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Intracellular Ca^{2+} Mobilization and Beta-hexosaminidase Release Are Not Influenced by 60 Hz-electromagnetic Fields (EMF) in RBL 2H3 Cells

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The effects of extremely low frequency electromagnetic fields (EMF) on intracellular Ca^{2+} mobilization and cellular function in RBL 2H3 cells were investigated. Exposure to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h did not produce any cytotoxic effects in RBL 2H3 cells. Melittin, ionomycin and thapsigargin each dose-dependently increased the intracellular Ca^{2+} concentration. The increase of intracellular Ca^{2+} induced by these three agents was not affected by exposure to EMF (60 Hz, 1 mT) for 4 or 16 h in RBL 2H3 cells. To investigate the effect of EMF on exocytosis, we measured beta-hexosaminidase release in RBL 2H3 cells. Basal release of beta-hexosaminidase was 12.3±2.3% in RBL 2H3 cells. Exposure to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h did not affect the basal or 1 μM melittin-induced beta-hexosaminidase release in RBL 2H3 cells. This study suggests that exposure to EMF (60 Hz, 0.1 or 1 mT), which is the limit of occupational exposure, has no influence on intracellular Ca^{2+} mobilization and cellular function in RBL 2H3 cells.

Key Words: EMF, Ca^{2+} mobilization, Exocytosis, Beta-hexosaminidase

INTRODUCTION

Many epidemiologic studies suggested the possibility that exposure to extremely low frequency electromagnetic fields (EMF) may be related to the risk of acute lymphoblastic leukemia in children [1-3]. There is a public concern about the possible adverse health effects associated with exposure to EMF. However, the mechanism of the interaction between EMF and cellular systems is still unclear.

We previously reported that there are no significant changes in phospholipase activity such as PLA_2, PLC and PLD in RAW 264.7 cells and RBL 2H3 cells exposed to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h [4]. However, cellular function may be affected by a variety of signaling pathways in addition to the phospholipase pathway. Considering that many cellular functions are closely related to the increase of intracellular Ca^{2+} concentration, it is plausible that EMF exposure may cause alterations in Ca^{2+} mobilization via both extracellular Ca^{2+} influx and intracellular Ca^{2+} release.

Ca^{2+} is a universal messenger that controls a variety of cell functions, including secretion. The increase of intracellular Ca^{2+} is caused by the influx of extracellular Ca^{2+} via Ca^{2+} channel, intracellular Ca^{2+} release via inositol-1,4,5-triphosphate (IP_3) [5,6] and inhibition of the Ca^{2+} pump that lowers the intracellular Ca^{2+} concentration. Intracellular Ca^{2+} mobilization can be regulated by a variety of exogenous stimulants. Melittin-induced increase of intracellular Ca^{2+} is related to PLC-mediated inositol triphosphate (IP_3) accumulation with receptor operated Ca^{2+} channels (ROCC) [7]. Ionomycin is a well-known ionophore and increases intracellular Ca^{2+} concentration through Ca^{2+}-release activated Ca^{2+} (CRAC) channels [8]. Thapsigargin also increases intracellular Ca^{2+} concentration via the inhibition of sarco/endoplasmic reticulum Ca^{2+} ATPase [9]. In this study, we investigated the effect of EMF on intracellular Ca^{2+} mobilization stimulated by melittin, ionomycin and thapsigargin and on beta-hexosaminidase release in RBL 2H3 cells.

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METHODS

Materials

Melittin, ionomycin, thapsigargin and ρ-nitrophenyl-N-acetyl-β-glucosaminide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's minimum essential medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Co. (Grand Island, NY, USA). Fura-2/AM was purchased from Enzo Life Sciences (Plymouth, PA, USA). Other reagents were

ABBREVIATIONS: EMF, extremely low frequency electromagnetic fields; ROCC, receptor operated Ca^{2+} channels; CRAC, Ca^{2+}-release activated Ca^{2+}.

purchased from Sigma Chemical Co. (USA).

Cell culture

Rat basophilic leukemia (RBL 2H3) cells were grown in Dulbecco's modified Eagle minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotic-antifungal mix (100 IU/ml penicillin G, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B) at 37°C in 5% CO_2.

EMF exposure system

EMF generation equipment was designed and constructed by Korea Electrotechnology Research Institute (Korea). Monitoring of magnetic field was conducted under observation of the current injected to the exposure system, because the magnetic field is proportional to the injected current. The field generator consisted of four square-shaped coils and one cage with three testing floors (top, middle and bottom floor).

The voltage fluctuation rate and the harmonic rate of the power quality using a power amp was under 1%. After fixing the magnetic field of the center of the middle floor to 1 mT, the fields at various points were measured. The spatial variation of the magnetic field was less than 3%. This strongly demonstrates that the field generator is well suited for a small-scale *in vitro* study. Using a water-jet cooling system, the temperature in the incubator at 1 mT was maintained at 37±0.3°C. Also, a magnetic field shielding system using ferrite material was adopted to shield the strong magnetic field in the outer regions of the EMF exposure system. The coils were turned on for at least 30 min before use, and the cells were exposed to 0.1 mT and 1 mT in 60 Hz magnetic field for 4 h and 16 h. All experiments were conducted under the same environmental conditions.

MTT assay

Cell viability was performed with an MTT-based colorimetric assay [10]. Cells in 96-well plates (1×10^5 cells/well) were exposed to EMF at 37°C for 4 or 16 h. 20 μl of MTT solution (5 mg/ml in phosphate buffered saline) was added and further incubated for 3 h. After aspirating the supernatant from the wells, 100 μl of dimethyl sulfoxide was added to dissolve the formazan crystals. The absorbance of each well was then read at 520 nm.

Measurement of intracellular Ca^{2+} mobilization

The intracellular Ca^{2+} level was measured using Fura-2/AM by monitoring a fluorospectrometer [11]. Briefly, culture medium was replaced and cells were washed 3 times with PBS. After that, cells were detached by trypsin and suspended with 10 ml Krebs solution, then loaded with Fura-2/AM to a final concentration of 2 μM and incubated at 37°C for 1 h. The loaded cells were washed twice with Krebs solution and centrifuged at 3,000×g for 10 min. The Fura-2 fluorescence was monitored on a Quanta Master (Qm4, Photon Technology International, NJ, USA.) at 37°C with excitation at 340 and 380 nm and emission at 500 nm. The ratio of F_{340}/F_{380} was recorded, and the maximum fluorescence ratio (R_{max}) was measured by using 0.1% Triton X-100. The minimum fluorescence ratio (R_{min}) was measured following depletion of external Ca^{2+} by addition of 5 mM EGTA/Tris pH8.5.

Measurement of beta-hexosaminidase release

To investigate the effect of EMF on exocytosis, we measured beta-hexosaminidase release in RBL 2H3 cells. RBL 2H3 cells exposed to EMF were washed with PIPES buffer 3 times and then suspended in PIPES buffer. The cells were stimulated with 0.5 μM melittin for 30 min at 37°C [12]. The cell suspension was centrifuged at 300×g for 10 min. 20 μl of the supernatant was incubated with an equal volume of substrate solution (2 mM ρ-nitrophenyl-N-acetyl-β-glucosaminide, 0.1 M citrate, pH 4.5) in duplicate in 96-well plates for 1 h at 37°C. The reaction was stopped by adding 200 μl of 0.1 M sodium carbonate buffer, pH 10.0 [13]. The absorbance was measured at 405 nm with fluorospectrophotometer (FL600 Microplate Fluorescence Reader, Bio-tek).

Statistical analysis

Results are presented as mean±S.D. and were analyzed statistically by analysis of variance (ANOVA). Differences between groups were determined with the Newman-Keul's test. The level of significance was set at less than 5% ($p<0.05$).

RESULTS

Effect of EMF on the viability of RBL 2H3 cells

To exclude the possibility that EMF may affect cellular viability, we first confirmed the cytotoxicity of EMF using an MTT assay. The exposure to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h did not produce any cytotoxic effects in RBL 2H3 cells (Fig. 1).

Effect of EMF on intracellular Ca^{2+} mobilization

To investigate the effect of EMF on the influx of extracellular Ca^{2+} via receptor operated Ca^{2+} channels (ROCC), we measured melittin-induced intracellular Ca^{2+} mobilization in RBL 2H3 cells exposed to EMF (60 Hz, 1 mT) for 4 or 16 h. Melittin dose-dependently increased the intracellular Ca^{2+} concentration (Fig. 2A). EMF did not af-

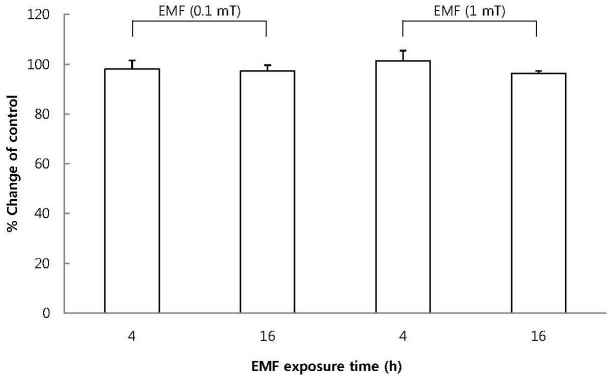

Fig. 1. The effect of EMF on cell viability of RBL 2H3 cells. The cells were exposed to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h and viability was measured with MTT assay. Results are indicated in mean±S.D. from four separate experiments.

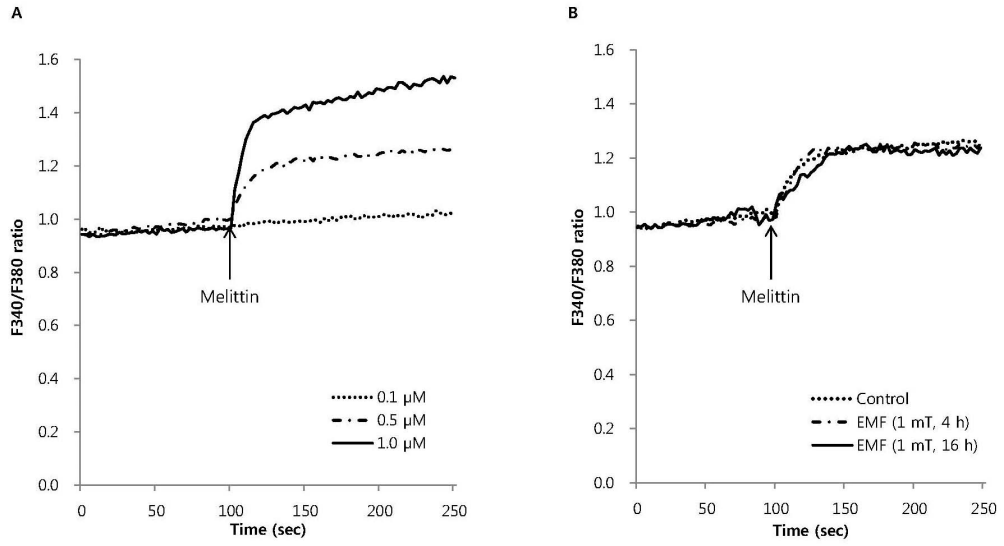

Fig. 2. The effect of EMF on the intracellular Ca^{2+} mobilization induced by melittin in RBL 2H3 cells. Intracellular Ca^{2+} mobilization induced by melittin was measured with Quanta Master in Fura-2AM-loaded RBL 2H3 cells. Melittin dose-dependently increased intracellular Ca^{2+} mobilization (A) and 0.5 μM melittin-induced intracellular Ca^{2+} mobilization was not affected by EMF (60 Hz, 1 mT) for 4 or 16 h (B). Results are the representative data of four separate experiments.

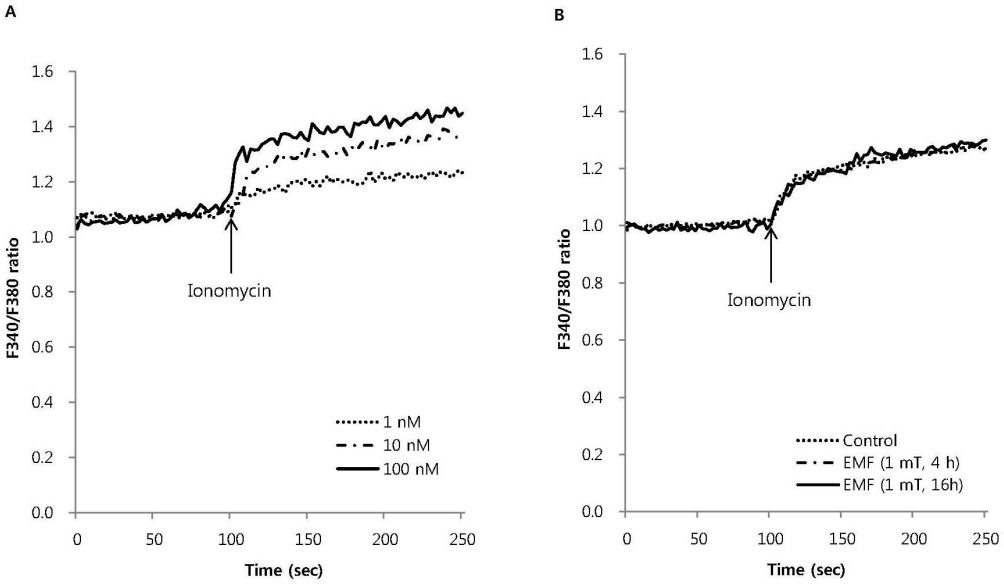

Fig. 3. The effect of EMF on intracellular Ca^{2+} mobilization induced by ionomycin in RBL 2H3 cells. Ionomycin dose-dependently increased intracellular Ca^{2+} mobilization (A) and 10 nM ionomycin-induced intracellular Ca^{2+} mobilization was not affected by EMF (60 Hz, 1 mT) for 4 or 16 h (B). Results are the representative data of four separate experiments.

fect the 0.5 μM melittin-induced intracellular Ca^{2+} mobilization (Fig. 2B). As an ionophore, ionomycin increased intracellular Ca^{2+} concentration in a dose-dependent manner (Fig. 3A), while thapsigargin significantly increased the concentration via inhibition of sarco/endoplasmic reticulum Ca^{2+} ATPase in RBL 2H3 cells (Fig. 4A). EMF did not influence the intracellular Ca^{2+} concentration induced by 10 nM ionomycin or 100 nM thapsigargin (Fig. 3B, 4B). EMF (60 Hz, 0.1 mT) for 4 or 16 h did not change the intracellular Ca^{2+} concentration induced by 0.5 μM melittin, 10 nM ionomycin or 100 nM thapsigargin (data not shown).

Effect of EMF on beta-hexosaminidase release in RBL 2H3 cells

To investigate the effect of EMF on exocytosis, we measured beta-hexosaminidase release in RBL 2H3 cells. Basal release of beta-hexosaminidase was 12.3±2.3% in RBL 2H3 cells. Melittin increased beta-hexosaminidase release in a dose-dependent manner, whereas ionomycin and thapsigargin did not cause beta-hexosaminidase release (Table 1). Exposure to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h did not affect the basal and 1 μM melittin-induced beta-hexosaminidase release in RBL 2H3 cells (Fig. 5).

DISCUSSION

The aim of this study was to investigate the effect of EMF on intracellular Ca^{2+} mobilization and exocytosis of beta-hexosaminidase in RBL 2H3 cells. Before measuring the exocytosis of beta-hexosaminidase, it was very important

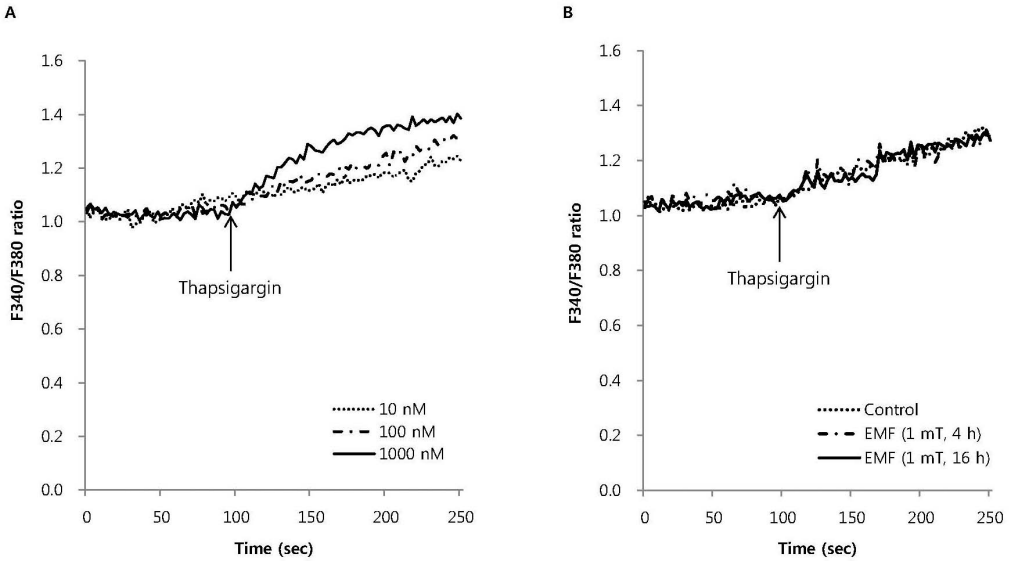

Fig. 4. The effect of EMF on intracellular Ca^{2+} mobilization induced by thapsigargin in RBL 2H3 cells. Thapsigargin dose-dependently increased intracellular Ca^{2+} mobilization (A) and 100 nM thapsigargin-induced intracellular Ca^{2+} mobilization was not affected by EMF (60 Hz, 1 mT) for 4 or 16 h (B). Results are the representative data of four separate experiments.

Table 1. Dose-response of beta-hexosaminidase release by melittin, ionomycin and thapsigargin in RBL 2H3 cells

Groups	Concentrations	% Release
Control		14.8±1.6
Melittin	0.1 μM	18.3±1.2*
	0.5 μM	28.8±4.1*
	1.0 μM	34.9±6.8*
Ionomycin	1 nM	13.1±2.1
	10 nM	14.5±3.6
	100 nM	15.2±1.7
Thapsigargin	10 nM	13.5±0.7
	100 nM	14.3±1.1
	1000 nM	16.0±2.3

*Significantly different from control ($p < 0.05$).

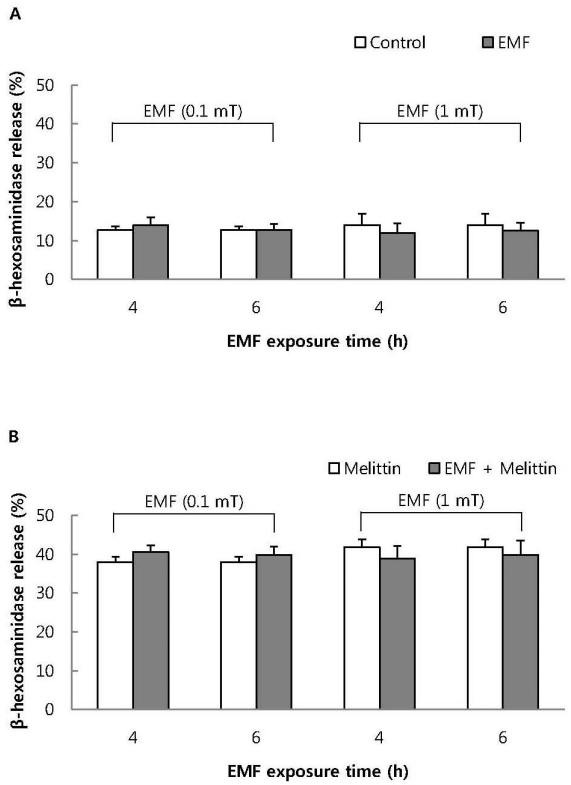

Fig. 5. The effect of EMF on basal (A) and 1 μM melittin-induced beta-hexosaminidase release (B) in RBL 2H3 cells. Both basal and 1 μM melittin-induced beta-hexosaminidase release were not affected by exposure to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h. Results indicate mean±S.D. from four separate experiments.

to confirm the cytotoxicity of EMF. Exposure to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h did not show any cytotoxic activity in RBL 2H3 cells. It has been reported that 0.5 mT EMF has no significant effect on proliferation of human peripheral blood mononuclear cells [14], but 20 mT EMF for up to 23 days could inhibit the growth of human mesenchymal stem cells [15]. The cytotoxicity of EMF may be dependent on the intensity of EMF. The current data did not reveal any cytotoxic activity in RBL 2H3 cells, suggesting that exocytosis of beta-hexosaminidase may be independent on cell membrane rupture by EMF.

Ca^{2+} is a universal messenger that controls various cellular functions, including secretion, contraction, proliferation and differentiation. Intracellular Ca^{2+} is increased by a variety of pathways, such as the influx of extracellular Ca^{2+}, release of intracellular Ca^{2+} stores [5,6] and inhibition of the Ca^{2+} pump that reduces intracellular Ca^{2+} concentration. In this experiment, melittin, ionomycin and thapsigargin each dose-dependently increased intracellular Ca^{2+} concentration. The increase of intracellular Ca^{2+} induced by these three agents was not affected by exposure to EMF (60 Hz, 1 mT) for 4 or 16 h in RBL 2H3 cells. The melittin-induced increase of intracellular Ca^{2+} was related to PLC-mediated inositol triphosphate (IP_3) accumulation with receptor operated Ca^{2+} channels (ROCC) [7]. Ionomycin is a well-known ionophore and increases intracellular Ca^{2+} concentration through Ca^{2+}-release activated Ca^{2+} (CRAC) channels [8]. Thapsigargin also increases intra-

cellular Ca^{2+} concentration via the inhibition of sarco/endoplasmic reticulum Ca^{2+} ATPase [9]. These data suggest that EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h does not influence intracellular Ca^{2+} mobilization via the influx of extracellular Ca^{2+}, the release of intracellular Ca^{2+} store or the Ca^{2+} pump in RBL 2H3 cells. However, there has been much debate regarding the effect of EMF on intracellular Ca^{2+} concentration: EMF has been shown to increase intracellular Ca^{2+} concentration [16,17], to decrease it [18] and to have no effect on it [19,20]. Such different effects of EMF on intracellular Ca^{2+} concentration may be due to the cell types used and the experimental conditions.

To investigate the effect of EMF on exocytosis, we measured beta-hexosaminidase release in RBL 2H3 cells. Exposure to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h did not affect the basal and 1 μM melittin-induced beta-hexosaminidase release in RBL 2H3 cells. This data agreed with the previous report that single exposure to EMF did not cause the degranulation of mast cells [21].

Guidelines on 50/60 Hz electromagnetic fields were issued by IRPA/INIRC in 1990. The basic hypothesis suggested that 50/60 Hz magnetic fields from external sources such as powerlines could be linked to an increased risk of childhood leukemia [1]. However, the mechanism underlying the interaction between EMF and cellular functions remains elusive. It is therefore necessary to assess the changes of intracellular Ca^{2+} and cellular function caused by EMF in order to understand the possible adverse effects of EMF in leukocytes. In this study, we used a kind of leukocyte, RBL 2H3 cells (rat basophilic leukemia cells), and exposed the cells to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h. It has been reported that the limit of EMF for general public exposure and occupational exposure are 0.2 mT and 1 mT, respectively [22]. The intensity of EMF used in this experiment is the limit of occupational exposure. Although further studies are necessary to identify the changes in intracellular Ca^{2+} mobilization and cellular function by EMF exposure, we suggest that EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h may have no influence on intracellular Ca^{2+} mobilization and cellular function in RBL 2H3 cells.

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