{2+} loading medium (K^+ medium with 3, 6, 9, or 12 $MgCl_2$) and passed through gauze to remove agglutinated cell debris.

2.1. *Mg^{2+}-loading*

Mg^{2+} loading was achieved by incubating the cells in Mg^{2+}-loading medium for 30 min at 37°C with the addition of 6 μM of the cation ionophore A23187 (Boehringer, Mannheim). To remove the ionophore, the cells were incubated 4 times for 5 min at 37°C in loading medium plus 1% bovine serum albumin. Thereafter, the cells were washed once with choline Cl medium (in mM: 150 choline Cl, 5 glucose, 50 sucrose, 30 HEPES/Tris, pH 7.4).

2.2. *Mg^{2+} efflux*

Mg^{2+} efflux was measured by incubating the cells at a concentration of 0.5% (v/v) at 22°C in NaCl medium (in mM): 150 NaCl, 5 glucose, 50 sucrose, 30 HEPES/Tris, pH 7.4. At 0, 10 and 20 min 1 ml aliquots of the cell suspensions were spun down (0.5 min, 10,000 × *g*), the supernatant was aspirated and 1 ml 10% trichloroacetic acid (TCA)/0.175% $LaCl_3$ was added to the sedimented cells and centrifuged. Mg^{2+} content of the TCA extract was measured by atomic absorption spectrophotometry (Philips, SP9).

The protein sediments were dissolved by heating with 1 N NaOH for 1 h and the protein contents were measured with the Pierce BCA Protein Assay [5].

Mg^{2+} efflux was calculated from the reduction of cellular Mg^{2+} content.

2.3. *Concentration of intracellular free Mg^{2+} ($[Mg^{2+}]_i$)*

Thymocytes were incubated in incubation medium (cell concentration: 10%, v/v), at 37°C for 30 min with 10 μM of the acetoxymethyl ester of Mag-Fura-2 (Molecular Probes, Eugene, OR, USA). Thereafter, the cells were washed once in incubation medium and stored for the experiments at 4°C for up to 4 h without any change in their response. Prior to fluorescence measurements, aliquots of the cells were washed once in incubation medium and suspended in 2 ml of the same medium.

When Mg^{2+}-loaded thymocytes were used, the acetoxymethyl ester of Mag-Fura-2 was added during incubation with the ionophore in the presence of 3 mM $MgCl_2$ for Mg^{2+} loading (see above) and the ionophore was washed off as described. The Mg^{2+}- and Mag-Fura-2-loaded cells were washed in choline Cl medium and used at once for determination of $[Mg^{2+}]_i$.

$[Mg^{2+}]_i$ was determined by measuring the fluorescence of the Mag-Fura-2-loaded cells in the fluorescence spectrometer LS 50 (Perkin-Elmer) at 22°C under stirring using the 'Intracellular Biochemistry' software (Perkin-Elmer).

Briefly, fluorescence was obtained at the excitation wavelengths of 335 and 378 nm (5 nm slit width) and the emission wavelength of 505 nm (5 nm slit width) by changing the excitation wavelength every 1.7 s. The ratio of the fluorescence intensities (R in Ref. [6]) excited at 335 and 378 nm were recorded and stored for further analysis.

For calibration of the signals, the cells were permeabilized with 20 μM digitonin in the presence of 2 mM EDTA to obtain the minimal fluorescence ratio (R_{min}, Ref. [6]). Thereafter, 50 mM $MgCl_2$ was added to obtain the maximal fluorescence ratio (R_{max}, Ref. [6]). $[Mg^{2+}]_i$ was calculated according to the formula of Grynkiewicz et al. [6], using a K_d of 1.5 mM for the Mg^{2+}-Mag-Fura-2 complex [7], after subtraction of the autofluorescence, which was identically measured in cells, not loaded with Mag-Fura-2. Collected data were plotted by means of the graphic software GRAPHPAD (ISI software).

3. RESULTS AND DISCUSSION

In non-loaded liver perfused with Mg^{2+}-free solution β-adrenergic stimulation induced net Mg^{2+} efflux which was measured from the increase of Mg^{2+} in the per-

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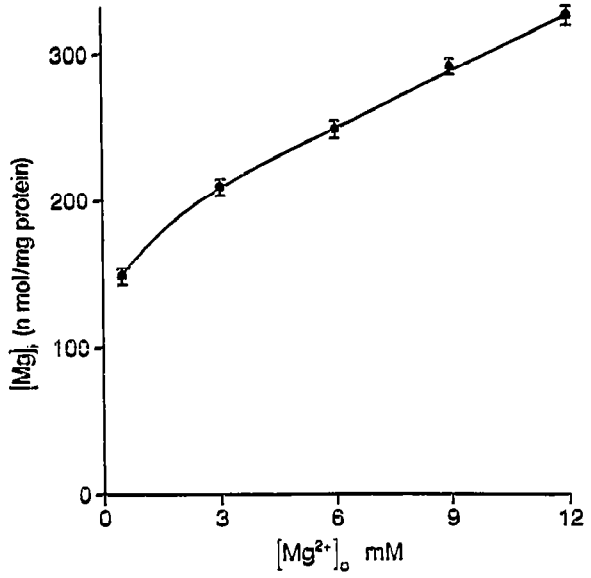

Fig. 1. Mg^{2+} content of rat thymocytes. The cells were loaded with Mg^{2+} by means of A23187 in the presence of 3, 6, 9 and 12 mM $MgCl_2$. Mean ± S.E.M. of 4 experiments.

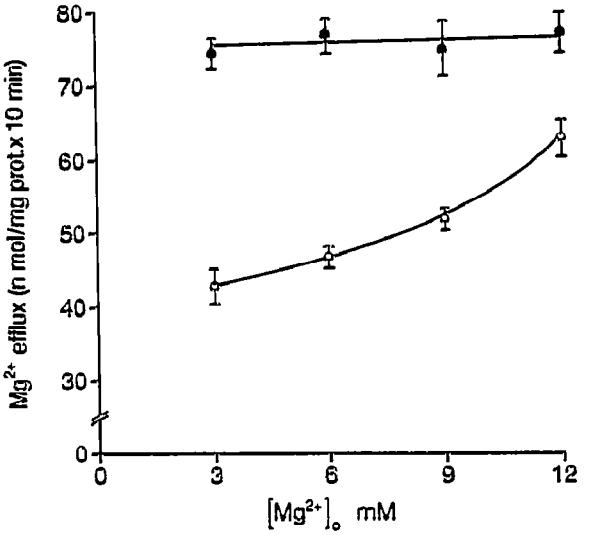

Fig. 2. Mg^{2+} efflux from Mg^{2+}-loaded rat thymocytes. The cells were loaded with Mg^{2+} as shown in Fig. 1. (●), Mg^{2+} efflux in the presence of 0.1 mM db cAMP; (○), Mg^{2+} efflux without addition of db cAMP. Mean ± S.E.M. of 4 experiments.

fusate. This Mg^{2+} efflux amounted only to 2% of total liver Mg^{2+} [4,8]. In non-loaded thymocytes 0.1 mM db cAMP induced a small net Mg^{2+} efflux, which was not significant (data not shown).

In thymocytes, because of the low cell concentration, Mg^{2+} efflux was measured from the decrease in cellular Mg^{2+} content. A minor loss of Mg^{2+} from non-loaded thymocytes similar to hepatocytes cannot lead to a significant difference in total cellular Mg^{2+}. Therefore, we measured Mg^{2+} efflux from Mg^{2+}-loaded thymocytes. In these cells Mg^{2+} efflux is operating via Na^+/Mg^{2+} antiport [1]. The increase of total Mg^{2+} by loading thymocytes at different extracellular Mg^{2+} concentrations ($[Mg^{2+}]_0$) is shown in Fig. 1. At the usual conditions [1] thymocytes were loaded at $[Mg^{2+}]_0$ = 12 mM. In these cells, 0.1 mM db cAMP increased Mg^{2+} efflux by 23% (Fig. 2).

In trout erythrocytes isoproterenol or cAMP increased Na^+/H^+ antiport by increasing the affinity of the Na^+/H^+ antiporter for intracellular H^+ [9]. Therefore, we tested whether cAMP also increased the affinity of the Na^+/Mg^{2+} antiporter for intracellular Mg^{2+}. For this purpose, we measured Mg^{2+} efflux from thymocytes which were loaded at an $[Mg^{2+}]_0$ of 3, 6, 9 and 12 mM. As shown in Fig. 2, the rate of Mg^{2+} efflux increased with elevated $[Mg^{2+}]_i$ (Fig. 2). A Hill plot of Mg^{2+} efflux showed that the Hill coefficient increases with Mg^{2+}-loading (Fig. 3). This may be explained by an increasing cooperativity at higher $[Mg^{2+}]_i$. After addition of 0.1 mM db cAMP, Mg^{2+} efflux was increased to approximately the same maximal rate, independent of Mg^{2+}-loading (Fig. 2). This result offers two explanations. First, it is possible that db cAMP increased the affinity of the Na^+/Mg^{2+} antiporter for intracellular Mg^{2+}, so that maximal Mg^{2+} efflux rates were obtained also at lower Mg^{2+}-loading. This finding is in agreement with the effects of cAMP on the activity of Na^+/H^+ antiport [9].

Second, the possibility exists that db cAMP released some Mg^{2+} which was stored in cell organelles of Mg^{2+}-loaded and non-loaded cells. This effect was proposed for hepatocytes, non-loaded with Mg^{2+}, in which cAMP induced Mg^{2+} efflux from mitochondria and in consequence Mg^{2+} efflux from the cells [10], probably via a transient increase in $[Mg^{2+}]_i$.

In order to ascertain whether a transient increase in $[Mg^{2+}]_i$ occurred in thymocytes, we measured the effect of 0.1 mM db cAMP on $[Mg^{2+}]_i$ in non-loaded and Mg^{2+}-loaded thymocytes. As shown in Fig. 4, in non-loaded and Mg^{2+}-loaded thymocytes, addition of 0.1

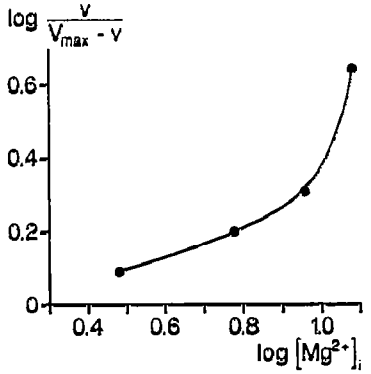

Fig. 3. Hill plot of Mg^{2+} efflux. Mg^{2+} efflux in the absence of db cAMP was plotted. Values taken from Fig. 2 (V_{max} represents Mg^{2+} efflux in the presence of db cAMP).

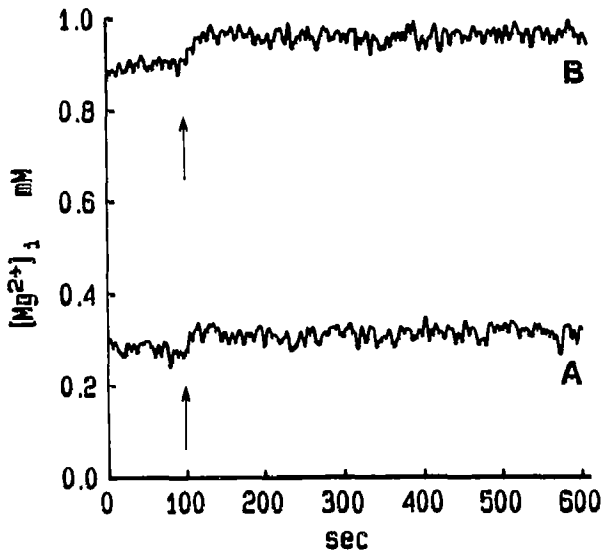

Fig. 4. Effect of db cAMP on the concentration of intracellular free Mg^{2+}. Arrows indicate the addition of 0.1 mM db cAMP to the cells. A, control cells, non-loaded with Mg^{2+}; B, cells loaded in the presence of 3 mM $MgCl_2$. A typical experiment was plotted for each curve.

mM db cAMP induced the same very small increase in $[Mg^{2+}]_i$ which amounted to 0.05 mM. In isolated hepatocytes, 0.1 mM db cAMP had no significant effect on $[Mg^{2+}]_i$ [8].

Since Mag-Fura-2 also reacts with Ca^{2+} (K_d of the Mag-Fura-2-Ca^{2+} complex is 53 μM [7]), we measured intracellular free Ca^{2+} ($[Ca^{2+}]_i$) by means of Fura-2 in analogy to the determination of $[Mg^{2+}]_i$. Under the same experimental conditions $[Ca^{2+}]_i$ amounted to 140 μM, and 0.1 mM db cAMP had no effect on $[Ca^{2+}]_i$ (data not shown). Therefore, the small increase in $[Mg^{2+}]_i$ was not caused by an increase in $[Ca^{2+}]_i$.

It is unlikely that the small rise in $[Mg^{2+}]_i$ by db cAMP has enhanced the rate of Na^+/Mg^{2+} antiport in thymocytes. Therefore, cAMP may have augmented the affinity of the Na^+/Mg^{2+} antiporter for intracellular Mg^{2+}, thus inducing a net Mg^{2+} efflux. Since $[Mg^{2+}]_i$ remained nearly constant during net Mg^{2+} efflux, the released Mg^{2+} must be substituted by Mg^{2+} release from intracellular Mg^{2+} stores, e.g. the mitochondria. In experiments with permeabilized hepatocytes cAMP induced Mg^{2+} efflux from this organelle [10].

It is likely that db cAMP induced phosphorylation of the Na^+/Mg^{2+} antiporter (or a protein related to this transport system). Also the Na^+/H^+ [11,12] and Na^+/Ca^{2+} antiporter [13,14] were phosphorylated by various activators.

Such a mechanism would be in agreement with properties of Na^+/H^+ and Na^+/Ca^{2+} antiport which, after phosphorylation, showed a higher affinity for intracellular H^+ [9] or Ca^{2+} [13].

However, addition of 1 μM okadaic acid to Mg^{2+}-loaded thymocytes had no effect on Mg^{2+} efflux, neither in the presence nor absence of db cAMP (data not shown). This result may indicate that the addition of 0.1 mM db cAMP causes maximal phosphorylation and that without db cAMP, the Na^+/Mg^{2+} antiporter may not be phosphorylated.

REFERENCES

[1] Günther, T. and Vormann, J. (1990) Magnesium Trace Elem. 9, 279–282.
[2] Vormann, J. and Günther, T. (1987) Magnesium 6, 220–224.
[3] Romani, A. and Scarpa, A. (1990) Nature 346, 841–844.
[4] Romani, A. and Scarpa, A. (1990) FEBS Lett. 269, 37–40.
[5] 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) Analyt. Biochem. 150, 76–85.
[6] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[7] Murphy, E., Freudenrich, C.C., Levy, L.A., London, R.E. and Lieberman, M. (1989) Proc. Natl. Acad. Sci. USA 86, 2981–2984.
[8] Günther, T., Vormann, J. and Höllriegl, V. (1991) Magnesium Bull., in press.
[9] Motais, R., Scheuring, U., Borgese, F. and Garcia-Romeu, F., in: J.M. Ritchie, P.J. Magistretti and L. Botis (eds.) Progress in Cell Research Vol. 1, Elsevier, 1990, pp. 179–193.
[10] Romani, A., Dowell, E., Fatholahi, M. and Scarpa, A. (1991) FASEB J. 5, A 1309.
[11] Livne, A.A., Sardet, C. and Pouyssegur, J. (1991) FEBS Lett. 284, 219–222.
[12] Bianchini, L., Woodside, M., Sardet, C., Pouyssegur, J., Takai, A. and Grinstein, S. (1991) J. Biol. Chem. 266, 15406–15413.
[13] Caroni, E. and Carafoli, E. (1983) Eur. J. Biochem. 132, 451–460.
[14] DiPolo, R. and Beauge, L. (1987) Biochim. Biophys. Acta 897, 347–354.