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Life Sciences 71 (2002) 581–590

Life Sciences

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Mitochondrial calcium response in human transformed lymphoblastoid cells

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Received 30 July 2001; accepted 1 February 2002

Abstract

Human lymphoblastoid cell line (LCL) transformed by Epstein-Barr Virus (EBV) is a unique cellular model for the study of human diseases. Although pathophysiological significance of mitochondrial calcium regulation is drawing attention, it is not known whether or not mitochondria in LCLs play a role in intracellular calcium signaling. In this study, role of mitochondria of the lymphoblastoid cell line in calcium signaling was examined. Intra-mitochondrial calcium concentration ($[Ca^{2+}]m$) was successfully measured using dihydro-Rhod-2, revealed by the decrease of fluorescence after application of carbonyl cyanide m-chlorophenylhydrazone (CCCP) and intracellular localization patterns imaged by florescent microscope. Platelet activating factor (PAF) concentration-dependently increased cytosolic calcium concentration ($[Ca^{2+}]i$), while no increase of $[Ca^{2+}]m$ was observed. In contrast, 10 μM thapsigargin increased $[Ca^{2+}]i$ as well as $[Ca^{2+}]m$. LCLs may be used for the study of possible pathophysiological role of mitochondrial calcium regulation in human diseases.

Keywords: Calcium; Mitochondria; Platelet activating factor; Thapsigargin; Carbonyl cyanide m-chlorophenylhydrazone

Introduction

The lymphoblastoid cell line transformed by Epstein-Barr Virus (EBV) has been used as a cellular model for human diseases [1–6], because lymphocyte is easily obtained from patients and transformed

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0024-3205/02/$ - see front matter
PII: S0024-3205(02)01725-3

cells can be maintained for a long time. Calcium signaling in the transformed lymphoblastoid cell lines (LCLs) has been examined to study pathophysiological basis of various common diseases such as hypertension [1], diabetes mellitus [2], Alzheimer's disease [3], Huntington's disease [3], and bipolar disorder [4]. LCLs have also been used for the study of mitochondrial function in mitochondrial encephalomyopathies [5] as well as neurodegenerative disorders [3,6].

Recently, a role of mitochondria in regulation of intracellular calcium signaling has been well characterized in excitable cells such as neuronal cells [7 8], myocytes [9], pancreatic β-cells [10] and Hela cells [11], and a possible pathophysiological role of mitochondrial calcium regulation in human diseases such as neurodegenerative disorders, mitochondrial encephalopathies, and mental disorders was suggested. However, whether or not LCLs can be used for the study of mitochondrial calcium signaling is not known, because mitochondrial calcium concentration in non-excitable cells such as LCLs has not been well studied.

Although LCLs transformed by EBV do not have B-cell antigen receptor-mediated calcium mobilization [12], platelet activating factor (PAF)-mediated calcium mobilization via phosphoinositide (PI) pathway is well characterized [13–16].

In this study, the role of mitochondria in calcium signaling of LCLs is characterized in order to clarify whether or not this cell line can be used for the clinical studies of mitochondrial calcium signaling in human diseases.

Material and Methods

Peripheral blood was drawn from healthy volunteers into 7 ml plastic tubes containing heparin sodium and lymphocytes were separated by standard protocols using Ficoll-Paque (Pharmacia-Upjohn Inc.). Written informed consent was obtained from the subjects. This study is approved by the Ethical Committee of the Brain Science Institute. These cells were cultured with RPMI 1640 medium (SIGMA) containing 20% fetal bovine serum (FBS) (GIBCO BRL), penicillin and streptomycin (50 ug/ml each) (GIBCO BRL), and filtered supernatant of the B95-8 cell culture infected by Epstein-Barr Virus (a gift from Dr. Fukushima, Shinshu Univ.). The cells were passaged every week until the cell line was established. Thereafter, the cells were passaged three times a week using similar medium except for the addition of 10% FBS. The cells were kept frozen before calcium measurement. The cells were thawed and used for the experiments within 2 months.

For cytosolic Ca^{2+}([Ca^{2+}]i) measurement, fura-2-AM (final concentration 1 μM) (Dojindo, Kumamoto, Japan) was added to the medium containing 2×10^6 cells/mm^3, and cells were incubated at 37 °C for 30 minutes, washed in serum free-RPMI 1640 medium, maintained at room temperature for 30 minutes, and dispersed in measurement buffer containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH = 7.4), and 5 mM glucose. The cells loaded with Fura-2-AM were measured by a fluorescence spectrometer F-2500 (Hitachi, Tokyo, Japan). The cells were maintained at 37 °C and gently mixed during measurement. The emission at 510 nm was monitored by excitation at 340 nm or 380 nm. After auto-fluorescence was subtracted, calcium concentration was calculated being calibrated by the Ca^{2+}-saturated condition after cell lysis by addition of 1% Triton X-100 5uL and the Ca^{2+}-free condition after adding 0.2 M EGTA (ethylene glycol-bis-[β-aminoethylether]-*N,N,N′,N′*-tetraacetic acid) 20uL. Kd was assumed to be 227 nM [17].

Mitochondrial Ca^{2+} concentration ($[Ca^{2+}]m$) was measured by dihydro-Rhod-2 [18]. Minimal amount of $NaBH_4$ was added to the 0.4 mM Rhod-2-AM (Dojindo) solution in dimethyl sulfoxide (DMSO). After the color disappeared, dihydro-Rhod-2-AM (final concentration 4 μM) was added to the medium containing 2×10^6 cells/mm^3 and incubated at room temperature for 60 minutes, washed in serum free-RPMI 1640 medium, maintained at 37 °C for 60 minutes and dispersed in the same measurement buffer.

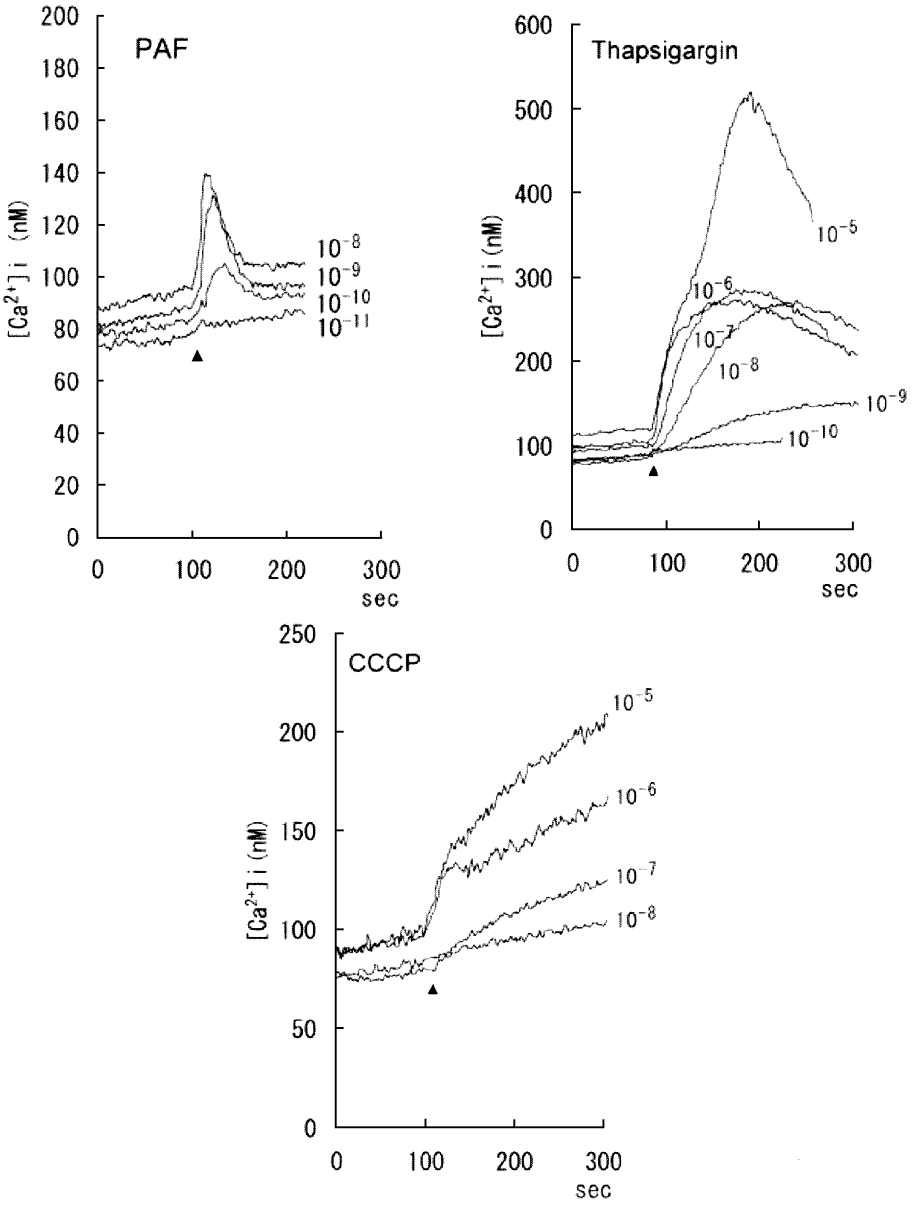

Fig. 1. Cytosolic calcium response evoked by PAF (upper left), thapsigargin (upper right), and CCCP (bottom), measured by Fura-2. All figures indicate a representative data set in one cell line.

The emission at 576 nm was measured by excitation at 553 nm. After baseline calcium concentration was measured, the following chemicals were applied to the cells; PAF (Calbiochem), CCCP (carbonyl cyanide m-cholorophenylhydrazone) and thapsigargin. PAF was used to examine the role of mitochondria in phosphoinositides-linked calcium mobilization. CCCP, a proton ionophore, was used for the examination of leak of Ca^{2+} due to loss of proton gradient across mitochondrial inner membrane.

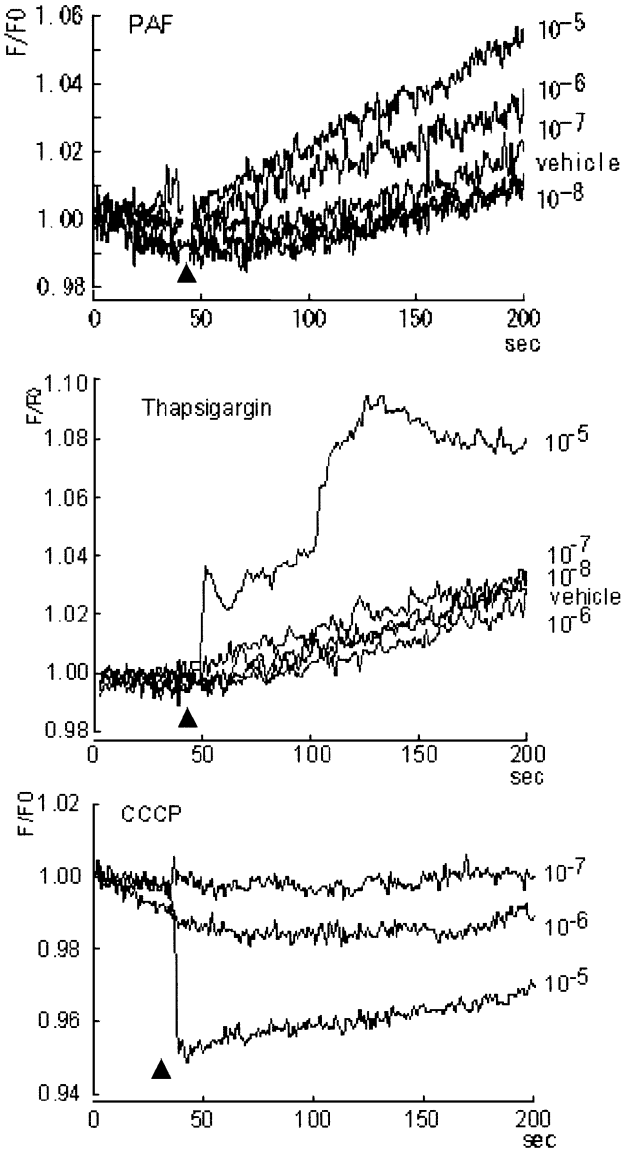

Fig. 2. Mitochondrial calcium response evoked by PAF (upper), thapsigargin (middle), and CCCP (bottom), measured by Rhod-2. F/F0 denotes the ratio of 576 nm fluorescence of Rhod-2 (F) divided by the initial value of F (F0). All figures indicate a representative data set in one cell line.

Thapsigargin is an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase and subsequently activates store-dependent calcium influx. This was used to increase cytosolic calcium concentration to the higher levels than that caused by PAF [19].

All experiments were performed in duplicate or triplicate in at least two cell lines.

Lymphoblastoid cells loaded with dihydro-Rhod-2 were also imaged in the same buffer at 24 °C on an Olympus IX-70 with a Uplan Apo 100 × objective. Samples were illuminated with the light through an emission filter (590DF35, OMEGA) using a CCD camera (MicroMax-1300Y/HS, Roper Scientific), controlled by MetaMorph 4.6 software (Universal Imaging). Deconvolution (non-neighboring method) was applied to the image.

Resting mitochondrial calcium concentration ([Ca^{2+}]m) was calculated being calibrated by the Ca^{2+}-saturated and Ca^{2+}-free conditions [11]. Ca^{2+}-saturated condition was achieved by addition of 1% Triton X-100 (5uL) and bromo-A23187 (SIGMA) (final concentration of 10 μM), a non-fluorescent calcium ionophore [20]. Ca^{2+}-free condition was achieved by adding EGTA (final concentration 20 mM) and $MnCl_2$ (final concentration 10 mM). The Kd of Rhod-2 in mitochondria was assumed to be 1 μM, which was examined in mitochondria of Hela cells [11]. [Ca^{2+}]m was calculated by the following equation.

$$[Ca^{2+}]m = Kd(F - Fmin)/(Fmax - F)$$

where, F is the fluorescence of Rhod-2, Fmax denotes the maximum fluorescence, and Fmin denotes the minimum fluorescence.

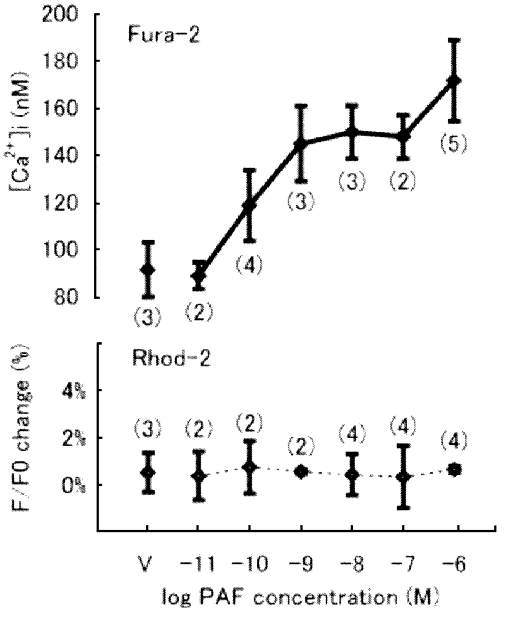

Fig. 3. Dose response curve of cytosolic (upper) and mitochondrial calcium concentration (lower) for PAF. Peak [Ca^{2+}]i after PAF stimulation were analyzed.

Results

Reliability of the measurement

Inter-assay intra-individual coefficient of variation (CV) of $[Ca^{2+}]i$ was examined by two experiments on different days in 5 cell lines derived from healthy volunteers (29–46 years old male) at one concentration each of the agents. Inter-assay CVs for $[Ca^{2+}]i$ measured by Fura-2 were 6.4% for basal level, 12.4% for peak level after 10^{-6}M PAF stimulation, 12.8% for peak level after 10^{-5}M thapsigargin, 19.4% for the initial increase after thapsigargin, and 6.1% for the calcium level 50 seconds after CCCP stimulation. Intra-assay CVs of $[Ca^{2+}]m$ was examined by two experiments on one day in these 5 cell lines. Intra-assay CVs for $[Ca^{2+}]m$ measured by Rhod-2 is 4.1% for peak of

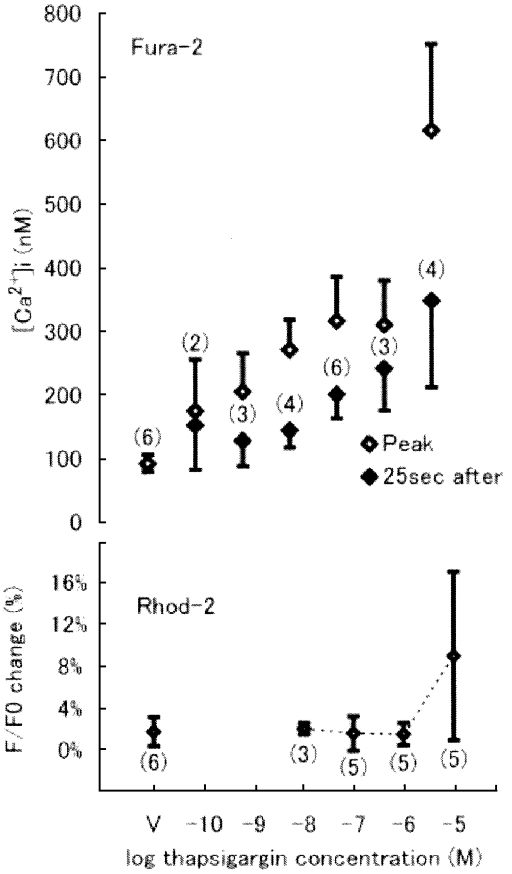

Fig. 4. Dose response curve of cytosolic (upper) and mitochondrial calcium concentration (lower) for thapsigargin. For $[Ca^{2+}]i$, both peak level and the average values of 24-26 sec after the stimulation were shown. For $[Ca^{2+}]m$, the ratio of average F/F0 value of 96–105 sec after the stimulation divided by the average F/F0 values before the stimulation were shown. Bars indicate standard deviation (SD). V: Vehicle. Numbers in parenthesis indicate the number of examinations.

fluorescence ratio (F/F0) after 10^{-5}M thapsigargin and 16.1% for decrease of F/F0 after 10^{-5}M CCCP stimulation.

Inter-individual variations of these variables were examined in these 5 cell lines in duplicate. Inter-individual CVs for $[Ca^{2+}]$i were 13.1% for basal level, 19.6% for PAF, 11.4% for peak after thapsigargin, 16.7% for initial increase after thapsigargin, and 10.5% for CCCP. Inter-individual CVs of $[Ca^{2+}]$m were 40.2% for thapsigargin and 43.8% for CCCP.

PAF

PAF concentration-dependently increased ($[Ca^{2+}]$i) (Fig. 1) with the EC_{50} of 3.8×10^{-10} M. PAF did not increase $[Ca^{2+}]$m in the concentration range of $10^{-11}-10^{-6}$ M (Fig. 2, Fig. 3). These data suggest that increase of cytosolic calcium mediated by PAF receptor-linked PI metabolism did not affect mitochondrial calcium concentration in LCLs.

Thapsigargin

Thapsigargin concentration-dependently increased $[Ca^{2+}]$i (Fig. 1) in the range of 10^{-9} to 10^{-5} M with no saturation (Fig. 4). It increased $[Ca^{2+}]$m only at 10^{-5} M (Fig. 4), the concentration which elevated $[Ca^{2+}]$i by more than 300 nM (Fig. 1).

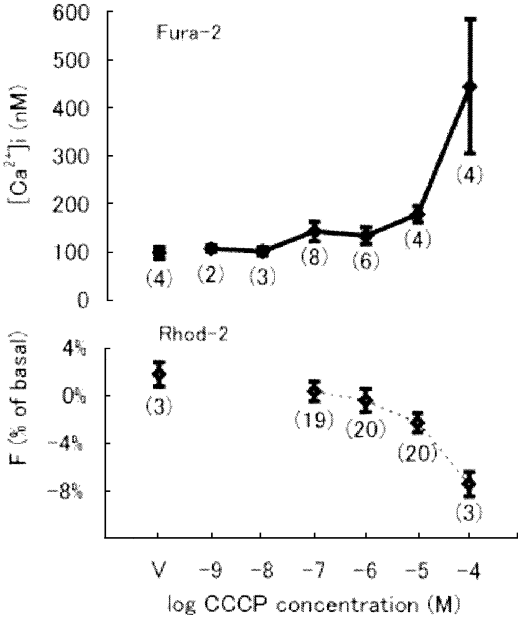

Fig. 5. Dose response curve of cytosolic (upper) and mitochondrial calcium concentration (lower) for CCCP. The average $[Ca^{2+}]$i 15–24 sec after the stimulation was shown. For $[Ca^{2+}]$m, the ratio of average F/F0 value of 45–54 sec after the stimulation divided by the average F/F0 values before the stimulation were plotted.

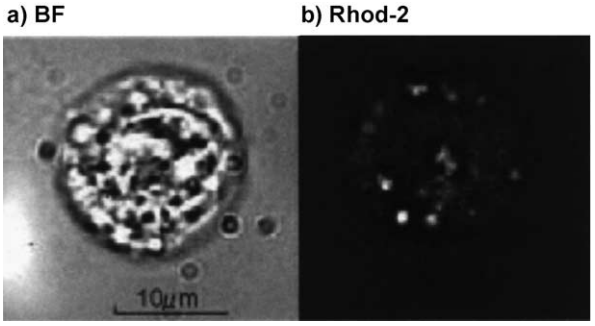

Fig. 6. Images of a lymphoblastoid cell loaded with dihydro-Rhod-2 by bright field (BF) (a) and fluorescence microscope (b). The Rhod-2 fluorescence is localized in mitochondria.

CCCP

CCCP concentration-dependently increased $[Ca^{2+}]i$ in the range of 10^{-9} to 10^{-4} M (Fig. 1) without saturation (Fig. 5). On the other hand, CCCP dose dependently decreased $[Ca^{2+}]m$.

Resting mitochondrial Ca^{2+} concentration

$[Ca^{2+}]m$ at rest was calculated to be 224.1 ± 26.9 nM (mean ± SD, n = 10), and it reached 379 nM (n = 1) after thapsigargin 10^{-5} M stimulation.

Localization of Rhod-2 signal

Rhod-2 image of lymphoblastoid cells showed patchy staining in cytosol. This pattern is compatible with preferential staining of mitochondria using dihydro-Rhod-2 (Fig. 6).

Discussion

In this study, intracellular mitochondrial calcium concentration was successfully measured in transformed lymphoblastoid cells using dihydro-Rhod-2, revealed by decreased fluorescence after stimulation by proton ionophore and images showing accumulation of the dye in mitochondria.

PAF-induced increase of $[Ca^{2+}]i$ was not accompanied by increase of $[Ca^{2+}]m$, suggesting that mitochondria may not participate in PAF-evoked calcium mobilization in LCLs unlike agonist stimulation in excitable cells. However, an effect of mitochondria on PAF evoked calcium response cannot be totally ruled out by the present results.

$[Ca^{2+}]m$ was increased when $[Ca^{2+}]i$ was elevated during thapsigargin 10 μM stimulation, which may suggest that mitochondria in LCLs have ability to accumulate calcium similarly to excitable cells.

This study has several limitations. In this study, thapsigargin response was determined in the presence of 1 mM Ca^{2+} throughout the incubation. This procedure does not resolve the store-dependent depletion and Ca^{2+} entry components of the response. This likely accounts for the nature of the dose response

curve we obtained following thapsigargin stimulation which spans 4 orders of magnitude and does not reach saturation, as well as large variance at 10^{-5} M stimulation. Thapsigargin inhibits some ion channels at concentration above 1 μM; thus the effects of thapsigargin on Ca^{2+} flux at 10^{-5} M are likely to be complex.

Absolute concentration determination is difficult by using Rhod-2, because it is not a ratio-metric dye. Therefore, mitochondrial calcium concentration presented in this study is quite preliminary and needs further validation. However, the value at which mitochondria begin to accumulate cytosolic calcium seemed to be comparable to the resting $[Ca^{2+}]$m measured in this study. The data suggested that $[Ca^{2+}]$m did not increased upon PAF induced $[Ca^{2+}]$i increases, while it did increase upon thapsigargin induced $[Ca^{2+}]$i increases. This may suggest that mitochondria in lymphoblastoid cells have privileged access to Ca^{2+} entered from plasma membrane Ca^{2+} channels [21]. Otherwise, this may be simply due to difference of $[Ca^{2+}]$i levels achieved by these two stimulations.

In this study, only transformed lymphoblastoid cells were examined. However, it is not known whether or not the results in these cells completely represent endogenous conditions since gene expression may be altered in these cells.

In spite of these limitations, this study suggests that mitochondria in human LCL can accumulate calcium similarly to excitable cells and this cell model may be used for the studies of possible pathophysiological significance of mitochondrial calcium regulation in various human diseases. We hypothesized that altered mitochondrial calcium signaling may participate in the pathophysiology of bipolar affective disorder [22]. This experimental system may clarify possible significance of mitochondrial calcium regulation in this disorder.

Acknowledgements

T.K. is supported by the Independent Investigator Award from the National Alliance for Research on Schizophrenia and Depression (NARSAD), a Research Grant from the Human Science Foundation, and a grant from Japanese Ministry of Science, Culture, and Technology. The authors are grateful to Dr Yoshimitsu Fukushima, Ms. Keiko Wakui, Dr. Takafumi Ishida, and Dr. Jeffrey P. Gardner, who gave valuable suggestions on cell culture. The authors are also thankful to Dr. Ariyuki Kagaya, Dr. Takehiko Yokomizo, Dr. Yoshihisa Kudo, and Dr Masamitsu Iino, for valuable suggestions on the calcium measurements.

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