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雑誌名	東京家政大学研究紀要 2 自然科学
巻	39
ページ	133-138
発行年	1999
出版者	東京家政大学
URL	http://id.nii.ac.jp/1653/00010665/

〔東京家政大学研究紀要　第39集　(1), p. 133～138, 1998〕

Cytosolic free calcium concentrations in Polymorphonuclear leukocytes from Magnesium-deficient rats

by

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(Received on September 30, 1998)

INTRODUCTION

Side by side with Ca,P,K,S,Na,Cl, and Si,Mg is an important element. Mg also has a great difference from Ca which has many similarities to Mg as element. That is because Mg has scant single and peculiar symptoms and signs and is often masked with coexisting other electrolytic abnormalies. In vivo, it is known that Mg has activating and catalyzing actions on enzymes, such as activation of phosphoric enzyme, reaction including ATP, action on many tissue phosphoric groups and so forth, playing a central role in the transfer, storage and utilization of energy. Especially, the correlation between Mg deficiency and cardiovascular diseases attracts attention. Arthura[1] reported that Mg deficiency caused thinning of peripheral arteries and arteriolae and subsequent hypertension. A study on the changes in the concentration of intracellular free Ca^{2+} during Mg deficiency was made in rat leukocytes with an aim at elucidating the defense mechanism of the body. It has been known that Ca^{2+} regulates various cellular functions. For direct measurement of intracellular Ca^{2+} concentration, an assay system using fluorescent probe quin2/AM was recently developed and its much detailed analysis has become possible. Andersoon[2] observed an increase in Ca^{2+} concentration following the stimulation with FMLP and indicated that the increase resulted from the influx of extracellular Ca^{2+} and Ca^{2+} release from its intracellular pool which were caused by stimulation with FMLP. Further, there was also a possibility that Ca^{2+} release from its pool occurs first after the stimulation with FMLP, resulting in Ca^{2+} influx. The present study was made to examine that Ca^{2+} is involved in the stimulus response of neutrophils of the Mg deficient rats.

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MATERIALS AND METHOS

1. Animal care and diets.

Twelve Sprague-Dawley strain male rats (120-150g body weight; Japan Clea Co., Tokyo) aged 5 weeks were individually housed in stainless steel wire bottom cages in a room maintained at 22-24℃ with 50% relative humidity. The room was lighted from 07:00-19:00. Animals were fed a basal diet (AIN-76TM) for 10 days. They were randomly assigned into two groups each containing 6 rats. As shown in Table 1, the basal diet was a purified casein-based diet based on AIN-76TM. Food and deionized water provided *ad libitum*.

2. Preparation of rat neutrophils

The rats were killed by exsanguination from abdorminal aorta using a syringe under light ether anesthesia on day 10 after fed with experimental diet. Blood samples were taken into a heparinized tube and diluted two fold with Hanks balanced salt solution(HBSS). Lymphoprep density gradient was used for the separation and sedimentation with a 1.5% (w/v) dextranT-500 solutions described by Suzuki[3]. Cells were isolated and stored in Dulbecco's phosphate

Table 1. Composition of experimental diets.

Constituents	Control	Deficient
	Amount (%)	
Casein	20.0	20.0
Cornstarch	15.0	15.0
Sucrose	50.0	50.0
Fiber	5.0	5.0
Corn oil	5.0	5.0
AIN Mineral mix	3.5	3.5 [1)]
AIN Vitamin mix	1.0	1.0
Choline bitartrate	0.2	0.2
DL-Methionine	0.3	0.3
Magnesium [2)]	500 ppm	14 ppm

These mixes were purchased from Oriental Yeast Co., Ltd., Tokyo and Wako Pure Chemical, Osaka.

1) AIN-76^{TM} mineral mix prepared without Mg (g/Kg): $CaHPO_4$, 500.0; NaCl, 74.0; $K_3C_6H_5O_7H_2O$, 220.0; K_2SO_4, 52.0; manganous carbonate, 3.5; ferric citrate, 6.0; $ZnCO_3$, 1.6; cupric carbonate, 0.3; KIO_3, 0.01; $CrK(SO_4)_2 \cdot 12H_2O$, 0.55; Sodium selenite, 860 μg.

2) MgO, 24.0; magnesium oxide was omitted from AIN-76^{TM} mineral mix and Mg was determined by atomic absorption spectrophotometry.

buffered saline (PBS). The trypan blue exclusion test exhibited 96% cell viability was observed in the final cell suspension.

3. Determination of cytosolic free Ca^{2+}

The content of Ca^{2+} of PMN were analyzed by using Rink's (fluorescent Ca^{2+} indicator, Quin 2/AM) method[4]. Spectrofluorimetric experiments were conducted on a HITACHI F-2000 fluorescence spectrophotometer (Ex339nm, Em 492nm). Double wave length excitation method were used in order to investigate how time-dependent changes in the fluorescence of quin 2/AM and FMLP dependent activation. PMN were stimulated by 10^{-7}M FMLP. A saline medium containing 145mM NaCl, 5mM KCl, 1mM$Na_2HPO_4 \cdot 12H_2O$, 1mM$CaCl_2$, 0.5mM $MgSO_4 \cdot 7H_2O$, and 5mM-glucose, buffered at pH7.4 with 10mM Hepes/$NaHCO_3$/100ml was used for all experiments. The hydrolysis of quin2/AM can display as monitor by changing of fluorescence spectrum. Quin2/AM was added to a final concentration of 30 μM, from a stock solution sus-

pended in dimethyl sulfoxide (DMSO, storage at −20℃). PMN were incubated for 20 min, after which quin2/AM was added the suspension was washed twice with PBS. Just before use, a sample of the cell suspension was centrifuged and resuspended in medium. Each cuvette was 5.0-10.0 x10^6 cells/ml and the cuvette holder was thermostated at 37℃ and equipped with a continuous stirring device. The fluorescence production of normal condition (F), after the addition of FMLP and 0.1%Triton X-100 (Fmax) for destroy of cell membrane were measured, respectively. Finally, 100mM ethylenebis tetraacetic acid (EGTA) was added in excess to chelate all calcium (Fmin). The formation of Ca^{2+} content was used as previously described by Rink[4] Ca^{2+} was calculated from the ratio of fluorescent intensities measured at 339nm excitation and 492nm emission using Rink's method. The formation was as follows;

$[Ca]i=Kd(F-Fmin)/(Fmax-F)$

An excellent fit is obtained to the theoretical behavior for simple 1 dye: 1 Ca binding with an effective dissociation constant Kd of 115nM. The values obtained using program for measurement of Ca^{2+} concentration were converted into Ca^{2+} concentration by the automatic data processing.

RESULTS

Body weight gain of Mg- deficient rats was shown as Fig.1. Table 2 shows the plasma Mg concentrations and white blood cell counts. The procedure was illustrated in Fig.2. Almost two dotted lines of fluorescence intensity are the effect of stimulation with FMLP and destroy of cell membrane by Triton. The Ca^{2+} level at time 0 was estimated from the fluorescence intensity following the addition of FMLP. The results of the examination of the activity of PMN and the involvement of Ca^{2+} indicated Ca^{2+} concentration after about one minute from the

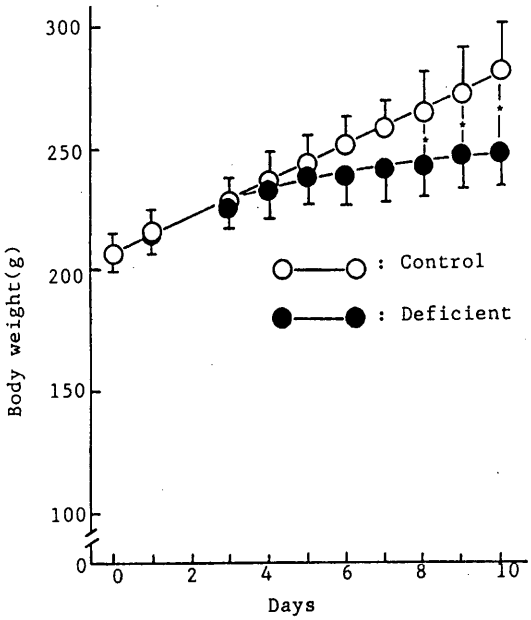

Fig.1 Body weight gain of magnesium deficient rats. The rats were fed AIN-76 purified diet for 10 day and then experimental diets for 10 days ad libitum. Results are expressed as means ± SD. * $P<0.05$.

stimulation by FMLP to be higher compared with the control group and the difference to be greater with time as shown in Fig.3. By scanning the changes in fluorescence intensity due to changing the Ca^{2+} concentration, its was shown that FMLP stimulus was transferred as a signal, resulting in a increase of the intracellular Ca^{2+} level. For measurement Ca^{2+} concentration, cell membrane was destroyed by adding Triton-x100 after stimulus response and the maximum Ca^{2+} level was determined. At the

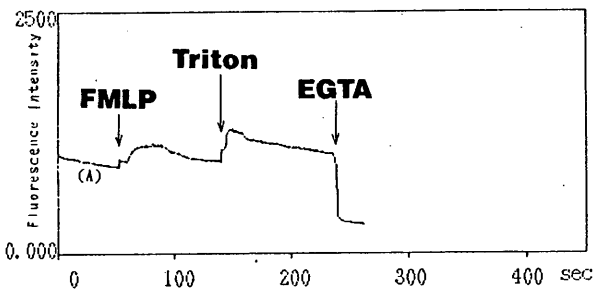

Fig.2 The procedure and Ca^{2+}-dependent fluorescence intensity by addition of FMLP. Triton. and EGTA.

Table 2. Plasma magnesium concentrations and white blood cell counts.

Day		4	7	10
Plasma Mg (μg/ml)	Cont.	12.73±0.81	13.58±3.49.	14.27±1.70
	Def.	7.94±1.79**	4.88±1.56**	2.68±0.32**
Total WBC (cells/mm^3)	Cont.	6,420±1,870	7,960±1,510	8,700±1,680
	Def.	7,860±1,590	25,230±11,780*	34,020±16,940**
Neutrophil (cells/mm^3)	Cont.	1,232±553	2,448±780	2,070±870
	Def.	1,091±637	6,904±2,703**	12,450±6,570**
Eosinophil (cells/mm^3)	Cont.	102±12	129±41	140±100
	Def.	99±16	986±386**	1,970±1,430**
Mononuclear (cells/mm^3)	Cont.	5,086±190	5,384±2,030	6,490±910
	Def.	6,671±221	15,217±5,956*	19,600±6430**

Results are expressed as mean ± SD. * $p<0.0$; * * $p<0.01$.

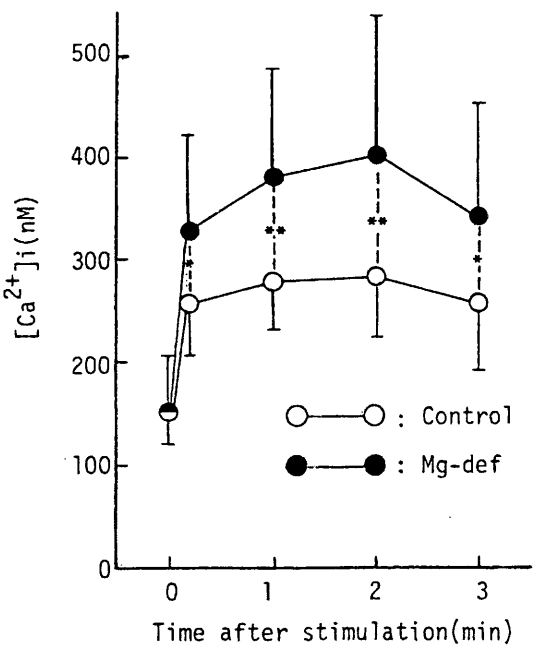

Fig.3 The concentration of Ca^{2+} in Mg-deficiency and control groups. Values are mean ±SD(n=6).
* $P<0.05$: * * $P<0.01$.

beginning of Ca^{2+} concentration was almost 150nM and is reach in approximately 400nM in Mg deficient group. Its amplitude is nearly 3-hold higher than that obtained in the normal condition. After about one minute from the stimulation by afmlpa in the Mg deficient group to be higher compared with control one in Ca^{2+} concentration.

DISCUSSION

From the above results, the present study suggests that the liberation from the intracellular storage site and the extracellular inflow are thought to be involved in the increase in intracellular Ca^{2+} concentration and the change immediately after the stimulation. The change with time are thought to reflect the liberation from the storage site and the intracellular inflow, respectively that Andersson suggested[2]. Korchak[5] has examined intracellular and extracellular calcium mobilization and correlated

these movements with the observed pattern of phosphoinositide remodeling using the three stimuli FMLP, concanavalinA, and phorbol myristate acetate (PMA). It was indicated that Ca^{2+}can be mobilized from intra-and extracellular pools; the pattern of calcium movements has been shown to very with the stimulus. In case of Mg deficiency, the intracellular Ca^{2+} concentration is increased not only immediately after the stimulation but with time, so that both the liberation from the storage site and also the extracellular inflow are thought to be elevated as shown in Fig.3. It was assumed that the opening control of Ca^{2+} channel and/or the metabolic turnover of inositolphospholipid might be involved in the increasing system for intracellular Ca^{2+} concentration, whereas a control system through cell membrane, some regulating factors of Ca^{2+} and/or a regulation system mediated by mitochondoria may be concerned in the reduction of Ca^{2+}. Intracellular Ca^{2+} has various effects on a wide range of cell functions, i.e., contractions of skeletal and smooth muscles, deformaion and movement of cells, cell division, phagocytosis and secretory activities of cells in the pancreas hypophysis and adrenals. Recently, it was suggested that Ca^{2+} is mediated in the release of transmitter substance from nerve endings and the membrane transforming mechanism of axons. It seemed essential for health care and protection of diseases to keep the homeostasis of Mg and other electrolytes. Further, it was found that Mg plays some important roles in various physiological activities such as peculiar membrane functions, nerve transmission, Ca^{2+} channel, ion transport and immune response. It is also necessary to investigate other fluorescent indicators such as fura-2 to measure Ca^{2+} concentration.

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SUMMARY

It has been investigated in order to clarify the relationship among magnesium(Mg) intake, Mg density mg/1000Kcal and various disease, Itokawa[6] reported that 150-300mg is optimal amount of Mg for recommended dietary intakes from evaluating dietary intake data in normal Japanese man. Recently, the amount of Mg intake tends to decrease by changes in the nutritional environment. Phagocytes such as polymorphonuclear leukocyte (PMN), monocyte or macrophage incorporate microorganisms and foreign matters intracellularly and kill them. This bacteriocidal process involves partially the degranulation which discharges the granular contents intraphagocytically or extracellularly and partially the formation of highly-toxic active oxygen by activating acid metabolism. PMN plays an important role in early vital defence mechanism though its phagocytosis on those microorganisms and bacteria which have invaded intracorporeally or its bacteriocidal action. On the other hand, Mg can be regarded as a natural weak $Ca2^{2+}$-antagonist since Mg has a competing effect on Ca^{2+}. Both elements are physiologically important minerals. In the present study, changes in free cytosolic Ca^{2+} concentration of isolated rat PMN cells following N-formyl-methionyl-leucyl-phenylalanine (FMLP) stimulation that a bacterial chemotactic peptide were measured using quin2 acetoxyl-methylester (quine2/AM) as an intracellular Ca^{2+} probe. Mg deficient group was higher than that of the control group in Ca^{2+} concentration. After about one minute from the stimulation by FMLP in the Mg deficient group to be higher compared with the control one in Ca^{2+} concentration. In Mg deficient rats, the intracellular Ca^{2+} concentration is increased not only immediately after the stimulation by FMLP but with time. It is considered the effect of the storage site and extracellular inflow on Ca^{2+} concentration.

これまでにMg摂取量（1000kcal当たりのMg濃度），種々の疾病等とMgの関係を明らかにするために検討がなされている。糸川は日本人におけるMgの目標摂取量として一日当たり150-300mgが適当であると報告している。近年、Mg摂取量は食環境の変化から減少傾向にあるとされている。ところで、多形核白血球（PMN)、単球、あるいはマクロファージの食作用は微生物や異物を体内に取り込みそれらを消化している。この殺菌作用の過程で食胞内あるいは細胞外の顆粒が一部脱顆粒を引き起こし活性酸化的代謝によって高い毒性を示す活性酸素を分泌している。PMNは体内に侵入した微生物、病原細菌に対して生体の一次防御反応において重要な役割を担っている。一方、MgはCaと弱い天然の拮抗作用を有していると考えられている。Ca、Mgともに生理学的に重要なミネラルであるが、本研究はラットより分離したPMNの遊離Ca濃度の変化を走化性ペプチドであるFMLPを刺激剤、quine2AMをプローブとして測定した。細胞内遊離Ca濃度はMg欠乏群では対照群より高値を示した。すなわち、Mg欠乏群においてFMLPによる刺激から約1分後には対照群と比較し高値を示した。Mg欠乏群では細胞内遊離Ca濃度はFMLPによる刺激直後ばかりではなく時間の経過とともに増加した。このことは、細胞内Ca濃度が貯蔵部位からと細胞外からの流入の両方が影響していると考えられた。