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SHORT COMMUNICATION

Defibrination Essential in the Assay of Ionized Magnesium in Mononuclear Cells

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Summary: Intracellular free (ionized) magnesium concentration was measured in mononuclear cells isolated from healthy volunteers by use of dual wavelength fluorescence (indicator: mag-fura-2). We found a free Mg^{2+} concentration of 1.28 ± 0.08 mmol/l in mononuclear cells isolated from heparinized blood. When we defibrinated blood samples prior to the isolation step we measured 0.78 ± 0.05 mmol/l of free Mg^{2+} in these cells. We conclude that this difference is caused by the platelets present in the heparinized specimens.

Introduction

Only 1% of the total body magnesium (Mg) is present in the extracellular fluid. Low plasma concentrations of Mg^{2+} have been reported in patients with autosomal inherited hypomagnesaemia (1) without symptoms of any significance. Measurement of the intracellular free magnesium content of mononuclear cells may contribute to a more accurate assessment of the magnesium status of patients with hypomagnesaemia (3) and the interpretation of the clinical symptoms. Therefore we developed a method for the measurement of intracellular ionized Mg^{2+} concentration. *Raju* et al. (2) developed the fluorescent probe mag-fura-2 (Furaptra) to measure intracellular free Mg^{2+} concentration. This probe – with a K_d of 1.5 mmol/l – is insensitive to pH and ionized Ca^{2+} in the physiological range. Because of the high amount of platelets in the isolated mononuclear cell fraction we studied the difference in the intracellular ionized Mg^{2+} concentration between the procedure with defibrination prior to the isolation step and without. The total cell volume of all platelets is approximately three times the total volume of all mononuclear cells. Moreover the concentration of the intracellular mag-fura-2 after loading the cells with this fluorescent probe may be different between both types of cell suspensions. The contributions of the fluorescence signals coming from platelets and mononuclear cells depends not only on the difference in the amount of cells and their volume but also on the difference in loading the fluorescent probe. This makes interpretation of the fluorescent signals difficult, if not impossible. Control over the contribution to the fluorescent signal by the platelets in the final cell suspension is therefore of great importance.

Materials and Methods

Mononuclear cells were isolated using the method of *Elin & Johnson* (3) from blood obtained from healthy persons using Ficoll-Paque (density 1.077 kg/l). We used either 20 ml heparinized or 30 ml defibrinated blood as described by *de Abreu* et al. (4). The mononuclear cells and platelets were counted on a Sysmex NE 8000 (TOA Medical Electronics Co, Kobe Japan). The viability of these cells was controlled by staining with trypan blue. After isolation the cells were loaded with the probe, using a solution containing 0.2 g/l pluronic-127 (Molecular Probes, Eugene, Oregon), 4.8 µmol/l mag-fura-2AM (Molecular Probes, Eugene, Oregon), 10 ml RPMI-1640 medium (Sigma, St Louis MO, USA) during 45 minutes at 37 °C. The cells were washed at room temperature in *Hank*'s balanced salt solution: 80 mmol NaCl, 42 mmol KCl, 5.6 mmol glucose, 0.5 mmol KH_2PO_4, 0.3 mmol Na_2HPO_4 per litre with an osmolality of 250 mosm/kg and a pH 7.4 adjusted with Tris. This solution was also used as medium of measurement.

We used a dual-wavelength excitation spectrofluorometer (Shimadzu RP-5000) with excitation wavelength alternating between 336 nm and 368 nm (bandwidth 5 nm). Fluorescent emission was measured at 495 nm (bandwidth 5 nm). After 10 minutes at 37 °C for temperature equilibration, the resting level of fluorescence was measured with a final cell density of approximately 3×10^9/l. To equilibrate magnesium over the cell membrane for calibration purposes, 7.5 µmol/l of the Mg^{2+}-H^+-ionophore: 5-bromo-A23187 (Molecular Probes, Eugene, Oregon) was used in the presence of 10 µl 10 mmol/l nigericine (Molecular Probes, Eugene, Oregon) to speed up the equilibration process (2). Zero Mg^{2+} concentration and various Mg^{2+} concentrations were set with EGTA and in steps from 0 mmol/l to 21.38 mmol/l respectively. The resting level of intracellular Mg^{2+} was calculated using the *Grynkiewicz* equation (5).

Results

Over 95% of mononuclear cells showed trypan blue exclusion before the loading procedure with mag-fura. Fifty-five percent

(n = 20) and 47% (n = 20) of mononuclear cells were recovered from heparinized and defibrinated blood respectively. Microscopic examination of the harvested cells in the final suspension shows the composition of the cell population as follows (mean ± 1 SD): lymphocytes 90 ± 3%; polynuclear cells 4 ± 2% and monocytes 6 ± 3%. There was no difference in the composition of the cell suspensions between the heparinized and defibrinated samples. In heparinized plasma we found a platelets/mononuclear cells ratio of 38 and in defibrinated plasma of 0.7.

The intracellular ionized Mg^{2+} content in mononuclear cells from heparinized and defibrinated blood is shown in table 1. Statistical analysis was performed by the *Student*'s t-test for the difference of the means between the two types of cell suspensions.

Discussion

The mean resting concentrations of intracellular ionized Mg^{2+} in mononuclear cells isolated from heparinized (1.28 mmol/l) or defibrinated blood (0.78 mmol/l) are significantly different ($p < 0.001$). We believe this is caused by the presence of platelets in the experiments with heparinized blood.

Supposing about the same intracellular conditions in platelets and mononuclear cells, the cell types will show the same calibration curve for the Mg-probe. However, the calculation of intracellular ionized Mg^{2+} concentration can have any outcome depending on relative cell volume and dye loading in the mixed cell population. The platelet concentration in the final cell suspension from heparinized blood was approximately 100×10^9/l wheras in the cell suspension from defibrinated blood the platelet concentration was only approximately 2×10^9/l. This signal coming from the intracellular free Mg^{2+} in platelets in both cell suspensions cannot be used as background fluorescence since the intensities change due to the measuring procedure.

Resting concentrations of intracellular free Mg^{2+} in platelets and mononuclear cells will probably not be the same, compounding the problem. *Matsuno* et al. (6) found a concentration of 0.54 ± 0.14 mmol/l cell water for ionized Mg^{2+} concentration in resting blood platelets. *Ng* et al. (7) reported 0.19 ± 0.02 mmol/l in mononuclear cells and these authors reported no data about the presence of platelets in their final suspension.

Considering the theoretical arguments and our experimental findings, we recommend eliminating the platelets prior to the isolation of mononuclear cells for the assay of the (ionized) Mg^{2+} concentration in cell suspensions using the Mg-sensitive probe mag-fura-2.

Tab. 1 The resting ionized Mg^{2+} in mononuclear cells

Mononuclear cells isolated from	Ionized magnesium	
	(mmol/l) mean ± SD	(fmol/cell) mean ± SD
heparinized blood n = 20	1.28 ± 0.08	0.13 ± 0.01
defibrinated blood n = 13	0.78 ± 0.05	0.08 ± 0.01

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