Effect of concanavalin A and extracellular magnesium on the concentration of intracellular free Ca^{2+} in thymocytes from normal and Mg-deficient rats

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Zusammenfassung

In den Thymozyten von Mg-Mangelratten war der Ca^{2+}-Gehalt verdoppelt. Die Konzentration an freiem Ca^{2+} ($[Ca^{2+}]_i$) unterschied sich nicht signifikant von der in normalen Thymozyten, d. h. in Mg-Mangel-Thymozyten ist mehr Ca^{2+} gespeichert als in normalen Thymozyten. Nach Zugabe von 8 µg/ml Concanavalin A wurde die $[Ca^{2+}]_i$ in normalen und Mg-Mangel-Thymozyten verdoppelt. Der Anstieg der $[Ca^{2+}]_i$ erfolgt durch Ca^{2+}-Influx. Dieser Ca^{2+}-Influx und die Zunahme von $[Ca^{2+}]_i$ wurden durch 0,1 mM Verapamil und 4 mM extrazelluläres Mg^{2+} nicht beeinflußt. Die im Mg-Mangel verminderte Immunreaktion ist nicht durch ein verändertes Ca^{2+}-Signal, sondern durch andere biochemische Mechanismen bedingt.

Summary

In thymocytes from Mg-deficient rats, total Ca^{2+} content was doubled. The concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was not significantly different from normal thymocytes. Thus, in Mg-deficient thymocytes, more Ca^{2+} is stored intracellularly than in normal thymocytes. After addition of 8 µg/ml concanavalin A, $[Ca^{2+}]_i$ was doubled both in normal and Mg-deficient thymocytes. The increase in $[Ca^{2+}]_i$ is produced by Ca^{2+} influx. This Ca^{2+} influx and the increase-in $[Ca^{2+}]_i$ was not affected by 0.1 mM verapamil or 4 mM extracellular Mg^{2+}. The diminished immune response in Mg deficiency is not caused by a defective Ca^{2+} signal. Other biochemical mechanisms must be responsible.

Résumé

La quantité totale de Ca^{++} a été doublée dans les thymocytes de rats présentant une carence en magnésium. La concentration intracellulaire de CA^{++} libre ($[Ca^{++}]_i$) n'était pas significativement différente de celle de thymocytes normaux. Cependant, dans les thymocytes déficients en Mg, la concentration intracellulaire de Ca^{++} était supérieure à celle de thymocytes normaux. Après addition de 8 µg/ml de concanavalline A, $[Ca^{++}]_i$ a doublé, tant dans les thymocytes normaux que dans ceux carencéx en Mg. L'augmentation de $[Ca^{++}]_i$ est due à l'influx de Ca^{++}. 0,1 mM de vérapamil ou 4 mM de Mg^{++} extracellulaire n'ont fait varier ni cet influx de Ca^{++} ni cette augmentation de $[Ca^{++}]_i$. La Diminution de la réaction immune en cas de carence en magnésium n'est pas due à une altération du stimulus des ions Ca^{++}, mais vraisemblablement à d'autres mécanismes biochimiques.

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Introduction

The alteration of cellular Ca^{2+} metabolism may play a major role in the pathobiochemical mechanism of Mg deficiency [5]. So, in Mg deficiency total intracellular $Ca^{2+}$is increased in various tissues [5] and in thymocytes [1]. Cell membrane permeability for ions [5] and compartmentation of intracellular Ca^{2+} [7] are changed. Ca^{2+} influx has been found to be increased [5].
A changed phospholipid metabolism [2] and an increased biosynthesis of prostaglandins [12] were determined as possible consequences of an altered Ca^{2+} metabolism in thymocytes from Mg-deficient rats. However, intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), which is a second messenger and regulator of many metabolic reactions, was not measured in Mg-deficient cells.
In the cascade of biochemical events that lead to mitosis, as can be studied by ConA stimulation of T-lymphocytes, an early stimulation of Ca^{2+} influx [9] and an increase of $[Ca^{2+}]_i$ for a few hours are essential [8, 9, 10, 13, 14].
In Mg deficiency, a reduction of humoral and cellular immunity are observed [4] and ConA stimulation of DNA synthesis in thymocytes is reduced by 40 % [6]. To evaluate the role of $[Ca^{2+}]_i$ in the diminished immune response in Mg deficiency, we measured $[Ca^{2+}]_i$ in thymocytes from Mg-deficient rats after stimulation by ConA. Since in ConA stimulation of lymphocytes an increase of Ca^{2+} influx is involved, we, additionally, tested whether an increase of extracellular Mg^{2+} concentration can act as a Ca^{2+} antagonist in this experimental system.

Materials and Methods

Female Wistar rats, weighing 70 g, were fed an Mg-deficient diet (Mg^{2+} content: 3 mmol/kg, Ca^{2+} content: 250 mmol/kg, Ssniff, Soest, FRG) and distilled water ad libitum for 8 – 10 weeks. Control rats were fed the same food supplemented with $MgCl_2$ to an Mg^{2+} content of 80 mmol/kg.
Thymocytes were isolated by sieving and washing in Hepes-buffered salt medium (HBS).

HBS contained (in mM): 145 NaCl, 5 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 glucose and 10 Hepes, pH 7.4. Usually, the thymocytes of two rats were pooled. The thymocytes were loaded with quin-2/AM according to Tsien et al. [15]. Briefly, the thymocytes (10^8 cells/ml) in HBS were incubated with 15 µM quin-2/AM (Sigma, freshly dissolved in dimethylsulfoxide, DMSO) for 10 min at 37 ° C. Fluorescences were recorded with a Perkin-Elmer 650-10 S fluorescence spectrophotometer. Standard monochromator settings were 339 nm excitation with 4 nm slits; 492 nm emission with 10 nm slits.

Under these conditions a constant flourescene (F) was measured for 1 – 2 min. After addition of 10 µl 5 mM digitonin (dissolved in DMSO) F_{max} was measured. For the measurement of F_{max} in Ca^{2+}-free tests, 1 mM $CaCl_2$ was added. For the measurement of F_{min} 10 µl 200 mM EGTA and 10 µl 2 M Tris were added resulting in a pH > 8.3. $[Ca^{2+}]_i$ was calculated according to

$$[Ca^{2+}]_i = K_D \times \frac{F - F_{min}}{F_{max} - F}.$$

For K_D a value of 115 nM was taken (15).

Cell viability was tested by trypan blue staining. Under these conditions less than 10 % thymocytes were stained.

Aliquots of the isolated thymocytes were washed twice in Ca^{2+}-free HBS, containing 0.1 mM EGTA, freeze-dried, and ashed in the Plasma Processor 200 E (Technics, GmbH). The ash was dissolved in 200 µl 0.1 N HCl and the Ca^{2+} content was measured in the atomic absorption spectrophotometer (Philips, SP 9) by microinjection. In the serum of the normal and Mg-deficient rats, the Mg^{2+} concentration was also measured by atomic absorption spectrophotometry.

Results and Discussion

The Mg-deficient rats had developed the usual symptoms of Mg deficiency: transiently red ears, a minor increase in body weight and drastically reduced serum Mg^{2+}-concentration (Tab. 1). The Ca^{2+} content of the thymocytes from Mg-deficient rats was twice the normal value (Tab. 1).

In normal rat thymocytes, a $[Ca^{2+}]_i$ of 145 nM was measured (Tab. 2). Other authors obtained similar results: 123 nM in lymphocytes from nodes and thymus [14], or 136 nM and 118 nM, respectively, for the same cells [15]. *Hesketh* et al. [8] measured 110 nM in thymocytes. A recalculation, using the usual dissociation constant of the quin-2-Ca^{2+} complex of 115 nM [14, 15], resulted in a free Ca^{2+} concentration of 142 nM.

Ten minutes after addition of 8 µg/ml ConA, $[Ca^{2+}]_i$ was increased by the factor 2.21. Identical results were obtained by other authors [8, 9, 10, 13, 14]. The increase in $[Ca^{2+}]_i$ by ConA is caused by increased Ca^{2+} influx, as it can be abolished by omission of extracellular Ca^{2+} and addition of EGTA during reincubation after loading the cells with quin-2.

In thymocytes from Mg-deficient rats, $[Ca^{2+}]_i$ was not significantly lower than in normal thymocytes and the increase of $[Ca^{2+}]_i$ by ConA was somewhat, but not significantly, lower than in normal thymocytes. Although total Ca^{2+} content was doubled in Mg-deficient thymocytes, $[Ca^{2+}]_i$ was not increased. This result indicates that in Mg-deficient thymocytes more Ca^{2+} is stored intracellularly than in normal thymocytes.

Tab. 1: Serum Mg^{2+} concentration and Ca^{2+} content in thymocytes from normal and Mg-deficient rats

	$[Mg^{2+}]$ in serum mM	$[Ca^{2+}]$ in thymocytes mmol/kg dry weight
normal rats	0.87 ± 0.02	0.44 ± 0.03
Mg-def. rats	0.30 ± 0.01	0.80 ± 0.05

In unstimulated and ConA-stimulated thymocytes from normal and Mg-deficient rats, $[Ca^{2+}]_i$ was independent of extracellular Mg^{2+} concentration within the tested range of up to 4 mM. This result indicates that the ConA-stimulated Ca^{2+} channel is not affected by Mg^{2+}. Interestingly, this Ca^{2+} channel was also not affected by 0.1 mM verapamil which inhibits Ca^{2+} influx in other cells, e. g. smooth muscle cells. Also in B-lymphocytes, verapamil had no effect on the increase of $[Ca^{2+}]_i$ by anti-Ig antibodies [3]. Probably, only voltage-dependent Ca^{2+} channels are sensitive to verapamil [11] or high extracellular Mg^{2+} concentration.

The quin-2-method is based on the intracellular incorporation of a rather high concentration of a Ca^{2+}-binding and Ca^{2+}-buffering fluorescent substance. Under normal conditions, extracellular Ca^{2+} is taken up intracellularly together with quin-2 when using this procedure. Therefore, this method measures the capacity of the $[Ca^{2+}]_i$-regulating system, which consists of Ca^{2+} influx, Ca^{2+} efflux and intracellular Ca^{2+} storage after intracellular uptake of quin-2 and Ca^{2+} and redistribution of Ca^{2+}.

Reincubation of thymocytes from Mg-deficient rats in Ca^{2+}-free medium yielded a lower value of $[Ca^{2+}]_i$, which indicates that in Mg-deficient thymocytes the Ca^{2+}-storing and Ca^{2+} pumping mechanisms may have an increased activity, resulting in a lower steady-state concentration of $[Ca^{2+}]_i$, when extracellular Ca^{2+} and thus Ca^{2+} influx are lacking.

In earlier experiments with thymocytes from Mg-deficient rats, ConA stimulation of DNA synthesis was reduced by 40 % [6]. However, as found in the present experiments, $[Ca^{2+}]_i$ and the increase of $[Ca^{2+}]_i$ by ConA were similar both in normal and Mg-deficient thymocytes.

These results show that the reduced immune response in Mg deficiency is not caused by a defective Ca^{2+} signal.

However, the role of the increase of $[Ca^{2+}]_i$ in the immune response, which only occurs when thymocytes are stimulated with phytohemagglutinin or ConA, has not been defined. In the physiological stimulation of thymocytes by interleukin 2, an increase of $[Ca^{2+}]_i$ does not result [10].

Therefore, it can be concluded that in Mg-deficient thymocytes there is a defect in the cascade of biochemical events, following the unchanged Ca^{2+} signal, which reduces blast transformation and stimulation of DNA synthesis. So, for example, the metabolism of membrane phospholipids [2] and prostanoids [12] was changed in thymocytes from Mg-deficient rats. Because of the heterogeneity of thymocytes, the defect may occur only in a part of the Mg-deficient thymocytes, the rest may function in a normal manner.

Tab. 2: Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in thymocytes from normal and Mg-deficient rats measured by the quin-2 method after reincubation in media with various $MgCl_2$ concentrations ($[Mg^{2+}]_o$) with and without 8µg/ml ConA. Media contained 1 mM $CaCl_2$ or no $CaCl_2$, as indicated. Values are given as mean ± SEM. Number of experiments in brackets

Reincubation		Ø ConA	8 µg/ml ConA	
$[Mg^{2+}]_o$	$[Ca^{2+}]_o$	$[Ca^{2+}]_i$	$[Ca^{2+}]_i$	+ ConA
mM	mM	nM	nM	Ø ConA
normal thymocytes				
0	1.0	141 ± 18 (5)	318 ± 32 (5)	2.26
0.5	1.0	145 ± 7 (12)	321 ± 11 (11)	2.21
1.0	1.0	146 ± 31 (5)	311 ± 25 (5)	2.13
2.0	1.0	142 ± 11 (5)	317 ± 29 (5)	2.23
4.0	1.0	140 ± 34 (5)	328 ± 39 (10)	2.34
0.5	0			
+ 0.1 mM EGTA		122 ± 11 (8)	136 ± 18 (8)	1.11
0.5	1.0			
+ 0.1 mM Verapamil		161 ± 18 (5)	359 ± 29 (11)	2.23
Mg-def. thymocytes				
0	1.0	123 ± 14 (3)	265 ± 28 (3)	2.15
0.5	1.0	134 ± 7 (13)	277 ± 24 (13)	2.07
1.0	1.0	135 ± 25 (3)	270 ± 15 (3)	2.00
2.0	1.0	134 ± 24 (3)	286 ± 57 (3)	2.13
4.0	1.0	139 ± 24 (3)	313 ± 48 (7)	2.25
0.5	0			
+ 0.1 mM EGTA		68 ± 6 (3)	75 ± 10 (10)	1.10
0.5	1.0			
+ 0.1 mM Verapamil		145 ± 11 (3)	288 ± 26 (13)	1.99

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