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Lysophosphatidylcholine induces Ca^{2+} mobilization in Jurkat human T lymphocytes and CTLL-2 mouse T lymphocytes by different pathways

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ABSTRACT

Lysophosphatidylcholine (LPC), an important compound in the immune system, regulates a variety of biological processes. We examined and compared the effect of exogenous LPC on intracellular Ca^{2+} overload in human Jurkat CD4+ T lymphocytes and mouse CTLL-2 CD8+ T lymphocytes. LPC caused a dose-dependent intracellular Ca^{2+} level ($[Ca^{2+}]_i$) increase in both Jurkat and CTLL-2 lymphocytes. Pretreatment of cells for 5 min with 30 μM of ruthenium red, a potent ryanodine receptor inhibitor, reduced the LPC-induced Ca^{2+} response in both Jurkat and CTLL-2 T lymphocytes. Moreover, pretreatment of cells with 100 μM 2-APB for 15 min, a cell-permanent IP_3 receptor inhibitor, reduced about two thirds of the LPC induced calcium response in both kinds of cells. However, preincubation of the cells with verapamil, an L-type Ca^{2+} channel blocker, did not affect the LPC-induced $[Ca^{2+}]_i$ increase in CTLL-2 lymphocytes but inhibited this in Jurkat lymphocytes by 26%. In Ca^{2+}-free medium, LPC produced 75.8% of the total $[Ca^{2+}]_i$ increase in CTLL-2 lymphocytes and 38% of the total $[Ca^{2+}]_i$ increase in Jurkat lymphocytes. These data suggested that the LPC-induced $[Ca^{2+}]_i$ increase in human Jurkat and mouse CTLL-2 cell lines occurs via different pathways.

1. Introduction

Lysophosphatidylcholine (LPC) is an intrinsic, intracellular messenger generated by the hydrolysis of membrane phosphatidylcholine by intracellular phospholipase A2 (PLA2). It regulates a variety of biological processes including cell proliferation, differentiation, tumor cell invasiveness, and inflammation (Spiegel and Milstien, 1995; Moolenaar, 1999). In the immune system, LPC promotes inflammatory effects, including monocyte chemotaxis, macrophage activation, and inducement of apoptosis in normal, activated lymphocytes (Jing et al., 2000). As a component of oxidized low density lipoprotein, LPC plays an etiological role in atherosclerosis and is implicated in the pathogenesis of the autoimmune disease, systemic lupus erythematosus (SLE) (Koh et al., 2000). LPC is also an important natural adjuvant for the immune system, inducing humoral and cellular immune responses (Perrin-Cocon et al., 2006). Most important of all, as determined by the expression of a subunit of the interleukin 2 receptor and thymidine incorporation into DNA (Asaoka et al., 1992), LPC greatly potentiates the activation of human resting T lymphocytes that is induced by a membrane permeant diacylglycerol plus a calcium ionophore.

Recently, an orphan G-protein-coupled receptor (GPCR), G2A, has been identified as a high-affinity receptor for LPC and is expressed predominantly in lymphoid tissues and lymphocytes (Kabarowski et al., 2001). G2A was originally isolated as a stress-inducible GPCR that induces the cell cycle arrest at G2/M periods when cells are serum-starved or DNA-damaged; its expression is induced by cellular activation and stress (Weng et al., 1998). LPC, interacting with its receptor, G2A, induces the elevation of the intracellular calcium concentration ($[Ca^{2+}]_i$) and also the transcriptional activation of the serum response factor via the MAPK pathway (Murakami et al., 2004).

Calcium ions (Ca^{2+}) function as a universal second messenger in virtually all eukaryotic cells, including those of the immune system. The elevation of intracellular free Ca^{2+} is an essential trigger for T cell differentiation (Crabtree, 1989) and activation (Weiss and Imboden, 1987; Premack and Gardner, 1992; Feske, 2007). The engagement of T cell receptors and the rise in $[Ca^{2+}]_i$ is followed by a number of downstream effects. Changes in motility and cytoskeletal reorganization occur soon after T cells contact antigen-presenting cells (Gelfand, 1990; Donnadieu et al., 1994).

Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; CRAC, calcium release-activated calcium current; FBS, fetal bovine serum; GPCR, G-protein-coupled receptor; IP_3, inositol trisphosphate; LPC, lysophosphatidylcholine; PLA2, phospholipase A2; SCID, severe combined immunodeficient disease; SLE, systemic lupus erythematosus; WAS, Wiskott–Aldrich syndrome.

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Over periods of minutes to hours, the amplitude, duration, and kinetic signature of Ca^{2+} signals increase the efficiency and specificity of gene activation events, such as IL-2, a cytokine essential for T-cell proliferation and the immune response (Crabtree, 1989; Gelfand, 1990; Negulescu et al., 1994; Rao, 1994). Furthermore, cell killing by cytotoxic T cells (Poenie et al., 1987; Haverstick et al., 1991), apoptosis of self-reactive T cells during development in the thymus (McConkey et al., 1992; Nakayama et al., 1992), induction of tolerance to self-antigens in mature peripheral T cells (Schwartz, 1990; Nghiem et al., 1994) and inhibition on n-type K^+ channels in both T and B lymphocytes, also occur after the elevation of $[Ca^{2+}]_i$.

It has been reported that LPC induces several cellular changes in Jurkat lymphocytes, including an increase of ROS generation in a PKCδ-dependent and GPCR-independent manner, a marked and immediate tyrosine phosphorylation, a decrease of MMP, and most importantly, an increase of $[Ca^{2+}]_i$ through Ca^{2+} influx (Im et al., 2006). Legradi et al. reported that the LPC-induced $[Ca^{2+}]_i$ increase in Jurkat T cells was dependent on PTX-sensitive G proteins (Légrádi et al., 2004). Inhibition of the Ser/Thr kinases and tyrosine kinases with staurosporine and genistein, respectively, suppressed the rise in $[Ca^{2+}]_i$. Staurosporine, the inhibitor of Ser/Thr kinases impedes the LPC-induced $[Ca^{2+}]_i$ elevation. Furthermore, activation of G2A by LPC increased $[Ca^{2+}]_i$ induced receptor internalization, activated ERK mitogen-activated protein kinase and modified migratory responses of Jurkat T lymphocytes (Kabarowski et al., 2001).

Although LPC which is involved in the activation of T lymphocytes and apoptosis of activated lymphocytes can cause an increase in $[Ca^{2+}]_i$ that is also very significant for immune response, the exact signal pathway of LPC-induced $[Ca^{2+}]_i$ increase is poorly defined for T lymphocytes, especially for cytotoxic T lymphocytes. In this paper we examine and compare the pathways of exogenous LPC on intracellular Ca^{2+} overload in human Jurkat CD4+ T lymphocytes and mouse CTLL-2 CD8+ T lymphocytes. Using the two cell lines as models of helper and cytotoxic T lymphocytes, we may postulate the possible different mechanism in activation of two kinds of T lymphocytes in which LPC-induced the Ca^{2+} changes is involved.

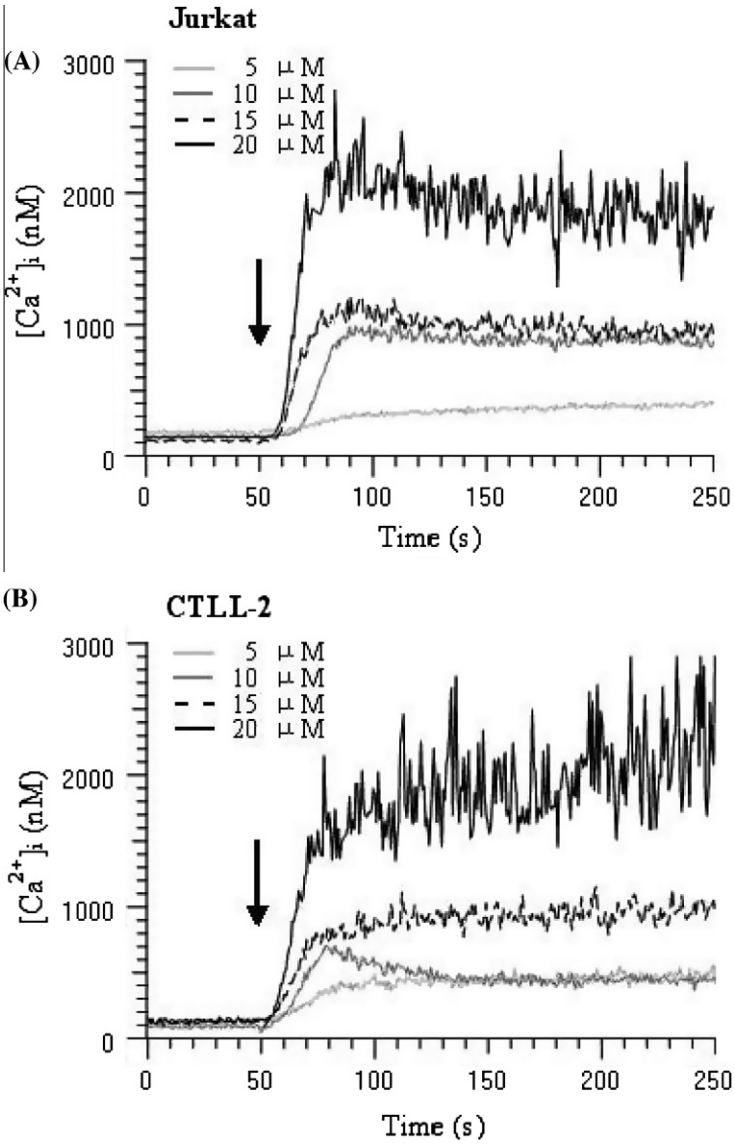

Fig. 1. Change in Ca^{2+} in response to different concentrations of LPC in Jurkat and CTLL-2 T lymphocytes. A (Jurkat) and B (CTLL-2): cells loaded with Fura-2/AM were stimulated by the addition of LPC in Ca^{2+}-containing medium. Arrows indicate the time of LPC addition. Traces were representative of at least 4 experiments.

2. Materials and methods

2.1. Materials

Jurkat (a clone of CD4+ leukemic T cells derived from *Homo sapiens*, ATCC number: TIB-152™) and CTLL-2 lymphocytes (a clone of cytotoxic CD8+ T cells derived from a C57BL/6 mouse (*Mus musculus*), ATCC number: TIB-214™) were obtained from the institute of Biophysics, Chinese Academy of Sciences (Beijing, China). RPMI 1640 medium was purchased from Invitrogen company (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Chuanye Bioscience Co. (Tianjing, China). Fura-2/AM, L-a-LPC (primary palmitate and stearate esters), IL-2, verapamil, ruthenium red and 2-aminoethoxydiphenyl borate (2-APB) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were purchased from the Beijing Chemical Co. (Beijing, China).

2.2. Cell culture

Jurkat lymphocytes were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37 °C in a humidified atmosphere containing 5% CO_2. CTLL-2 lymphocytes were cultured in the same conditions except that 30 U/ml of rat IL-2 was added to the culture medium. Cells were passaged three times a week and used in confluent culture in all the subsequent experiments.

2.3. Cell viability determination

Cell viability was determined using the dye exclusion assay. After the cells were challenged with different concentrations of LPC for 1 min, 0.02% Trypan blue (final concentration) was added to the cell suspensions, and the percentage of viable cells seen within 3 min recorded with an inverted microscope.

2.4. Intracellular Ca^{2+} measurements

$[Ca^{2+}]_i$ was determined using the Ca^{2+}-sensitive fluorescent indicator Fura-2/AM. Cells were washed, resuspended in Hepes-buffered medium (120 mM NaCl, 5 mM KCl, 8 mM $MgCl_2$, 32 mM sucrose, 2 mM $CaCl_2$, and 10 mM Hepes, pH 7.2) at a concentration of 1×10^7 cells/ml, and then incubated with 2.5 μM Fura-2/AM for 15 min at 37 °C. After being washed twice, cells were resuspended in the same Hepes buffer at a concentration of 1×10^6 cells/ml. The intensity of fluorescence was monitored at 510 nm (5 nm slit) with excitation alternating between 340 and 380 nm (5 nm slit) using a F4500 dual wavelength fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

$[Ca^{2+}]_i$ concentration was calculated by the FL Solutions 2.0 Intracellular Cation Scan software based on the formula described by Grynkiewicz et al. (1985), assuming a dissociation constant K_d of 224 nM. Maximal and minimal fluorescence values were obtained at the end of experiment by the sequential addition of 0.1% Triton X-100 and 5 mM EGTA.

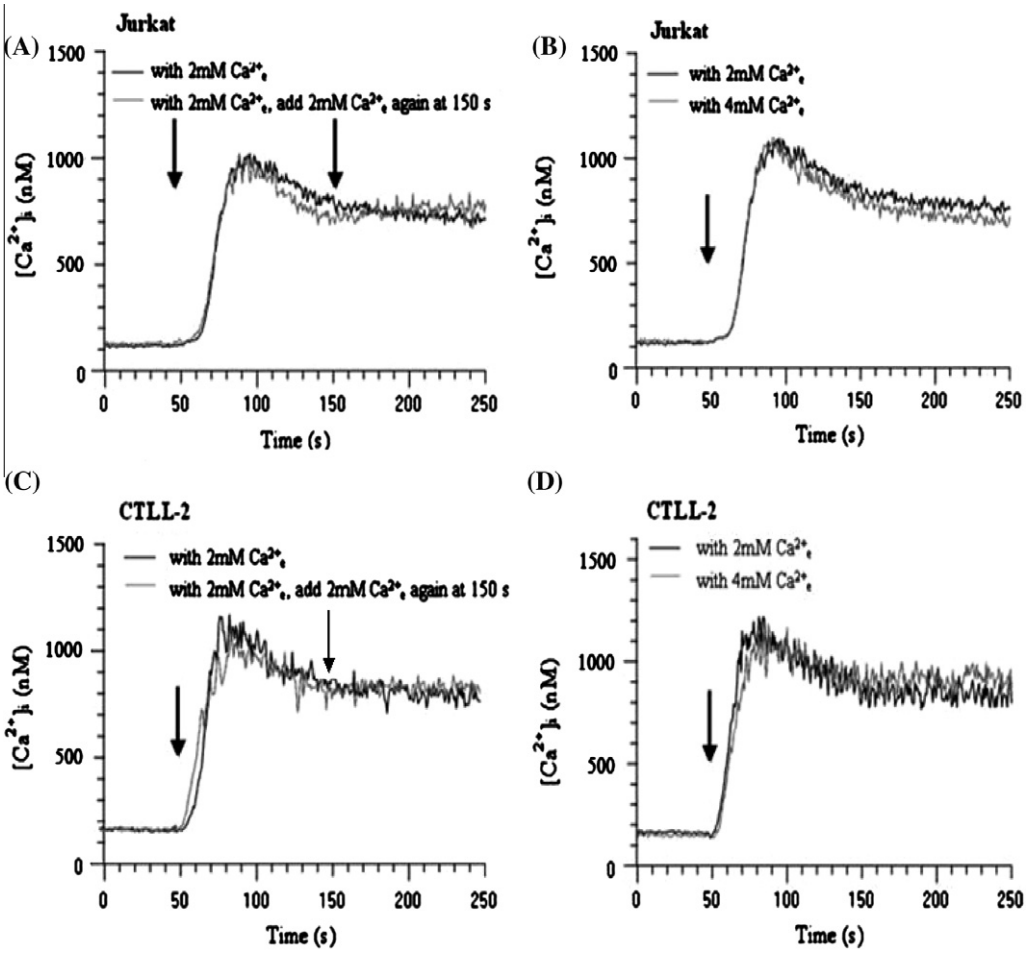

Fig. 2. Effect of varying the concentration of extracellular Ca^{2+} on the LPC-induced Ca^{2+} response in Jurkat (A and B) and CTLL-2 (C and D) T lymphocytes. (A and C) 2 mM Ca^{2+} present during the first 150 s then increased to 4 mM 100 s after LPC stimulation (first arrow) in one sample. (B and D) Fura-2/AM loaded cells were stimulated by the addition of 15 μM LPC in the presence of 2 mM or 4 mM extracellular Ca^{2+} in the medium. Traces are representative of at least three experiments.

In some experiments, Fura-2/AM loaded cells were washed and suspended in Ca^{2+}-free medium which contained similar components as the Hepes-buffered medium except that $CaCl_2$ was omitted. In Ca^{2+} restoration experiments, Ca^{2+} was initially absent from the medium but was restored to 2 mM after 100 s of LPC stimulation.

2.5. Drug treatments

In order to determine the pathway mediating LPC-induced Ca^{2+} mobilization, cells were adjusted to the same density as 1×10^6/ml and pretreated with some Ca^{2+} signaling protein inhibitors before being challenged with 15 μM LPC.

Drug treatments were conducted in the following modes: in some experiments, cells loaded with Fura-2/AM were preincubated with an L-type Ca^{2+} channel blocker (verapamil, 20 μM) or an inhibitor of the inositol trisphosphate (IP_3) receptor (2-APB, 100 μM) for 15 min. In another series of experiments, cells were pretreated with 30 μM ruthenium red, a ryanodine receptor inhibitor, for 5 min, and then stimulated with 15 μM LPC. For control samples, Fura-2/AM loaded cells were pretreated with same

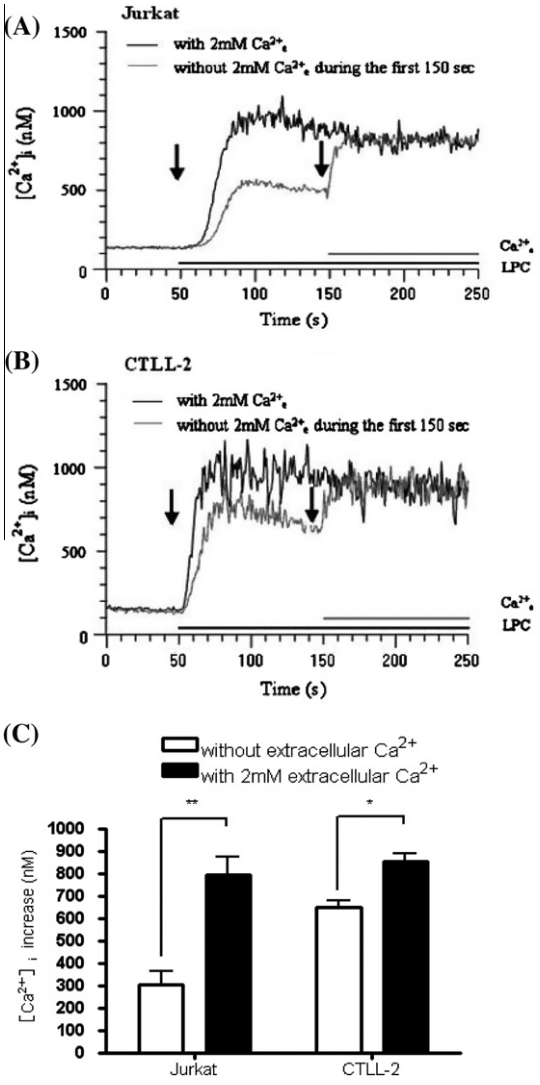

Fig. 3. Effects of external Ca^{2+} on the LPC-induced Ca^{2+} response in Jurkat and CTLL-2 T lymphocytes. Fura-2/AM loaded cells were stimulated by the addition of 15 μM LPC in the presence or absence of extracellular Ca^{2+}. A (Jurkat) and B (CTLL-2): typical traces of $[Ca^{2+}]_i$ responses and restoration. 2 mM Ca^{2+} was present (black) or absent (grey) during the first 150 s. Then Ca^{2+} was restored to 2 mM as indicated (second arrow) in the calcium-free medium 100 s after LPC stimulation (first arrow). Solid bars indicate the presence of LPC (black) and Ca^{2+} (grey). Traces were representative of at least three experiments. C: Average $[Ca^{2+}]_i$ increase above basal level in the presence (■) or absence (□) of external Ca^{2+}. Data are means ± SD from at least 4 separate experiments. $^*P < 0.05$; $^{**}P < 0.01$.

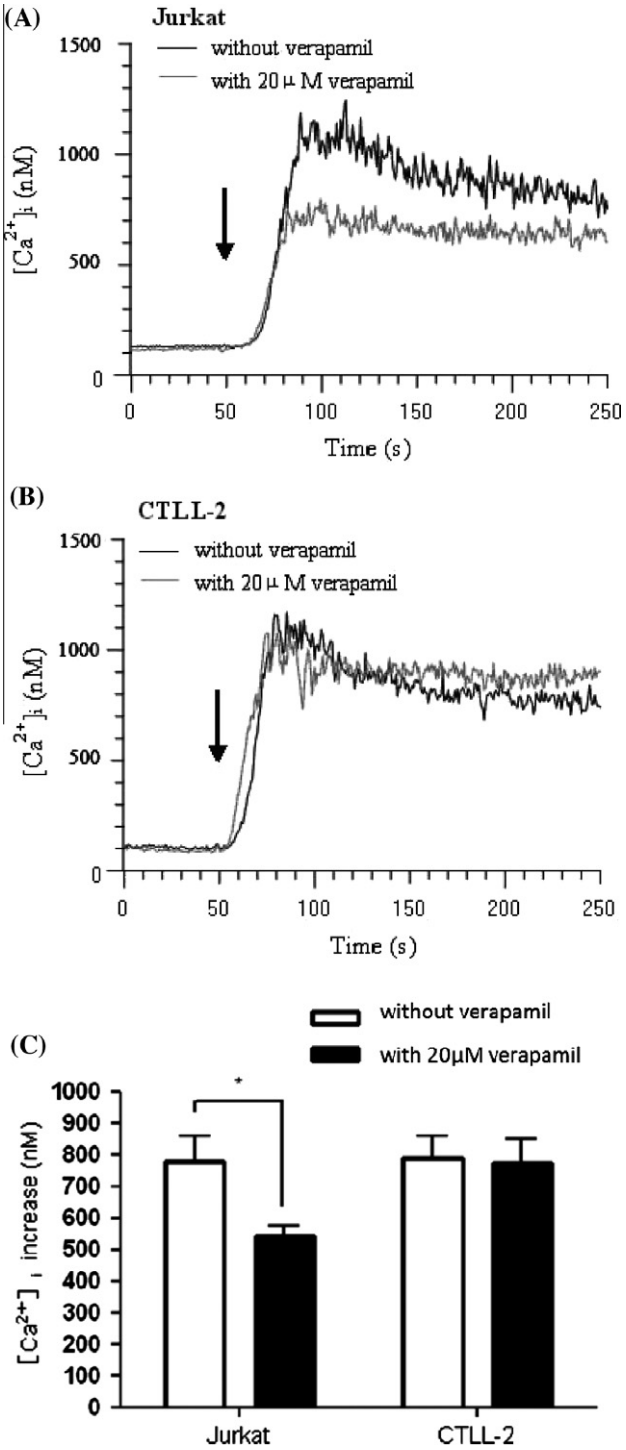

Fig. 4. Effect of verapamil on LPC-induced $[Ca^{2+}]_i$ increase in Jurkat and CTLL-2 T lymphocytes. Fura-2/AM loaded cells were pretreated with vehicle (as control) or with 20 μM verapamil for 15 min, then stimulated with 15 μM LPC in Ca^{2+}-containing medium. A (Jurkat) and B (CTLL-2): typical Ca^{2+} traces by LPC in the absence (black) or presence (grey) of verapamil. Arrows indicate the time of LPC addition. Traces were representative of at least 4 experiments. (B) Average $[Ca^{2+}]_i$ increase above basal level in the absence (□) or presence (■) of verapamil. Data are means ± SD from at least 3 separate experiments. $^*P < 0.05$.

amount of vehicle, Hepes-buffered medium, for the same time as the drug treated samples.

2.6. Statistical analysis

Comparisons between different treatment groups was performed using Student's two-tailed unpaired *t*-test or a one-way analysis of variance (Dunnett's test). Values of $P < 0.05$ were considered significant. All values are given as means ± SD.

3. Results

3.1. Ca^{2+} signals induced by LPC

We first examined the effect of different concentrations of LPC on Ca^{2+} mobilization in Jurkat and CTLL-2 lymphocytes. As shown in Fig. 1, LPC concentration induced a gradual increase in $[Ca^{2+}]_i$, which remained elevated in Ca^{2+}-containing medium in both Jurkat and CTLL-2 lymphocytes. In Jurkat cells, the level of $[Ca^{2+}]_i$ keep stable for 10 s after LPC was added, then rose for about 20 s and become stable and constant. In contrast, the $[Ca^{2+}]_i$ increase in CTLL-2 cells was more rapid than seen in Jurkat cells, since the increase appeared almost as soon as LPC was added to the suspension and kept rising for about 30 s before becoming constant. Concentrations of LPC > 15 μM could cause large Ca^{2+} responses potent enough to stimulate downstream Ca^{2+}-sensitive processes. However, cell viability tested by trypan blue exclusion showed that 20 μM LPC caused 10% cells to die while 15 μM LPC had no significant effect on cell viability (1% cells died). Hence, 15 μM LPC was used in all the subsequent studies.

3.2. The effect of external calcium concentration

To test the effect of external calcium concentration, the relationship between extracellular calcium concentration and LPC-induced increase in $[Ca^{2+}]_i$ was investigated. As shown in Fig. 2, the LPC-induced increase of $[Ca^{2+}]_i$ above the basal value was independent of the concentrations of Ca^{2+} (2 and 4 mM) in the culture medium in both Jurkat and CTLL-2 lymphocytes (Fig. 2A and B).

3.3. Sources of Ca^{2+} signals mediated by LPC

To test whether the increases in $[Ca^{2+}]_i$ induced by LPC were dependent on Ca^{2+} influx from the external medium, or on Ca^{2+} release from internal stores, we measured the effect of 15 μM LPC on Ca^{2+} response in the presence or absence of extracellular Ca^{2+} ($[Ca^{2+}]_e$). The amplitude of $[Ca^{2+}]_i$ increase induced by LPC in Jurkat and CTLL-2 lymphocytes decreased by 61.6% and 24.2%, respectively in the absence of $[Ca^{2+}]_e$ compared to the response in the presence Ca^{2+} (Fig. 3C). Similar results were observed in the Ca^{2+}

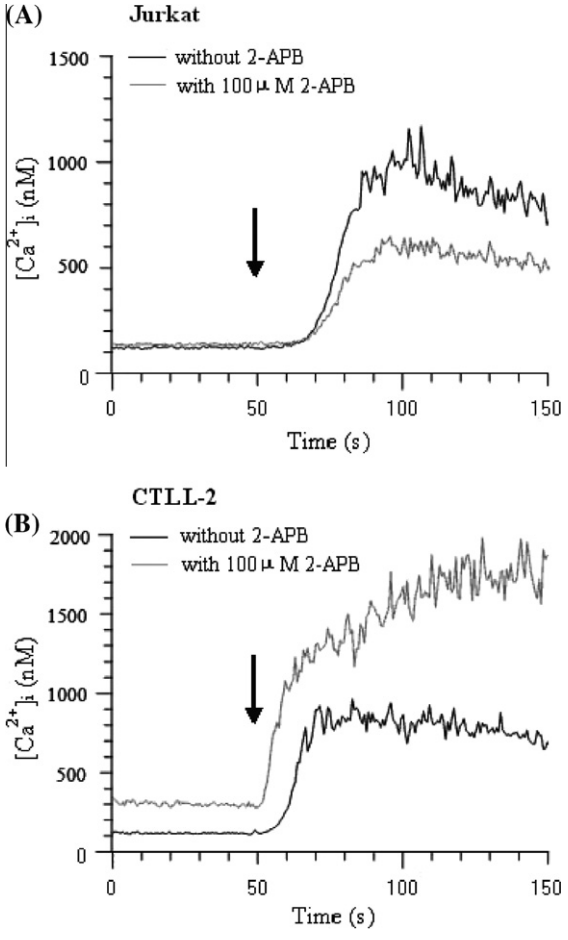

Fig. 5. Effects of 2-APB on LPC-induced $[Ca^{2+}]_i$ increase in Jurkat and CTLL-2 T lymphocytes. Fura-2/AM loaded cells were pretreated with vehicle (as control) or with 100 μM 2-APB (grey) for 15 min, then stimulated with 15 μM LPC in Ca^{2+}-containing medium. A (Jurkat) and B (CTLL-2): typical Ca^{2+} traces by LPC in the absence or presence of 2-APB. Arrows indicate the time of LPC addition. Traces were representative of at least three experiments.

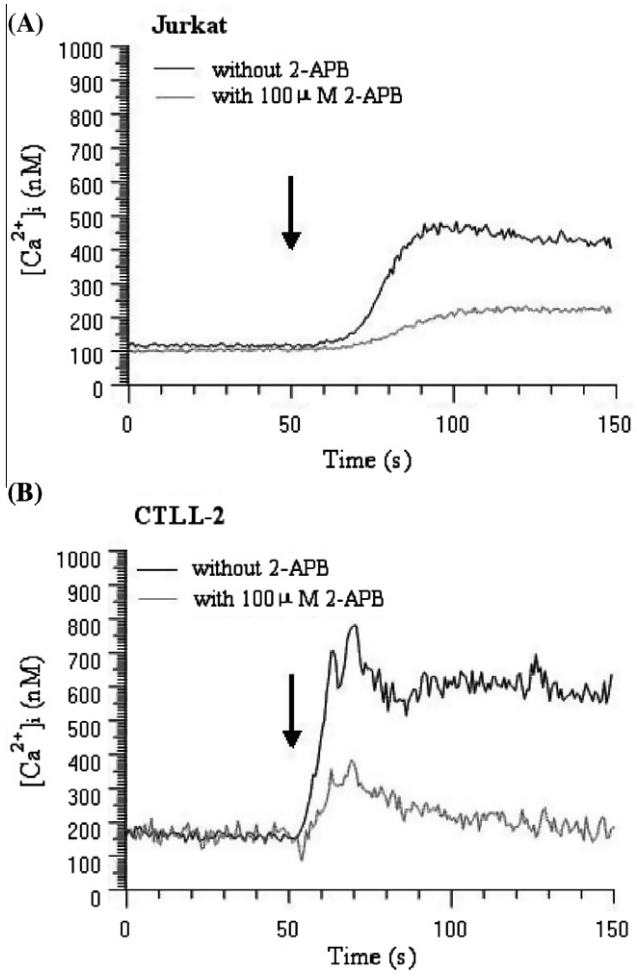

Fig. 6. Role of IP_3R on LPC-induced Ca^{2+} response in Jurkat and CTLL-2 T lymphocytes. Fura-2/AM loaded cells were pretreated with vehicle (as control) or with 100 μM 2-APB (grey) for 15 min, then stimulated with 15 μM LPC in Ca^{2+}-free medium. A (Jurkat) and B (CTLL-2): typical Ca^{2+} traces by LPC in the absence or presence of 2-APB. Traces were representative of at least three experiments.

restoration experiments. A further increase in $[Ca^{2+}]_i$ in both cell types, which we attributed solely to Ca^{2+} entry from the external medium, was observed when 2 mM Ca^{2+} was added to the normally Ca^{2+}-free buffer after LPC stimulation (Fig. 3A and B).

3.4. Effect of the L-type Ca^{2+} channel on LPC-induced Ca^{2+} increase in lymphocytes

To identify the signaling pathway of Ca^{2+} signals induced by LPC, we investigated the effect of verapamil, an L-type Ca^{2+} channel blocker, on LPC-induced Ca^{2+} elevation. Pretreatment of cells with 20 μM verapamil for 15 min did not affect LPC-induced Ca^{2+} response in CTLL-2 lymphocytes but inhibited the response by 25.7% in Jurkat lymphocytes compared to that in the absence of verapamil (Fig. 4A–C).

3.5. Role of 2-APB in LPC-induced Ca^{2+} release

In calcium-containing medium, pretreatment of cells with 100 μM 2-APB for 15 min inhibited about half of the LPC-induced Ca^{2+} release in Jurkat lymphocytes, but strengthened the LPC-induced calcium release in CTLL-2 lymphocytes (Fig. 5).

3.6. Role of inositol trisphosphate receptors in LPC-induced Ca^{2+} release

The possible involvement of the $G_{i/o}/G_{q/11}$ G proteins–phospholipase C–IP_3 receptor pathway in LPC-induced Ca^{2+} increase was examined in both Jurkat and CTLL-2 lymphocytes. In order to exclude the influence of extracellular Ca^{2+}, we conducted these experiments in Ca^{2+}-free medium. As shown in Fig. 6, pretreatment of cells with 100 μM 2-APB, a cell-permanent IP_3 receptor inhibitor, for 15 min inhibited the LPC response by about two thirds in both Jurkat and CTLL-2 T lymphocytes compared to that in the absence of 2-APB.

3.7. Role of ryanodine receptors in LPC-induced Ca^{2+} release

We next tested the role of ryanodine receptors, another class of intracellular Ca^{2+} releasing channels, in the LPC-induced Ca^{2+} response. Experiments were conducted in Ca^{2+}-free medium to minimize the Ca^{2+} entry interference. Pretreatment of cells for

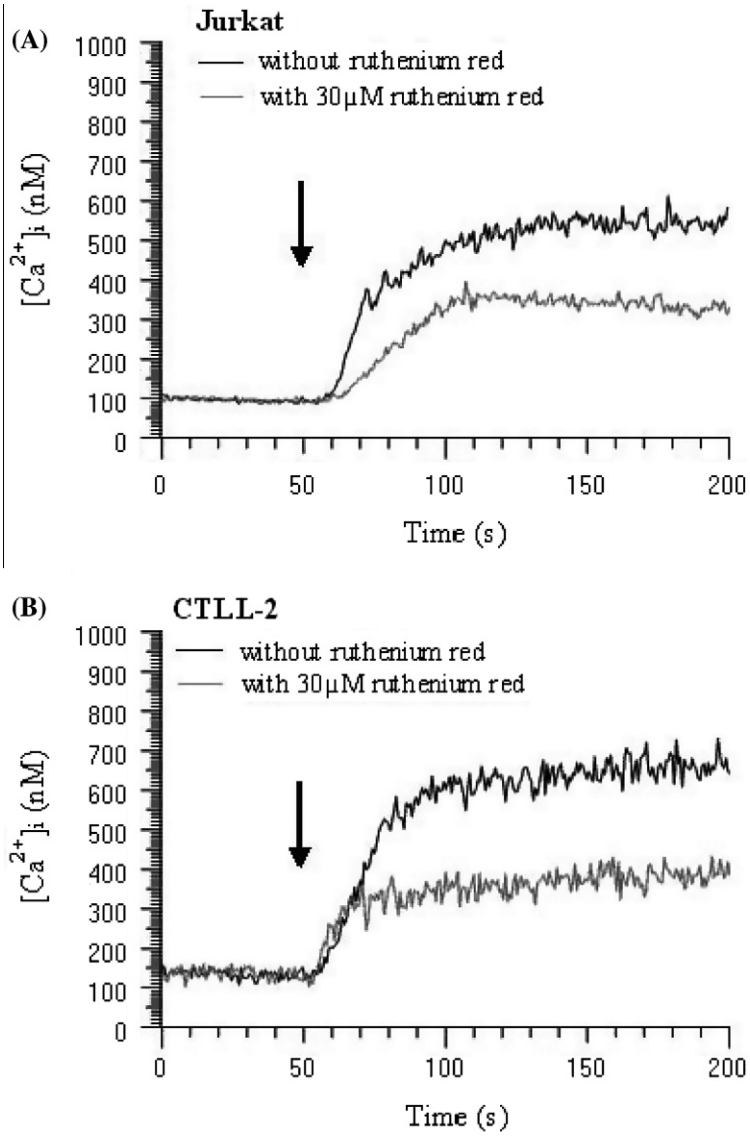

Fig. 7. Role of ryanodine receptors on LPC-induced Ca^{2+} response in Jurkat and CTLL-2 T lymphocytes. Cells loaded with Fura-2/AM were preincubated with vehicle (black) or with 30 μM ruthenium red (grey) for 5 min, then stimulated with 15 μM LPC in Ca^{2+}-free medium. A (Jurkat) and B (CTLL-2): typical Ca^{2+} traces by LPC in the absence or presence of ruthenium red. Traces were representative of at least three experiments.

5 min with 30 μM ruthenium red, a potent ryanodine receptor inhibitor, reduced the LPC-induced Ca^{2+} response in both lymphocytes (Fig. 7).

4. Discussion

In both Jurkat and CTLL-2 lymphocytes, LPC caused a dose-dependent increase in $[Ca^{2+}]_i$ that had nothing to do with the external calcium concentration in the culture medium when it is higher than 2 mM. However, although this increase in $[Ca^{2+}]_i$ mostly depended on Ca^{2+} influx from the external medium in Jurkat lymphocytes, it primary depended on Ca^{2+} release from internal stores in CTLL-2 lymphocytes. Moreover, preincubation of cells with the L-type Ca^{2+} channel blocker verapamil did not affect the LPC-induced Ca^{2+} increase in CTLL-2 lymphocytes but inhibited the response by 26% in Jurkat lymphocytes. In Ca^{2+}-free medium, pretreatment of cells with the potent ryanodine receptor inhibitor ruthenium red reduced the LPC-induced Ca^{2+} response in both kinds of lymphocytes. Moreover, pretreatment of cells with the cell-permanent IP_3 receptor inhibitor 2-APB reduced about two thirds of the LPC-induced calcium response in both Jurkat and CTLL-2 T lymphocytes. These results indicate that LPC-induced $[Ca^{2+}]_i$ mobilization was induced by different mechanisms in each kind of cells, which highlight a possible difference in activation of CD4+ and CD8+ T lymphocytes. In Jurkat human CD4+ T lymphocytes $[Ca^{2+}]_i$ mobilization probably occurred via an influx of external Ca^{2+}. In CTLL-2 mouse CD8+ T lymphocytes, the increase in $[Ca^{2+}]_i$ primary depended on Ca^{2+} release from internal stores and followed a more rapid action. Because pretreatment with verapamil had no obvious effect on LPC-induced Ca^{2+} increase we hypothesize that LPC is unable to activate L-type Ca^{2+} channels in the CTLL-2 T lymphocytes.

It is well known that IP_3 diffuses to the endoplasmic reticulum, binds IP_3 receptor and thus releases Ca^{2+} from the endoplasmic reticulum in almost all types of cells. 2-APB has been described as an antagonist of IP_3 receptors and has been used to block IP_3 receptor-induced $[Ca^{2+}]_i$ increase in various types of cells. However, 2-APB can also act as a partial activator of the IP_3 receptor, inducing some release of Ca^{2+} (Bootman et al., 2002). In our study, in calcium-containing medium, it was found that 2-APB strengthened the LPC-induced calcium release in CTLL-2 lymphocytes but inhibited about half of the calcium release in Jurkat lymphocytes. It is quite possible that 2-APB act mainly as antagonist of IP_3 receptors in Jurkat lymphocytes but also act as activator of the IP_3 receptor in CTLL-2 lymphocytes. On the other hand, it has been shown that this compound can also act on calcium release-activated calcium (CRAC) channels, and on store-operated calcium (SOC) channels in liver cells, through mechanisms independent of IP_3 receptors. In rat basophilic leukemia cells and Jurkat cells, CRAC current had a biphasic dependence on 2-APB concentration; it was potentiated by low micromolar concentrations of 2-APB and inhibited by higher levels of 2-APB (Prakriya and Lewis, 2001). Therefore, it is quite possible that 100 μm of 2-APB is high enough to inhibit Ca^{2+} release in Jurkat T lymphocytes while it is low enough to increase Ca^{2+} release in CTLL-2 T lymphocytes. This suggests that 100 μm 2-APB may regulate LPC-induced calcium release by different mechanisms in these two kinds of lymphocytes independent of IP_3 receptors, but related to CRAC and SOC channels.

LPC was recently found to be a signal produced by apoptotic cells following caspase-3 mediated activation of the intracellular calcium-independent PLA2 (Lauber et al., 2003). The functionally available concentration of LPC appears to be tightly controlled during the acute phase in response to tissue injury and cellular death (Roshak et al., 2000). In the immune system, impaired Ca^{2+} signaling in T cells has been linked to several inherited immunodeficiency diseases, such as severe combined immunodeficient (SCID), Wiskott–Aldrich syndrome (WAS), and some autoimmune and inflammatory diseases. Therefore, our results provide some useful information for further study of apoptosis and inherited immunodeficiency diseases.

Acknowledgments

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