Changes in free cytoplasmic magnesium following activation of human lymphocytes

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Activation of lymphocytes with 10 μM ionomycin leads to a rapid increase in the concentration of free cytoplasmic calcium ([Ca2+]i) and, at a slower rate, also to an increase in the cytoplasmic free magnesium concentration ([Mg2+]i). The ionomycin-induced Mg2+-mobilization response is dependent on the influx of extracellular Ca2+. After receptor-mediated lymphocyte activation, induced by mitogens or anti-receptor antibodies, a Mg2+-mobilization response does occur in a small fraction of the cells. Simultaneous measurement of [Ca2+]i and [Mg2+]i, in individual cells showed that the receptor-triggered Mg2+-mobilization response is restricted to cells that have a high [Ca2+]i. It can therefore be concluded that a high [Ca2+]i induces the release into the cytoplasm of Mg2+ from intracellular stores.

INTRODUCTION

Ligation of the antigen receptors of B-lymphocytes and T-lymphocytes by specific antigen or anti-receptor antibody leads to hydrolysis of PtdIns(4,5)P2 by a phospholipase C, generating diacylglycerol and Ins(1,4,5)P3 [1–3]. Ins(1,4,5)P3 mediates the release of intracellular stored Ca2+ into the cytoplasm; the influx from extracellular Ca2+ is probably mediated by InsP2 and InsP3 [4]. Under conditions of elevated concentrations of cytoplasmic Ca2+ ([Ca2+]i), diacylglycerol stimulates the translocation and activation of protein kinase C [5].

Most cellular receptors which activate phospholipase C are coupled to this enzyme via a GTP-binding protein (G-protein). The primary structure of the antigen receptor on T-lymphocytes and that on B-lymphocytes precludes direct interaction with G-proteins, but, owing to association with src-family tyrosine kinases, more likely is coupled to PLC activation via a phosphorylative event. Both the T-cell receptor and the antigen receptor on B-lymphocytes are complex hetero-oligomeric structures, composed of different subunits. The core receptor, responsible for ligand binding, consists of membrane immunoglobulin in B-lymphocytes and of the αβ T-cell receptor in most T-lymphocytes. Non-covalently associated with membrane immunoglobulin are disulphide-linked heterodimers composed of α-β subunits which contain sites that are tyrosine-phosphorylated after receptor ligation [6]. The T-cell receptor is associated with the CD3 complex, encompassing γ, δ, ε and ζ subunits. The associated structures of the antigen receptors on both T- and B-lymphocytes are required for efficient cell surface expression. In T- and B-lymphocytes, the cytoplasmic domain of the antigen–receptor complexes are associated with the src-family tyrosine kinases, fyn and lyn respectively, which are activated after receptor cross-linking [7,8].

The transient change in [Ca2+]i is up to now the only biochemical event in the complex process of receptor-triggered cell activation that can reliably be measured at the single-cell level (see [9–11] for review). The role of (changes in the intracellular concentration of) other bivalent cations in early events of cell activation is less clear.

Mg2+ ions are the most abundant bivalent cations in cells. Their concentration is critical for many cellular functions, such as DNA transcription, protein synthesis, glycolysis and oxidative phosphorylation [12]. Magnesium also appears to be an essential factor for a number of enzymes in the phosphatidylinositol shunt [13–16]. Recently it has been reported that the activity of a novel phosphatidylinositol-specific phospholipase C, which catalyses the conversion of phosphatidylinositol into diacylglycerol and inositol 1-phosphate, is regulated by Mg2+ [17]. This enzyme activity is found exclusively in membrane preparations of large B-cells, and not in small dense B-cells [17]. Since these data suggest a potential regulatory role for Mg2+ in receptor-mediated lymphocyte activation, we were interested in potential changes in [Mg2+]i in T- and B-cells following activation. The availability of mag-indo-1, a Mg2+-sensitive fluorochrome, made this a feasible goal.

MATERIALS AND METHODS

Cells

Peripheral-blood mononuclear cells were isolated by Ficoll Isopaque density-gradient centrifugation of heparinized blood from healthy adult donors, and were washed and suspended in RPMI-1640 medium (Gibco, Grand Island, NY, U.S.A.). T cells were separated from non-T-cells by rosetting with 2-aminoethylisothiouronium bromide–treated sheep red blood cells (EAET), followed by density-gradient centrifugation. The T-cell fraction was treated with NH4Cl to induce lysis of EAET. Non-T-cells consisted in general of 55–65% surface Ig+ B cells, less than 3% EAET-rosetting T cells, and 15–30% monocytes.

The Burkitt lymphoma cell line BL41 gpl-1 (kindly given by Dr. C. D. Gregory, Department of Cancer Studies, University of Birmingham, Birmingham, U.K.) was grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal-calf serum.

Loading procedures

Cells were washed twice and suspended in RPMI-1640 medium at a concentration of 10 × 106/ml. Mag-indo-1/AM (Molecular Probes, Eugene, OR, U.S.A) was dissolved in dimethyl sulph-
oxide (Merck, Darmstadt, Germany) at a concentration of 1 mM and was added directly to the cells in a final concentration of 2 μM. Cells were incubated with mag indo-1/AM for 20 min at 37 °C. Cells were washed with RPMI 1640 and resuspended at 1 × 10^6/ml in assay buffer (145 mM NaCl, 5 mM KCl, 1 mM NaHPO4, 5 mM glucose, 10 mM Hepes, pH 7.4) at 5 °C. Next, cells were washed with RPMI 1640 and resuspended in 1 × 10^6/ml assay buffer (145 mM NaCl, 5 mM KCl, 1 mM NaHPO4, 5 mM glucose, 10 mM Hepes, pH 7.4) at 5 °C.

For measurement of [Ca2+], [Mg2+], and [Mg2+] in, cells were loaded simultaneously with 4 μM fluo-3/AM, 0.4 μM SNARF-1/AM (seminalphthorhodafluor), and 2 μM mag indo-1/AM and otherwise processed as described above. The combination of fluo-3 and SNARF-1 allows use of the ratio of the fluorescence intensity of both fluorochromes as a parameter for changes in [Ca2+]. This procedure decreases the effects of variation in fluo-3 fluorescence intensity caused by variation in fluo-3 uptake [18].

For determination of the mag indo-1 fluorescence spectrum, mag indo-1-loaded cells were washed and resuspended at a concentration of 0.5 × 10^6/ml in assay buffer. Then 10 μl of this suspension was added to 2 ml of graded Mg2+ buffers or graded Ca2+ buffers. The buffers were identical in composition with the assay buffer as indicated above, apart from a variable concentration of MgSO4 or CaCl2. Cells were lysed by addition of 10 μl of 10% Triton X-100. Emission spectra were recorded on a Hitachi F-401 0 fluorimeter with excitation wavelength set at 355 nm (10 nm band-pass). The emission spectrum, ranging from 360 to 550 nm with a 10 nm band-pass setting, was recorded at 20 °C.

Flow-cytometric analysis of [Mg2+] and [Ca2+].

Flow-cytometric analysis of [Mg2+] with the Mg2+-sensitive dye mag indo-1 AM was carried out with a FACS Analyser flow-cytometer (Becton Dickinson) equipped with an Ushio 102 D mercury arc lamp. SP 375 and BP 360G filters were used for excitation. Fluorescence emissions were separated by a 440 DCLP beam splitter (Omega) into two component emissions, which were collected through a 405/22 nm band-pass filter (FL1; violet) and a 485/22 nm band-pass filter (FL2; blue). Fluorescence intensity data, as well as volume and side-scatter data, were collected continuously and stored in list mode by using Consort 30 software (BD). Data analysis was performed with INCA and Convert software [19].

In experiments in which [Mg2+] and [Ca2+] in individual cells were determined simultaneously were performed on a dual-laser FACSStar Plus flow cytometer (Becton Dickinson). Fluo-3 and SNARF-1 were excited at 488 nm through a 5 W Coherent argon laser (Innova 90−5; Innova, Palo Alto, CA, U.S.A.) operated at 250 mW. Fluorescence emissions were separated by a 600 nm dichroic mirror into two component emissions, which were collected through a 525 nm band-pass filter (FL1; fluo-3) and a 610 nm band-pass filter (FL2; SNARF-1). Mag indo-1 was excited at 351−363 nm with a 3 W Spectra Physics series 2000 argon-ion laser (Innova) operated at 100 mW. Fluorescence emission light was separated by a 505 nm band-pass filter (which when placed under an angle of 45° functions as a 450 nm dichroic) and collected through a 405/22 nm band-pass filter (violet; FL3) and a 485/22 nm band-pass filter (blue; FL4). For each experiment, fluorescence and scatter signals from up to 50,000 cells were collected in list mode by using FACSStar Plus research software. Stored data were converted into Consort 30 format, and FL1: FL2 and FL3: FL4 ratio values (indicative of [Ca2+] and [Mg2+], respectively) were calculated by using Convert software [19].

RESULTS

Mag indo-1 is a Mg2+-sensitive fluorochrome

The specificity of mag indo-1 for Mg2+ within the physiological Ca2+ range was studied by recording the fluorescence spectrum of mag indo-1 in graded Mg2+ and Ca2+ buffers. To that end, Burkitt lymphoma cells were loaded with 2 μM mag indo-1 AM, lysed in 0.1% Triton X-100, and suspended in graded Mg2+ or graded Ca2+ buffers. Fluorescence emission spectra (excitation 355 nm, emission range 360–550 nm) of these samples were recorded on a Hitachi spectrofluorimeter. With increasing Mg2+ concentrations, the mag indo-1 fluorescence spectrum shifts towards lower wavelengths, resulting in an increase in the 405 nm: 485 nm fluorescence ratio (Figure 1). The mag indo-1 fluorescence spectrum is not influenced by Ca2+ in concentrations up to 1 mM (Figure 1).

Figure 1 Mag indo-1 fluorescence emission ratio

Mag indo-1-loaded B cells were lysed in graded Mg2+ buffers (containing 1 mM CaCl2) or graded Ca2+ buffers (containing 2.5 mM MgSO4). Fluorescence emission spectra (360–550 nm) were recorded on a Hitachi F-4010 fluorimeter (excitation 355 nm). Shown is the 405: 485 nm fluorescence ratio at different Mg2+ (●) and Ca2+ (○) concentrations.

Figure 2 Changes in [Ca2+] and [Mg2+] in T-lymphocytes after activation with 2 μM ionomycin (arrow)

Peripheral-blood T-lymphocytes were loaded with either indo-1 or mag indo-1. Changes in 405:485 nm fluorescence ratio were measured on a FACS Analyser and analysed with INCA software. Shown is the mean change in 405:485 nm fluorescence ratio with time (256 channels resolution).
Ionomycin-induced Mg\textsuperscript{2+} mobilization is dependent on extracellular Ca\textsuperscript{2+}

Once it was established that the spectral properties of mag-indo-1 are identical with those of the Ca\textsuperscript{2+} indicator indo-1, [Mg\textsuperscript{2+}], in individual cells could be analysed by flow cytometry using a 'conventional' indo-1 filter set-up. Peripheral-blood B- or T-lymphocytes were loaded with mag-indo-1, suspended in Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-containing assay buffer, and [Mg\textsuperscript{2+}] was measured on a FACS Analyser. Activation of B- or T-lymphocytes with the Ca\textsuperscript{2+} ionophore ionomycin leads to an increase in [Mg\textsuperscript{2+}], (Figure 2). Apart from the fact that the mag-indo-1 fluorescence spectrum is not affected by changes in [Ca\textsuperscript{2+}] (see above), the kinetic difference between the response observed in indo-1 and mag-indo-1-loaded cells excludes the possibility that the shift in the mag-indo-1 fluorescence spectrum is caused by changes in [Ca\textsuperscript{2+}].

The ionomycin-induced Mg\textsuperscript{2+} mobilization appeared to be largely independent of extracellular Mg\textsuperscript{2+}, since cells washed and suspended in Mg\textsuperscript{2+}-free assay buffer showed a near-identical Mg\textsuperscript{2+}-mobilization response after activation with ionomycin as compared with cells in complete assay buffer (Figure 3). Activation of cells in Ca\textsuperscript{2+}-free assay buffer, however, totally abrogates the Mg\textsuperscript{2+} response (Figure 3). It therefore appears that high [Ca\textsuperscript{2+}], is required to cause mobilization of Mg\textsuperscript{2+} from intracellular stores (presumably mitochondria).

Changes in [Mg\textsuperscript{2+}], after ligation of the antigen receptor on lymphocytes

The above data on changes in [Mg\textsuperscript{2+}], were obtained in ionomycin-activated cells. Because we were interested in potential changes in [Mg\textsuperscript{2+}], after a physiological stimulus, in the next series of experiments mag-indo-1-loaded lymphocytes were activated by ligation of the antigen receptor. In peripheral-blood T-lymphocytes, the dose-dependency of changes in [Ca\textsuperscript{2+}], and [Mg\textsuperscript{2+}], after activation with ionomycin was compared with that after activation with concanavalin A (ConA). The highest concentration of ConA used induced a small, but reproducible, increase in overall [Mg\textsuperscript{2+}], (Figure 4).

The antigen receptor on B-lymphocytes was ligated with anti-Ig antibodies. In splenic B cells, activation with anti-\(\delta\) antibody results in an increase in [Mg\textsuperscript{2+}], from 0.5 mM (before) to 0.6 mM (after). Comparable small, but significant, increases in [Mg\textsuperscript{2+}], were observed in Burkitt-lymphoma cells activated with anti-\(\mu\) antibodies. (G. T. Rijkers and A. W. Griffioen, unpublished work). It should be noted that in both T- and B-lymphocytes the changes in overall [Mg\textsuperscript{2+}], were caused by a [Mg\textsuperscript{2+}], response in a small proportion of the cells, whereas in the majority of cells [Mg\textsuperscript{2+}], did not change after cell activation. This is in sharp contrast with receptor-mediated changes in [Ca\textsuperscript{2+}], in which cases a Ca\textsuperscript{2+}-mobilization response is observed in virtually 100% of cells [7,9].

Simultaneous measurement of [Ca\textsuperscript{2+}], and [Mg\textsuperscript{2+}],

Data presented thus far suggest that a Mg\textsuperscript{2+}-mobilization response occurs only in those cells in which ligation of the antigen receptor has led to high [Ca\textsuperscript{2+}]. In order to address this issue directly, T cells were loaded with fluo-3 and mag-indo-1, and changes in [Ca\textsuperscript{2+}], and [Mg\textsuperscript{2+}], were measured simultaneously on a dual-laser FACStar Plus flow cytometer. Initial experiments were performed with low concentrations of ionomycin (1 \(\mu\)M)
376

**DISCUSSION**

A significant proportion of mobilized Ca²⁺ results from influx of extracellular Ca²⁺. For Mg²⁺ mobilization this clearly is not the case, because Mg²⁺-mobilization profiles were nearly identical in both kinetics and magnitude whether the response was induced in Mg²⁺-containing or Mg²⁺-free buffers. This indicates that virtually all cytoplasmic Mg²⁺ is derived from intracellular stores, most probably mitochondria.

Intracellular Ca²⁺ that is mobilized after ligation of the antigen receptor on either T- or B-lymphocytes plays multiple regulatory roles in receptor-mediated trans-membrane signalling. First, increased [Ca²⁺], mediates the activity of Ca²⁺/calmodulin-dependent kinases and, indirectly, also the activity of protein kinase C. Second, mobilized Ca²⁺ may amplify PtdInsP₃ hydrolysis. Data presented in this paper indicate that high [Ca²⁺] may also mediate mobilization of free cytoplasmic Mg²⁺, which is in accordance with data from other cellular systems [20,21].

It has been reported recently that epidermal growth factor increases [Mg²⁺], in myocytes [22] and in fibroblasts [23]. Previous studies on changes in [Mg²⁺], in lymphocytes after more or less physiological cell activation have failed to show significant changes in [Mg²⁺], [24]. The Mg²⁺-mobilization response that did occur after ligation of the antigen receptor indeed only concerned a small proportion of the cells and would have remained unnoticed without the technique of flow-cytometric analysis.

The physiological role of a transient increase in [Mg²⁺], in the process of lymphocyte activation remains speculative. The Mg²⁺-dependency of a wide variety of enzymes that can be demonstrated in cell-free systems does not rigidly predict that changes in overall cytoplasmic [Mg²⁺] actually modulate enzyme activity. Still it has been hypothesized that increased [Mg²⁺], may trigger additional hydrolysis of phosphatidylinositol, thus leading to excess diacylglycerol production [17]. In this view it could be expected that the cellular response following ligation of the antigen receptor differs in cells that do or do not show a Mg²⁺-mobilization response. We are currently addressing this issue by sorting cells on the basis of their Mg²⁺-mobilization response.

It can be concluded that, in addition to [Ca²⁺], [Mg²⁺], may change transiently following lymphocyte activation.

that induce a lasting increase in [Ca²⁺], in lymphocytes and increased [Mg²⁺], in only a fraction of the cells. It appeared that the ionomycin-induced increase in [Ca²⁺], in cells which did show a Mg²⁺-mobilization response was significantly higher than in those cells in which [Mg²⁺], remained at resting levels (Figure 5).

Subsequently, similar experiments were performed with T-cells (loaded with fluo-3, SNARF-1 and mag-indo-1) activated with ConA. In this case also, a Mg²⁺-mobilization response was observed only in those cells, in which activation with ConA had resulted in high [Ca²⁺] (Figure 6). It can therefore be concluded that lymphocyte activation through ligation of the antigen receptor may lead to changes in [Mg²⁺], in those cells in which high [Ca²⁺], is reached.

**Figure 5** Mg²⁺-mobilizing cells have high [Ca²⁺].

Peripheral-blood T-lymphocytes were loaded with fluo-3 and mag-indo-1, washed and activated with 1 µM ionomycin. Fluor-3 525 nm fluorescence intensity (488 nm excitation; a) and mag-indo-1 405: 485 nm fluorescence ratio (360 nm excitation; b) were measured. The fraction of cells that responded to ionomycin stimulation with an increase in [Mg²⁺] was gated, as were those cells in the corresponding time interval in which [Mg²⁺], remained at resting levels (boxed areas in b). Relative [Ca²⁺], of cells with resting [Mg²⁺] (continuous line) and of cells with increased [Mg²⁺], (broken line) is shown in (c).

**Figure 6** Simultaneous measurement of [Ca²⁺], and [Mg²⁺], in T-lymphocytes activated with ConA

Peripheral-blood T-lymphocytes were loaded with fluo-3, SNARF-1 and mag-indo-1, washed and activated with 2.5 mg ConA/ml. The ratio of fluo-3 525 nm fluorescence intensity and SNARF-1 630 nm fluorescence intensity (both at 488 nm excitation) as well as the mag-indo-1 405: 485 nm fluorescence ratio (360 nm excitation) were measured. The relation between the relative [Mg²⁺], (mag-indo-1 405: 485 nm ratio) and relative [Ca²⁺], (fluo-3:SNARF-1 ratio) is shown in dot-plot format. Most of the cells with high [Mg²⁺], also have a high [Ca²⁺].
Changes in cytoplasmic Mg$^{2+}$ in human lymphocytes

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