60 Hz electric field upregulates cytosolic Ca^{2+} level in mouse splenocytes stimulated by lectin

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ABSTRACT

The effect of a 60 Hz electric field (EF) on alteration of cytosolic free Ca^{2+} level ($[Ca^{2+}]_c$) was examined in mouse splenocytes stimulated by lectins, namely concanavalin A or phytohemagglutinin. In order to understand the role of EF on alterations in $[Ca^{2+}]_c$ and to determine whether EF exposure increased cell mortality the splenocytes were cultured under the EFs of 6 or 60 μA/cm^2 for 30 min or 24 hrs. Cell mortality was less than 2 % in experimental all conditions. Then the effect of the EF (6 μA/cm^2) on $[Ca^{2+}]_c$ in the splenocytes stimulated with the lectin was examined. $[Ca^{2+}]_c$ in the splenocyte was not changed by the EF (6 μA/cm^2) exposure alone. While, a lectin-induced $[Ca^{2+}]_c$ elevation in the EF exposed cells was significantly higher than that of the EF sham-exposed cells ($P< 0.05$: ANOVA, $P< 0.05$: paired *t* test). Moreover the enhanced increase of $[Ca^{2+}]_c$ in the EF exposed-, lectin stimulated-cells was only observed in the presence of extracellular Ca^{2+}. The EF dependent upregulation of $[Ca^{2+}]_c$ persisted after EF exposure ($P< 0.05$: paired *t* test). The results clearly indicate that Ca^{2+} influx across the plasma membrane is responsible for the enhanced increase of $[Ca^{2+}]_c$ in the EF exposed-, lectin stimulated-cells, and that EF has persistent effect on the cells. Although the precise mechanisms of the EF dependent

upregulation of $[Ca^{2+}]_c$ is not fully elucidated, the present results demonstrate that the 60 Hz EF (6 μA/cm^2) affects $[Ca^{2+}]_c$ during cell activation via a Ca^{2+} influx pathway induced by lectin stimulation.

Key words: concanavalin A, phytohemagglutinin, alternating current, extremely-low-frequency

INTRODUCTION

All terrestrial living organisms are constantly exposed to electromagnetic fields (EMF) in varying degrees. Physiological studies establishing the possible efficacy and mechanism of action of exposure to micro-current field induction are minimal. Several earlier studies have reported findings largely focused on the exogenous EMF as the relevant measure/metric inducing biological responses. Documented EMF-induced responses include changes in cytosolic calcium concentration [Barbier et al., 1996; Blackman et al., 1985, 1988; Cho et al., 1999; Onuma et al., 1986, 1988; Kim et al., 1998; Lyle et al., 1988; Walleczek et al., 1990], and other cellular responses such as protein expression [McLeod et al., 1987], and cell surface receptor redistribution and cytoskeletal reorganization [Cho et al., 1994, 1996; Onuma et al., 1988], all of which are believed mediated by changes in calcium metabolism. Liburdy [1992] observed similar increases in calcium transport in magnetically-induced and pure EF-exposed rat thymocytes implying the usefulness of EF as a relevant metric for biological responses. Cassarizza et al. [1989 a and b] and Cadossi et al. [1991, 1992] reported that pulsed EMF increases a proliferation of lymphocyte stimulated by lectin of under optimum concentration.

In 1972, the Ministry of Health and Welfare of Japan approved the manufacturing of this treatment apparatus as a system for physical therapy instrument and equipment with the indications of shoulder stiffness, insomnia, chronic constipation, and headache under the brand name of Hakuju AC High Voltage Electric Field Health Device, Healthtron (Approval No.: 14700BZZ00904) [Harakawa et al., 2002]. Maximum value of 6 μA/cm^2 as the induced current density by application of these devices into human body is expected using a human phantom (data not shown). Thus, the sinusoidal waveform of 60 Hz and current density of 6 μA/cm^2 is applied in this study. And because the $[Ca^{2+}]_c$ of mouse splenocyte, which is appropriate to assess a change of $[Ca^{2+}]_c$ [Cadossi et al 1992], stimulated with lectin reached a plateau by 4-8 minute after stimulation, the exposure period was set around 8 min. In view of the limited information available today on the effect(s) of EF on cytosolic free Ca^{2+} level $[Ca^{2+}]_c$ compared to that of the EMF, the present study, using a laboratory-devised exposure dish system, assayed increases in the number of calcium $[Ca^{2+}]_c$-high cells in lectin-stimulated mouse splenocytes exposed to 60 Hz (6 μA/cm^2) EF. The purpose of this evaluation was to examine one possible chemically mediated mechanism of action by which one could explain certain physiologic phenomenon observed when living

systems are exposed to EF.

MATERIALS AND METHODS

Electric Field Exposure System

The EF exposure system was composed of four parts; namely the field exposure dish made of polycarbonate; the function generator (SG-4101, IWATSU Co. Ltd., Tokyo, Japan); the digital multi-meter (VOAC-7411 IWATSU, Tokyo, Japan); and, the controller (Hakuju Co. Ltd., Tokyo, Japan). The field exposure dish was composed of the lid, the dish and the doughnut-shape insert (internal diameter: 12 mm) (Fig. 1). An EF was generated in between the two round-shape platinum electrodes (the cell culture space) by the function generator, and was finely adjusted by using the controller and the digital multi-meter. The field strength of 60 Hz was determined by measuring a current density within the cell culture space of the field exposure dish. The current density was calculated by the expression:

Current density = I/S

where “I” is the supplied current (μA), and S is the area (cm^2) of the cell culture space.

S = 0.6 x 0.6 x 3.14 = 1.1304

And

$$1/S = 0.085$$

Thus, the current density can be calculated by:

$$\text{Current density } [\mu A/cm^2] = 0.085 \times I\ [\mu A]$$

Prior to the EF exposure, approximately 1.5 ml of the assay buffer (137 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 5 mM glucose, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1 % (w/v) BSA and 10 mM HEPES pH 7.4) was poured into the electrode chamber. In order to avoid contact of the cells and the lower electrode, polycarbonate membrane (Isopore, MILLIPORE, MA, USA) was placed in between the dish and the insert. Approximately 1 ml of the cell suspension was poured into culture well/space and covered with a lid.

Cell preparation

Female BALB/c mice, 4-7 wk old obtained form CLEA Inc. (Tokyo, Japan), maintained in a conventional animal room equipped with air-conditioning device were splenectomized under anesthesia, and cell suspensions of splenocytes were prepared. To examine cell viability, the cells were cultivated in Dulbecco's modified Eagle's medium (SIGMA, MO, USA) supplemented with 10 % fetal bovine serum (FBS). The

cells were maintained in Hank's balanced salt solution (HBSS) (SIGMA, MO, USA) during examination for $[Ca^{2+}]_c$ which was carried out within 4 hr after cell preparation. Cells were stored at 4˚C prior to use.

Determination of the viability of EF-exposed cells

Mouse splenocytes (5 x 10^6 cells/ml) were exposed to 60 Hz either at 6 µA/cm^2 or 60 µA/cm^2 EF for 30 min and 24 hr, at 37 ˚C in 5 % CO_2. The sham (control) cells were left on the field exposure dish for 30 min and 24 hr but were not exposed to EF. The cell suspensions harvested from the field exposure dish at the end of 30 min, and 24 hr exposure time were stained with 2.5 µg/ml propidium iodide for 30 min at 4 ˚C, and percent dead cells were analyzed by flow cytometry.

Cell preparation for assay of $[Ca^{2+}]_c$-high cells and lectins used

Splenocytes (10^6 cells/ml) were incubated for 20 min at 37 ˚C in HBSS containing 2.5 µM fluo-3-acetoxylmethyl (Molecular Probes, USA) [Vandenberghe et al., 1990]. The cell suspension was then diluted 5 times with HBSS containing 1 % FBS, incubated for 40 min at 37 ˚C, washed 3 times with the assay buffer, and the cells were then suspended in the assay buffer at a concentration of 1 x 10^6/ml. Throughout the cell preparation, the cell suspensions were mixed gently.

Considering the reported synergetic interaction between EMF and mitogen [Walleczek et al., 1990], concanavalin A (ConA) (Seikagaku Co., Tokyo, Japan) and phytohemagglutinin (PHA) (SIGMA, MO, USA) were used.

Experimental design to determine the effect of 60 Hz (6 μA/cm^2) EF on the generation of [Ca^{2+}]$_c$-high cells

Taking into account the results of the viability test for exposed murine splenocytes earlier assayed, we chose to use the optimum culture and exposure conditions (60 Hz, 6 μA/cm^2 EF) in carrying out the following five experiments: (1) cells suspended in HEPES-buffered saline (BS) + 1 mM $CaCl_2$ were exposed to EF for a total of 40 min, and 12.5 μg/ml of ConA was added after the first 8 min of exposure. The control groups consisted of EF-unexposed cells containing ConA, and EF-exposed cells without ConA. Percent [Ca^{2+}]$_c$-high cells was checked at certain time points; (2) cells in HEPES-BS + 1 mM $CaCl_2$ were exposed for a total of 12 min, and different concentrations (1 ng- 12.5 μg/ml) of ConA was added after the first 4 min of exposure. The control group was essentially the same as that of the experimental group but without EF-exposure; (3) cells in HEPES-BS + 1 mM $CaCl_2$ were exposed for a total of 8 min, and 5 μg/ml of PHA was added after the first 4 min of exposure. The control

groups consisted of EF-unexposed cells containing PHA, and EF-exposed cells without PHA; (4) cells suspended in HEPES-BS without $CaCl_2$ were exposed for a total of 12 min, and different concentrations (1 ng - 5 μg/ml) of ConA was added after the first 4 min of exposure time. The control group was essentially the same as the experimental group but without EF exposure; and (5) to evaluate the persistent effect of EF exposure cells suspended in HEPES-BS + 1 ml $CaCl_2$ were exposed for a total of 4 min, after which different concentrations (0.025 - 12.5 μg/ml) of ConA was added, and the generation of $[Ca^{2+}]_c$-high cells for the next 8 min without EF exposure was monitored with flow cytometry. The control was essentially the same as the experimental group but without any EF-exposure. To assess whether an effects in $[Ca^{2+}]_c$ by EF-induced are influenced by extracellular calcium ion level, two different type assay buffer with or without $CaCl_2$ was used in this study.

Flow cytometry analysis

The cytofluorimetric measurement was conducted with the EPICS XL^{TM} and the system 2^{TM} software (BECKMAN COULTER INC., CA). The sample cells were auto gated and selected by data of a front scatter and sides scatter as a lymphocyte

region (50-70 % of all sample). The FL-3 filter was applied into the study to assess the viability of cells. PI fluorescence was expressed in arbitrary fluorescence intensity units and drawn the histogram as the number of cells versus the log scaled FL-3. The histogram was expressed bimodal pattern and the peak of high intensity side on FL-3 axis suggests dead cells. The FL-1 filter was applied into the study to assess the $[Ca^{2+}]_c$. Fluo-3 fluorescence was expressed in arbitrary fluorescence intensity units and drawn the histogram as the number of cells versus the liner scaled FL-1. On each study, arbitrary region, which covered 250 to 1024 unit of FL-1 including about 15 % of all lymphocyte regions, was programmed on the software before the pivotal measurement. According to increasing of the intensity of Fluo-3 fluorescence, the number of cells within arbitrary region increases, concomitantly. In this study, the cells within this region were defined as $[Ca^{2+}]_c$-high cell and the change of percentage of $[Ca^{2+}]_c$-high cell were used as the parameter to assess the alteration of $[Ca^{2+}]_c$. One measurement was conducted with the low level of flow speed and was programmed to stop at 5,000 cells count on lymphocyte legion.

All experiments described in this article were conducted in accordance with the Guiding principles for the Care and Use of Research Animals promulgated by Obihiro

University.

Statistical Analysis

Statistical analysis of cell viability was performed using a Student's *t* test. Data for effect by exposure to EF in $[Ca^{2+}]_c$ among groups was analyzed by ANOVA, Student's *t* test and paired *t* test. All computations for the statistical analysis were carried out in MS-EXCEL® Japanese Edition (Microsoft Office software: Ver. 9.0.1, Microsoft Japan Inc. Tokyo, Japan).

RESULTS

More than 98% of the cells were viable after exposure to either 6 μA/cm^2 or 60 μA/cm^2 EFs in all three replicates (Fig. 2).

The number of $[Ca^{2+}]_c$-high cells increased significantly in both EF-exposed and unexposed cell suspension containing 12.5 μg/ml ConA (Fig. 3). Those in EF-exposed cell suspension without ConA remained essentially unchanged. The ConA-induced response was noted immediately and reached a saturation point within 5-8 minutes after the addition of the mitogen. The differences between EF exposed and unexposed ConA-induced cells were insignificant ($P > 0.05$).

Results of EF-exposed cell cultures containing different concentrations of ConA with and without $CaCl_2$ are summarized in Figure 4. In the presence of 1mM $CaCl_2$, the EF significantly enhanced ConA dependent $[Ca^{2+}]_c$ rise ($P< 0.01$: ANOVA) relative to the control (Fig. 4A). Although the increase in $[Ca^{2+}]_c$-high cells was more substantial in the 0.675 - 5.0 µg/ml ConA stimulated groups, only the 1.25 µg/ml and 2.5 µg/ml ConA-induced cells showed significant differences with the control ($P< 0.05$: paired *t* test). ConA-dependent $[Ca^{2+}]_c$ rise was negligible in the Ca^{2+}-free cell condition in both the control and the EF-exposed groups (Fig. 4B).

To determine whether the EF-dependent $[Ca^{2+}]_c$ upregulation was limited to ConA, PHA-stimulated cells were also examined. Both EF-exposed and unexposed cells containing PHA registered significant increases in $[Ca^{2+}]_c$-high cells (Fig. 5). The increase in EF-exposed cells however was significantly greater than that measured in the ($P< 0.05$: paired *t* test) unexposed group.

The addition of 3.125-12.5 µg/ml of ConA to cell suspensions either unexposed or earlier exposed to EF for 4 min showed significant increase in $[Ca^{2+}]_c$-high cells compared to those cell stimulated with 0.025 µg/ml of ConA (Table 1). Cells stimulated with 3.125 and 6.25 µg/ml ConA exhibited sustained increase in $[Ca^{2+}]_c$-high

cells which leveled off at about 8 min post-ConA stimulation, while cell cultures stimulated with higher concentration of ConA (12.5 µg/ml) showed a decline in $[Ca^{2+}]_c$-high cells approximately 4 min post-ConA stimulation. The enhancing effect of EF exposure was significantly demonstrable at 2-4 min only in the presence of 6.25 µg/ml of ConA ($P< 0.05$: paired *t* test).

DISCUSSION

Splenocytes exposed to field strength of either 60 µA/cm^2 or 6 µA/cm^2 of the 60 Hz EF appeared undamaged and did not exhibit greater cell mortality than those in the unexposed condition. Cell viability of >98% may be suggestive of cells properly maintained under normal and optimum physiologic conditions. The EF-exposed and lectin-induced cells showed generally higher percent increases in $[Ca^{2+}]_c$-high cells compared to the unexposed control, implying enhanced effect of the EF by either ConA or PHA, on $[Ca^{2+}]_c$ influx. Significant elevation in $[Ca^{2+}]_c$-high cells was noted however at lectin concentrations 1.25 - 6.25 µg/ml, with the increase apparent within the first 1 - 2 min, and saturation points reached within 4 to 8 min post-lectin addition.

Both ConA and PHA were effective cell mitogens. Our results corroborate

earlier findings of mitogen-dependent increases in calcium uptake in rat thymocytes exposed to 60 Hz EF for 60 min [Walleczek et al., 1990; Liburdy, 1992]. And our results are similar to findings by Cassarizza et al. [1989 a and b] and Cadossi et al. [1991, 1992] that PEMF-induced influence on lymphocyte was limited in the addition of hypo-optimum concentration of lectin. However, it is new finding that EF of the sinusoidal waveform also affects to lectin-induced biological parameter of lymphocyte. Recently, Cho et al. [1999] reported a fourfold increase in $[Ca^{2+}]_i$ influx in human hepatoma cells exposed to 1-10 Hz EF for 30 min in the absence of mitogen. They however recorded a delayed saturation point relative to our findings and those reported by Liburdy [1992], further substantiating the role of EF in enhancing the effect of mitogen on calcium transport. The two possibility raise and are candidate as a target of future study that the coupling with mitogen play an integral part on starting of effect of EF and that it exist an effect of EF alone in fact, whose level is below a limit of detection on our method, and it is enhanced by mitogen. Based on the kinetics of [Ca2+]i influx, Liburdy [1992] concluded that the mechanisms responsible for EF-induced increases in calcium metabolism and microfilament reorganization may largely depend on the cell type exposed and on the mode of exogenous EF applied.

We observed markedly inhibited lectin-stimulated increase in $[Ca^{2+}]_c$-high cells and the EF-induced responsiveness of the cell with depletion of extracellular free Ca^{2+} in the medium, implying a negligible influence of the EF on the release of calcium from intracellular stores. Direct stimulation by an induced current, and indirect stimulation by the perception of extremely low frequency (ELF) field on the body surface have been proposed as possible triggers of the effect of ELF EMF on a living body [Kato et al., 1989; Weigel et al., 1987]. While, magnetic field can generally penetrate the cell membrane, the ELF EF cannot because of the presence of the lipid bilayer (plasma membrane) that functions as an electrical insulator [Lee et al., 1993; Poo 1981]. Thus, direct activation of internal Ca^{2+} stores by exogenous EF is unlikely. Present findings concur with earlier reports of Liburdy [1992] implying the influence of the EF primarily on enhancement of the transport of extracellular supply of calcium across the plasma membrane, with the calcium channel as the site of interaction. The degrees to which EF interacts directly on the structure of the ligand-gated calcium channel or with inositol-1,4,5-triphosphate and its phosphorylation product that regulates the opening of the channel requires further examination and is beyond the scope of this study.

The increase in $[Ca^{2+}]_c$-high cells ($P< 0.05$: paired t test) was significant only

in EF pre-exposed cells at 2 and 4 min after ConA (6.25 μg/ml) stimulation, suggesting the persistence of the effect of EF on calcium influx. Since the $[Ca^{2+}]_c$ measurement was carried out only within a limited period after the addition of ConA, we could not further assess how long the EF effect persisted. Further investigation would be appropriate to determine the extent of EF persistent effect, and its mechanism(s) in future related studies.

In conclusion, the results clearly indicate that Ca^{2+} influx across the plasma membrane is responsible for the enhanced increase of $[Ca^{2+}]_c$ in the EF exposed-, lectin stimulated-cells, and that EF has persistent effect on the cells. Although the precise mechanisms of the EF dependent upregulation of $[Ca^{2+}]_c$ is not fully elucidated, the present results demonstrate that the 60 Hz EF (6 $\mu A/cm^2$), whose level is estimated to be induced within human body by an application of some clinical physical device utilized exposure to EF, affects $[Ca^{2+}]_c$ during cell activation via a Ca^{2+} influx pathway induced by lectin stimulation.

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Figure legends

Fig. 1. Schematic diagram of the field exposure dish. Except for two electrodes, all parts were made of polycarbonate.

Fig. 2. Cell viability after the EF exposure. Open bar and broken bar show the sham exposed and the 30 min EF exposed cells, respectively. Filled bar shows the 24 h EF exposed cells. Each value indicates the average ± SEM (n = 3).

Fig. 3. Effect of the EF exposure on ConA-dependent $[Ca^{2+}]_c$ rise in mouse splenocytes (I). The $[Ca^{2+}]_c$ was measured by flow cytometry on 5 x 10^3 cells in each measurement during 40 min EF exposure. At 8 min of EF exposure, ConA (12.5 μg/ml) was added. Squares, circles and triangles indicate $[Ca^{2+}]_c$ of ConA stimulated cells, EF exposed cells, and EF exposed-, ConA stimulated-cells, respectively (n=1). Each value indicates percentage of the $[Ca^{2+}]_c$-high cell.

Fig. 4. Effect of the EF exposure on ConA-dependent $[Ca^{2+}]_c$ rise in mouse splenocyte

(II). $[Ca^{2+}]_c$ was measured on 2 x 10^4 cells after the 12 min EF exposure. ConA (0.01 - 5 μg/ml) was added at 4 min of the EF exposure. Splenocytes were maintained with 1 mM Ca^{2+} (A) or without Ca^{2+} (B) during measurements. Filled bars and open bars indicate EF exposed cells and sham exposed cells. Each value indicates percentage of the $[Ca^{2+}]_c$-high cell. The values indicate the average ± SEM (n= 6). *: The values in the EF-exposed cell are significantly higher than those of sham-exposed cell by ANOVA (P<0.05). †: Two values at this point are significantly different by paired *t*-test (P< 0.05).

Fig. 5. Effect of the on PHA dependent $[Ca^{2+}]_c$ rise in mouse splenocytes. PHA was added to the cell after 4 min EF exposure. Each value indicates percentage of the $[Ca^{2+}]_c$-high cell. *: There are significant difference between two groups (Paired *t*-test: P< 0.05, n = 4).

Table 1.

Title: Persistent effect of the EF on $[Ca^{2+}]_c$ of mouse splenocytes.

*: Paired *t*-test: P< 0.05, n = 4.

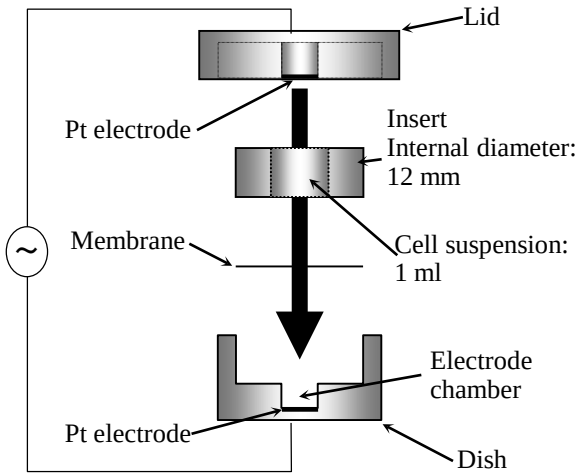

Figure 1

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60 Hz electric field upregulates cytosolic Ca^{2+} level in mouse splenocytes stimulated by lectin

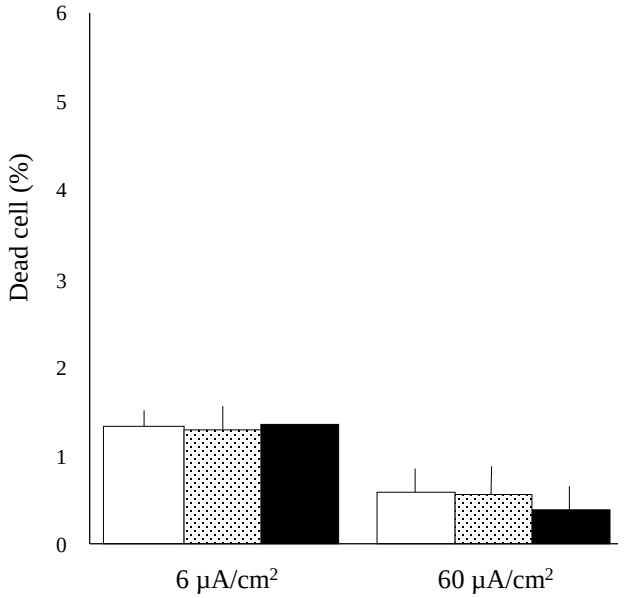

Figure 2

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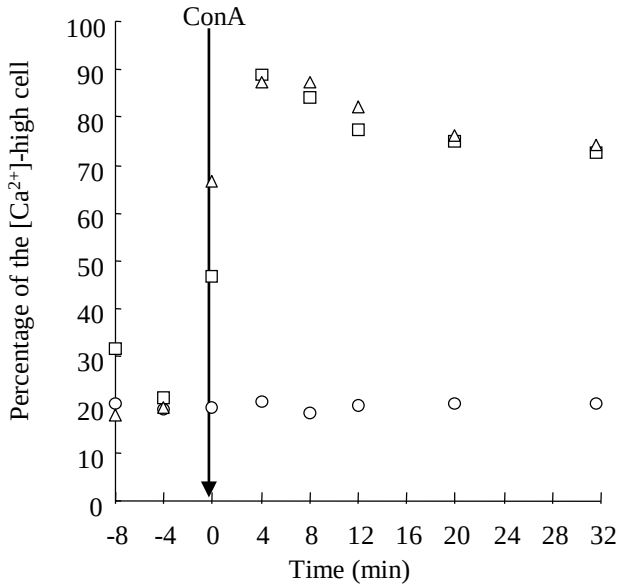

Figure 3

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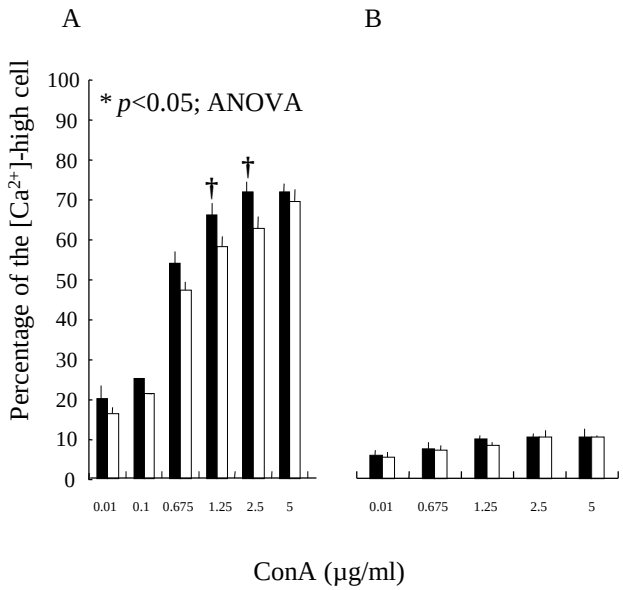

Figure 4

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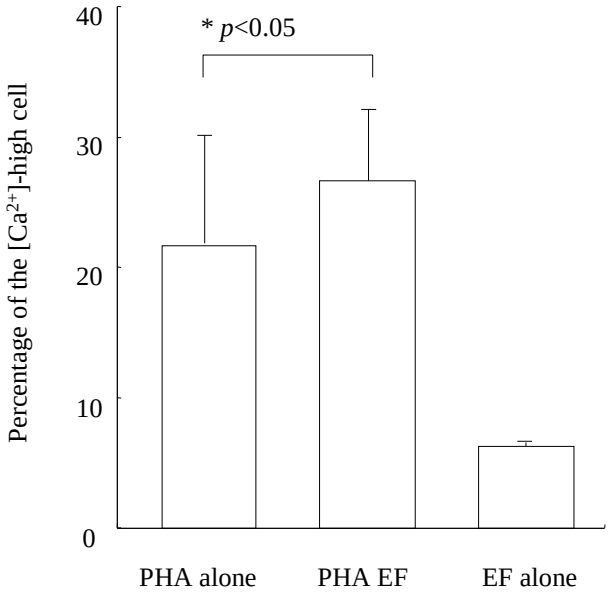

Figure 5

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Treatment Type	Time after exposure to EF (min)	Final Concentration of additive Con A (μg/ml)							
		12.5		6.25		3.125		0.025	
		Average	SD	Average	SD	Average	SD	Average	SD
ConA alone	2	39.2	8.5	28.9	6.2	24.2	7.0	19.6	5.7
	4	55.6	6.0	47.3	5.6	39.5	8.5	20.3	4.7
	6	54.5	4.6	54.3	5.1	49.5	7.6	20.4	4.8
	8	50.3	3.7	55.1	5.3	53.5	6.3	20.7	5.1
ConA+EF	2	40.8	9.5	31.1 *	7.4	25.2	7.0	17.9	4.6
	4	56.3	7.9	50.9 *	8.5	41.9	9.6	18.9	5.5
	6	54.2	5.8	56.2	7.1	51.6	8.3	19.5	5.4
	8	49.9	4.0	55.0	5.5	54.3	6.5	19.9	5.5

Table 1
Title:Persistent effect of the EF on $[Ca^{2+}]_c$ of mouse splenocytes.

Table 1

Harakawa, S. et al.
60 Hz electric field upregulates cytosolic Ca^{2+} level in mouse splenocytes stimulated by lectin