Submicromolar La^{3+} concentrations block the calcium release-activated channel, and impair CD69 and CD25 expression in CD3- or thapsigargin-activated Jurkat cells

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The calcium release-activated channel (CRAC) opened in Jurkat cells activated either with CD3 monoclonal antibody or the endoplasmic reticulum Ca^{2+} -ATPase blocker, thapsigargin, is blocked by La^{3+} with an IC_{50} of 20 nM. Similarly, the entry of Mn^{2+} , used as a surrogate for Ca^{2+} , is also blocked by submicromolar La^{3+} concentrations. La^{3+} seems to play its role simply by plugging the CRAC because this ion does not penetrate the cells,

as demonstrated by chelation experiments with EGTA. Blocking the Ca^{2+} influx in activated Jurkat cells results in a lack of expression of CD25, a chain of the interleukin-2 receptor and of CD69, a marker of T-cell activation. By contrast, the very early steps of the T-cell signalling pathway such as the release of Ca^{2+} from intracellular stores and the subsequent inhibition of phosphatidylserine synthesis are not affected by La^{3+} .

INTRODUCTION

In Jurkat T cells, activation of the T-cell receptor (TCR)–CD3 complex results in recruitment of tyrosine kinases that are members of the *src* family including *fyn* and *lck*. It has been proposed that the *src* family of tyrosine kinases activate a phospholipase C isoform, $\gamma 1$, which in turn stimulates phosphoinositide hydrolysis, leading to the generation of inositol 1,4,5-trisphosphate (IP_3) [1–3]. IP_3 triggers intracellular calcium release [4,5]. Emptying of the intracellular calcium stores generates a Ca^{2+} influx through a calcium release-activated channel (CRAC), possibly opened by a calcium influx factor (CIF) as described by Randriamampita and Tsien [6]. The first steps involved in T-cell activation are bypassed when using the endoplasmic reticulum Ca^{2+} -ATPase blocker, thapsigargin [7,8]. This compound passively decreases the Ca^{2+} level in the endoplasmic reticulum, allowing the CRAC to open. The effects of La^{3+} on Ca^{2+} signalling have been previously tested in lacrimal cells (in the range 30–500 μM) [9], in salivary acinar cells at 1 mM [10] and in C6-2B glioma cells at 200 μM [11]. At these high concentrations, La^{3+} has been shown to inhibit membrane Ca^{2+} -ATPase and Ca^{2+} entry in cells treated with thapsigargin. We show here that in Jurkat T lymphocytes, submicromolar La^{3+} concentrations block capacitative Ca^{2+} entry as well as Mn^{2+} entry (when Mn^{2+} was used as a surrogate for Ca^{2+}). Activation of T cells via the TCR can be monitored by measuring the expression of CD25 and CD69. CD25 is a 55 kDa protein that forms a low-affinity receptor for interleukin-2 (IL-2). CD25, when associated with the 75 kDa component of the IL-2 receptor, forms a heterodimer of high affinity for this interleukin. CD69, a disulphide-linked dimeric structure including a 33 kDa and a 27 kDa chain, is the earliest molecule that appears *de novo* on the lymphocyte surface on activation. We show here that blocking Ca^{2+} entry induced by TRC activation results in a lack of surface expression of both CD25 and CD69.

MATERIALS AND METHODS

Cells

The human T-cell line Jurkat was kindly supplied by Dr. A. M. Schmitt-Verhulst (Centre d'Immunologie, Marseille-Luminy, France). Cells were cloned by limiting dilution. Clone D was selected on the basis of its IL-2 production when activated with phytohaemagglutinin and phorbol 12-myristate 13-acetate. Cells were cultured in RPMI 1640 medium (Seromed, Lille, France) supplemented with 5% (w/v) fetal calf serum, 50 i.u./ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, 2 mM L-glutamine and 1 mM pyruvate.

Measurements of changes in cytosolic $[\text{Ca}^{2+}]$

The assay of cytosolic $[\text{Ca}^{2+}]$ was performed by using Indo-1 (Calbiochem) [12]. Cells ($5 \times 10^6/\text{ml}$) were loaded by using 5 μM Indo-1 at 37 °C in the dark for 1 h and then washed and resuspended in Hepes buffered saline (140 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 10 mM glucose, 20 mM Hepes, 0.1% BSA, pH 7.4). When necessary, calcium was omitted. The analyses were performed on a fluorescence-activated cell sorter (FACStar Plus, Becton Dickinson) fitted with two argon lasers, one tuned to 488 nm (250 mV) to gate out debris and damaged cells according to the dual-scatter dot plot, and the other to UV (100 mV, 351–364 nm) to excite Indo-1 fluorescence. The fluorescence intensities at 480 and 400 nm, corresponding to the free Indo-1 and the complex Ca^{2+} –Indo-1 concentrations respectively, were measured. Calculation of the ratio of fluorescence at 400 nm to that at 480 nm allows the evaluation of changes in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) independently of the cell size and the intracellular Indo-1 concentration. The flow rate was set to 1000 cells/s and the mean ratio of 3000 cells was noted every 30 s.

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic Ca^{2+} concentration; CRAC, calcium release-activated channel; IL-2, interleukin-2; IP_3 , inositol 1,4,5-trisphosphate; mAb, monoclonal antibody; PtdSer, phosphatidylserine; TCR, T-cell receptor.

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Measurement of Mn^{2+} entry

Mn^{2+} uptake was monitored by the quenching of the Indo-1 fluorescence at 430 nm, which is insensitive to variations in $[Ca^{2+}]_i$.

Phosphatidylserine synthesis

Jurkat cells (2×10^6) were maintained in 0.5 ml of a buffer (pH 7.4) containing 137 mM NaCl, 2.7 mM KCl, 2.5 mM glucose, 20 mM Hepes, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ at 37 °C in the presence of 4 μ Ci/ml [3H]serine (0.37–1.1 TBq/mmol, Amersham, Les Ulis, France) and effectors as indicated in the legends to the Figures. After incubation for 0–2 h the cells were rapidly sedimented in an Eppendorf centrifuge, the supernatants discarded and the cell phospholipids extracted with chloroform/methanol by the method of Bligh and Dyer [13]. The lipid extracts were analysed by TLC in a solvent system composed of chloroform/methanol/acetic acid/water (75:45:12:3). Authentic phospholipid standards (Sigma) were run in parallel and detected with iodide vapours. Radioactivity in lipid spots was determined by using an automatic linear radiochromatography analyser (Berthold).

Measurements of CD69 and CD25

Cells (10^6 /ml) were incubated for 24 h in RPMI 1640/5% fetal

calf serum, in the presence of phorbol 12-myristate 13-acetate (10^{-8} M) and CD3 (2 μ g/ml) or thapsigargin (10^{-7} M), with or without the Ca^{2+} entry blockers SK&F 96365 (3 μ M) or La^{3+} (1 μ M), then washed and tested for the expression of CD25 and CD69 antigen. CD25 monoclonal antibody (mAb) conjugated to phycoerythrin and CD69 mAb conjugated to fluorescein isothiocyanate (Becton Dickinson) were used to detect the expression of their respective antigens by cytofluorimetry.

RESULTS

La^{3+} blocks CD3-induced Ca^{2+} entry

When assayed in a medium containing 1 mM Ca^{2+} , CD3 mAb induces a strong increase in $[Ca^{2+}]_i$. This rise is the result of two effects: a release of Ca^{2+} from the intracellular compartment and a Ca^{2+} influx. The effect of different concentrations of La^{3+} on CD3-induced Ca^{2+} signals is shown in Figure 1A. It appears that La^{3+} inhibits the sustained elevation of $[Ca^{2+}]_i$ but allows a transient increase, presumably due to the release of Ca^{2+} from intracellular stores. The inhibition of the $[Ca^{2+}]_i$ plateau occurred with an IC_{50} of 40 nM (Figure 1B). When stimulations occurred in a low- $[Ca^{2+}]$ medium (about 10^{-5} M, owing to contaminations) the initial rise of $[Ca^{2+}]_i$ was not affected by La^{3+} (Figure 1C); on the contrary, the second $[Ca^{2+}]_i$ increase after the addition of 1 mM Ca^{2+} in the medium was greatly dependent on the presence

Figure 1 Inhibition of Ca^{2+} influx in CD3-activated Jurkat cells

(A) Cells maintained in medium containing 1 mM Ca^{2+} were treated at time 0 with different concentrations of La^{3+} and 5 μ g/ml CD3 monoclonal antibody. $[Ca^{2+}]_i$ was followed by measuring the change in the 400/480 nm fluorescence ratio of Indo-1. (B) The level of the Ca^{2+} plateau at time 5 min plotted against the La^{3+} concentration. (C) Cells maintained in a nominally Ca^{2+} -free medium were treated at time 0 with different concentrations of La^{3+} and 5 μ g/ml CD3 monoclonal antibody. At time 4 min 30 s, 1 mM $CaCl_2$ was added to the cell suspension and $[Ca^{2+}]_i$ was followed for an additional 2 min. (D) The slope of the second $[Ca^{2+}]_i$ rise plotted against La^{3+} concentration.

Figure 2 Changes in $[\text{Ca}^{2+}]_i$ induced in Jurkat cells by thapsigargin (A) or CD3 monoclonal antibody (B)

The fluorescence of Indo-1 was measured in cells treated either with thapsigargin or CD3 alone ($\square$), in cells treated with 500 nM La^{3+} added to the cell suspension at time 0 just before CD3 or thapsigargin ($\blacksquare$) and in cells treated with either CD3 or thapsigargin at time 0 and then with 500 nM La^{3+} at time 4 min (indicated by an arrow) ($\bullet$, $\circ$). The Ca^{2+} concentration in the medium was 1 mM. The Figure is representative of three independent experiments.

of La^{3+} , with an IC_{50} of 20 nM (Figure 1D). $[\text{Ca}^{2+}]_i$ elevation was inhibited by La^{3+} when the cells were preincubated for 5 min before the CD3 treatment and also when La^{3+} was applied 3 min after CD3 mAb (Figure 2B). Similar results were obtained with cells treated with the endoplasmic reticulum Ca^{2+} -ATPase blocker, thapsigargin (Figure 2A). In previous papers we have shown that emptying intracellular stores of Jurkat cells inhibited the base-exchange enzyme system responsible for phosphatidylserine (PtdSer) synthesis [14,15]. Measuring PtdSer synthesis was thus used as a tool to verify the status of intracellular Ca^{2+} stores [16,17]. PtdSer synthesis was inhibited both by CD3 mAb and thapsigargin and this process was not influenced by La^{3+} even at high concentrations (results not shown).

La^{3+} does not act by penetrating Jurkat cells

It is accepted that La^{3+} does not penetrate cells even at very high concentrations. To verify this point, cells were first treated with 1 μM La^{3+} , followed immediately by CD3 mAb. Under these conditions, only a peak of Ca^{2+} due to the release of Ca^{2+} from intracellular stores was seen (Figure 3). Then, at 4 min, 10 μM EGTA was added to the cell suspension. At this concentration, EGTA was unable to chelate a significant amount of Ca^{2+} that was present at 1 mM but was quite able to chelate 1 μM La^{3+} owing to its very high affinity for that ion. Under these experimental conditions, the effect of La^{3+} was fully reversed by EGTA

Figure 3 EGTA (10 μM) reverses the effect of La^{3+}

Jurkat cells were treated at time 0 with 10⁻⁷ M thapsigargin (A) or CD3 mAb (B) alone ($\square$) or in the presence of 500 nM La^{3+} ($\blacksquare$). At time 4 min (indicated by an arrow), 10 μM EGTA was added ($\bullet$) to chelate La^{3+} , and $[\text{Ca}^{2+}]_i$ was followed for an additional 3 min. The Ca^{2+} concentration in the medium was 1 mM. Similar results were obtained from two independent experiments.

and the Ca^{2+} entry recovered its previous ability. Identical results were observed with cells activated either with CD3 mAb or thapsigargin (Figures 3A and 3B).

La^{3+} blocks Mn^{2+} entry

Mn^{2+} can be used as a Ca^{2+} surrogate to estimate Ca^{2+} influx through the plasma membrane [18–20]. Figure 4 shows that Mn^{2+} entry into Jurkat cells as measured by the quenching of Indo-1 fluorescence at 430 nm is inhibited by La^{3+} . Similar results were obtained whether the cells were activated with CD3 mAb or thapsigargin.

La^{3+} inhibits CD69 and CD25 expression

It is well known that the membrane expression of CD69 and CD25 reflects the state of activation of T lymphocytes. To see whether blocking the Ca^{2+} channel modifies T-cell activation, we measured the expression of both CD69 and CD25 on cells activated for 24 h in the absence or presence of 1 μM La^{3+} . Figure 5 shows that La^{3+} blocks the membrane expression of these two activation markers, indicating that Ca^{2+} influx is necessary to T-cell activation. SK&F 96365, a known inhibitor of Ca^{2+} entry [21], had a similar, although lower, effect at 3 μM (Figure 5). That concentration was enough to abrogate the CD3- and thapsigargin-induced plateau of $[\text{Ca}^{2+}]_i$ increase by 90%. Higher concentrations could not be used owing to the toxicity of this compound (results not shown).

DISCUSSION

In the present study, the efficacy of Ca^{2+} entry compared with release from intracellular stores at activating Jurkat cells was studied. We first demonstrated that La^{3+} was able to inhibit Ca^{2+} influx in the absence of modifications of Ca^{2+} release from intracellular compartments. This was verified both in CD3- and thapsigargin-treated cells in which submicromolar La^{3+} concentrations totally blocked the Ca^{2+} influx without changes in Ca^{2+} release from the internal Ca^{2+} stores. The biosynthesis of PtdSer through the Ca^{2+} -dependent base-exchange enzyme system located in the endoplasmic reticulum reflects the status (full/empty) of the Ca^{2+} stores. We have thus used this original tool to follow the Ca^{2+} content of the Ca^{2+} stores. The inhibition of PtdSer synthesis induced by either CD3 or thapsigargin was not affected by La^{3+} , indicating that this ion does not interfere with the process leading to Ca^{2+} release. Because CRAC also allows Mn^{2+} to penetrate the cells [18–20], we have measured the uptake of Mn^{2+} in CD3- and thapsigargin-treated cells. It was found that Mn^{2+} entry was also blocked by La^{3+} . As Mn^{2+} enters the thapsigargin-stimulated cells it is very likely that this ion penetrates via CRAC, because thapsigargin does not open other channel types. Given the fact that La^{3+} does not penetrate Jurkat cells, as confirmed by the reversion of its effect by low EGTA concentrations, it was concluded that La^{3+} specifically blocks

Figure 4 La^{3+} blocks Mn^{2+} entry into Jurkat cells

The entry of Mn^{2+} into Jurkat cells was measured by the quenching of Indo-1 fluorescence at 430 nm. Controls included untreated cells ($\square$) and cells treated at time 0 with $1 \mu\text{M}$ La^{3+} ($\blacksquare$). (A) Cells were additionally treated at time 0 with CD3 mAb alone ($\circ$) or in the presence of La^{3+} ($\bullet$). (B) Cells were treated with 10^{-7} M thapsigargin alone ($\triangle$) or in the presence of $1 \mu\text{M}$ La^{3+} ($\blacktriangle$). The fluorescence of Indo-1 at 430 nm was followed during a first period of 3 min to show the complete Ca^{2+} independence of Indo-1 fluorescence at 430 nm; then $100 \mu\text{M}$ Mn^{2+} was added (indicated by an arrow) and the quenching of fluorescence was monitored for an additional 5 min. During the whole experiment cells were in medium containing 1 mM Ca^{2+} . These results are representative of three independent experiments.

Figure 5 Expression of CD69 and CD25 in activated Jurkat cells

The expression of CD69 (left panel) and CD25 (right panel) was measured in controls and also in either CD3- or thapsigargin-treated cells. The effects of La^{3+} ($1 \mu\text{M}$) and SK&F 96365 ($3 \mu\text{M}$) were studied. Measurements were made by cytofluorimetry as indicated in the Materials and methods section. Results are expressed as means $\pm$ S.D. for two experiments done in triplicate.

Ca^{2+} influx by plugging the CRAC. From these conclusions it was of interest to see whether some T-cell activation steps could be blocked by La^{3+} . As shown in Figure 5, La^{3+} impaired CD25 and CD69 expression at the cell surface, indicating that the CRAC that maintains a high $[\text{Ca}^{2+}]_i$ is necessary for the expression of these two activation markers. This effect of La^{3+} is very probably due to Ca^{2+} entry blockade because: (1) it was observed in cells treated with thapsigargin, which bypasses the early signalling cascade triggered by TCR stimulation, and (2) a similar inhibition was obtained independently with the Ca^{2+} channel blocker SK&F 96365. Conversely, it can be concluded that the first activation signals such as tyrosine kinase activation, phospholipase $\text{C}\gamma 1$ phosphorylation, phosphatidylinositol bisphosphate hydrolysis and IP_3 -induced Ca^{2+} release from intracellular stores are not sufficient to induce CD25 and CD69 expression.

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