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Effect of Extremely Low Frequency Electromagnetic Fields (EMF) on Phospholipase Activity in the Cultured Cells

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This study was conducted to investigate the effects of extremely low frequency electromagnetic fields (EMF) on signal pathway in plasma membrane of cultured cells (RAW 264.7 cells and RBL 2H3 cells), by measuring the activity of phospholipase A_2 (PLA_2), phospholipase C (PLC) and phospholipase D (PLD). The cells were exposed to the EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h. The basal and 0.5 μM melittin-induced arachidonic acid release was not affected by EMF in both cells. In cell-free PLA_2 assay, we failed to observe the change of $cPLA_2$ and $sPLA_2$ activity. Also both PLC and PLD activities did not show any change in the two cell lines exposed to EMF. This study suggests that the exposure condition of EMF (60 Hz, 0.1 or 1 mT) which is 2.4 fold higher than the limit of occupational exposure does not induce phospholipases-associated signal pathway in RAW 264.7 cells and RBL 2H3 cells.

Key Words: EMF, Phospholipase A_2, Arachidonic acid, Phospholipase C, Phospholipase D

INTRODUCTION

There is a public concern about the possible adverse health effects associated with exposure to extremely low frequency electromagnetic fields (EMF), this has been highlighted following suggestions of an epidemiological study that first pointed to a possible relationship between residential high-voltage power lines and childhood leukemia [1]. Epidemiologic studies suggested a correlation between leukemia and EMF exposure in electrical workers [2], while others have showed that no such correlation exists [3].The mechanism of the interaction between EMF and cellular systems is still unclear. However, hypothetically changes can be initiated at the cell surface, affecting surface constituents such as membrane-receptor complexes. Considering that many of the signal transducers are implicated in the process of cellular functions, it is plausible that EMF exposure may cause the alteration in signal transduction pathway in cell membranes.

Phospholipases such as phospholipase A_2 (PLA_2), phospholipase C (PLC) and phospholipase D (PLD) cleave the phospholipids present in cell membrane and play an important role in the modulation of cellular function. The position of cleavage on the glycerol backbone identifies the phospholipase family and generates unique and specific products, some of which have second messenger function. PLA_2 cleaves the sn-2 ester bond of cellular phospholipids, producing arachidonic acid (AA) and lysophospholipid. AA is the biosynthetic precursor for the eicosanoid family of potent inflammatory mediators. Eicosanoids play a role in a wide range of physiological and pathological processes such as immune responses, inflammation, and pain perception [4]. PLA_2 activation plays a key role in inflammatory responses of neutrophils, macrophages and mast cells [5,6]. PLC hydrolyzed phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol [7,8], and resulting IP_3 increases intracellular Ca^{2+} concentration by releasing Ca^{2+} from intracellular stores [9,10]. PLD principally catalyzes the hydrolysis of phosphatidylcholine, resulting in the formation of phosphatidic acid, which is a precursor of diacylglycerol [11,12]. Nowadays, it is recognized that PLD plays an important role in modulating cellular functions that require long term activation of protein kinase C, because phosphatidylcholine is the major phospholipid in cell membrane [13]. In this study, we investigated the effect of EMF on cellular signal pathway, by observing the changes of phopholipase (PLA_2, PLC and PLD) activity in RAW 264.7 cells and RBL 2H3 cells exposed to EMF.

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METHODS

Materials

Melittin, bromoenol lactone (BEL), dithiothreitol (DTT), arachidonyl trifluoromethyl ketone ($AACOCF_3$), phorbol

ABBREVIATIONS: AA, arachidonic acid; EMF, extremely low frequency electromagnetic fields; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D.

12-myristate 13-acetate (PMA), PLA_2 from honey bee venom were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle minimum essential medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Co. (Grand Island, NY, USA). 10-pyrene phosphatidylcholine (10-pyrene PC) was purchased from Molecular Probes (Leiden, Netherlands). [^{3}H]arachidonic acid ([^{3}H]AA) was obtained from NEN (Boston, USA). 1-Palmitoyl-2-[^{14}C]arachidonyl phosphatidylcholine ([^{14}C]AA-PC), [^{3}H]phosphatidylinositol ([^{3}H]PI) and [^{3}H]oleic acid were obtained from Perkin Elmer (Boston, USA). 1,2-Dioleoyl-3-phosphoethanol was obtained from Avanti Polar Lipid (Alabaster, USA). Other reagents were purchased from Sigma Chemical Co. (USA).

Cell culture

The murine macrophage (RAW 264.7) cells and 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 with 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 exposure system, because magnetic field is proportional to the injected current. The field generator consists of four square-shaped coils and one cage with three testing floor (top, middle and bottom floor).

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

Measurement of [^{3}H]AA release

The cells at the density of 10^6 cells/ml exposed to 0.1 mT and 1 mT at 60 Hz EMF for 4 h and 16 h. After exposure, the cells were incubated with [^{3}H]AA (0.2 μCi/ml) in medium containing 0.5% FBS for 2 h at 37°C. The cells were washed twice with Krebs buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 10 mM HEPES (pH 7.4), 1.8 mM $CaCl_2$, and 5 mM glucose) containing 0.5 mg/ml bovine serum albumin to trap the liberated [^{3}H]AA. The cells [^{3}H]AA release was induced by melittin for 30 min in 37°C. The radioactivity of [^{3}H]AA released in the low-serum medium (0.5%) was measured by scintillation counting [14]. Data are expressed as % release (radioactivity (cpm) in supernatant / radioactivity (cpm) in supernatant and pellet×100).

Preparation of PLA_2

After the cells exposed to EMF, washed and sonicated in 10 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl, 2 mM EGTA, 100 μM leupeptin, 150 μM aprotinin, and 1 mM Na_3VO_4. The lysates were centrifuged at 10,000 × *g* for 30 min at 4°C and the supernatants were stored at −70°C until used to supply $sPLA_2$ and $cPLA_2$ [15].

$cPLA_2$ assay with [^{14}C]AA-PC

$cPLA_2$ activity was assayed by measuring [^{14}C]AA hydrolyzed from [^{14}C]AA-PC. The reaction mixture containing 0.025 μCi [^{14}C]AA-PC, 100 mM Tris-HCl (pH 8.5), 10 μM bromoenol lactone (BEL) as an $iPLA_2$ inhibitor [16], 5 mM $CaCl_2$ and 1 mM dithiothreitol (DTT) as an $sPLA_2$ inhibitor [17] was incubated for a given time with cell-derived $cPLA_2$.

Each reaction mixture stopped by adding 560 μl of modified Doles reagent (n-heptane/isopropyl alcohol/1 N-H_2SO_4 = 400/390/10) [18]. After centrifugation, 150 μl of the upper phase was transferred to a new tube, to which 800 μl of n-heptane and silica gel (10 mg) was added. The mixtures were mixed and centrifuged again for 2 min, after which 800 μl of supernatant was moved into 4.0 ml of scintillation solution and counted for radioactivity in a Packard Tri-carb liquid scintillation counter. The specific activity in picomoles per minute per milligram of protein (pmol/min/mg) was obtained by dividing the activity by the amount of enzyme protein. To calculate the specific activity, protein was analyzed with a BCA (bicinchoninic acid) protein assay kit.

$sPLA_2$ assay with 10-pyrene PC

$sPLA_2$ activity was measured with a pyrene-labeled phosphatidylcholine in the presence of serum albumin using a spectrophotometer [19]. The cell derived $sPLA_2$ was incubated with a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 2 μM 10-pyrene PC, 0.1% bovine serum albumin, and 6 mM $CaCl_2$ for 20 min. The fluorescence was measured using excitation (345 nm) and emission (398 nm) wavelengths with a spectrophotometer (FL600, Microplate Fluorescence Reader, Bio-Tek).

PLC assay

The cells were exposed to EMF (0.1 mT and 1 mT at 60 Hz magnetic field for 4 h and 16 h), washed with PBS and suspended in 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 1 mM EDTA, 0.5% deoxycholate and 0.1% SDS, 100 μM leupeptin and 1% Triton X-100. Cell lysates were centrifuged at 20,000 × *g* for 30 min at 4°C and the supernatant was used as the PLC.

PLC activity was assayed with [^{3}H]PI as substrate. PI-hydrolyzing activity was assayed in a 200 μl reaction mixture containing 0.02 μCi [^{3}H]PI, 50 mM Hepes (pH 7.0), 3 mM $CaCl_2$, 2 mM EGTA, 0.1% sodium deoxycholate, and cell-derived PLC. The reaction was performed at 37°C for 30 min and stopped by adding 1 ml of chloroform : methanol : HCl (100 : 100 : 0.6, v/v/v), followed by 0.3 ml of 1 M HCl containing 5 mM EGTA. After brief centrifugation, 0.5 ml of the upper aqueous phase was assayed for [^{3}H] radioactivity by liquid scintillation counter [20].

PLD assay

The cells (10^6 cells/ml) exposed to 1 mT at 60 Hz EMF for 4 h and 16 h. After exposure, the cells were incubated with [^{3}H]oleic acid (2 μCi/ml) in medium containing 0.5% FBS for 3 h at 37°C. Thereafter, the labeling medium was replaced, and the cells were washed twice with Krebs buffer. The protein kinase C activator, PMA was added in the presence of 0.5% ethanol for 30 min in 37°C. The reactions were terminated by addition of 1 ml ice-cold methanol to the plates. The cells were scraped off from the plates, and the phospholipids were extracted and assayed [21]. For phosphatidylethanol, the lipid extracts (lower chloroform phase) were separated on Silica Gel 60 plates (Merck) using the organic phase of mixture of ethylacetat e : 2,2,4-trimethylpentane : acetic acid : water (13 : 2 : 3 : 10, v/v/v/v) as the mobile phase. Lipids were localized by iodine staining and identified by 25 μg of 1,2-dioleoyl-3-phosphoethanol as a standard. The areas corresponding to the phosphatidylethanol standard were scraped into scintillation vials, and the radioactivity was measured using liquid scintillation spectrometry.

Statistical analysis

Results are represented as mean±S.D. and were analyzed statistically with analysis of variance (ANOVA), and 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 basal and melittin-stimulated AA release in [^{3}H]-AA labeled cells

To investigate the effect of EMF on PLA_2 activity in cellular system, we measured basal and melittin-induced AA release in [^{3}H]AA-labeled RAW 264.7 cells and RBL 2H3 cells. Melittin, an endogenous PLA_2 activator [22], significantly increased AA release in RAW 264.7 cells and RBL 2H3 cells by 1.8 and 2.0 fold, respectively (Figs. 1, 2). EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h did not influence on the basal and 0.5 μM melittin-induced [^{3}H]AA release in both cell groups (Figs. 1, 2).

The change of PLA_2 activity derived from the cells exposed to EMF

To investigate the change of PLA_2 activity derived from the cells exposed to EMF, the hydrolysis of [^{14}C]AA-PC was measured. RAW 264.7 and RBL 2H3 cell derived-PLA_2 were significantly inhibited in the presence of 10 μM $AACOCF_3$ by 90.7% and 90%, respectively but was not influenced by

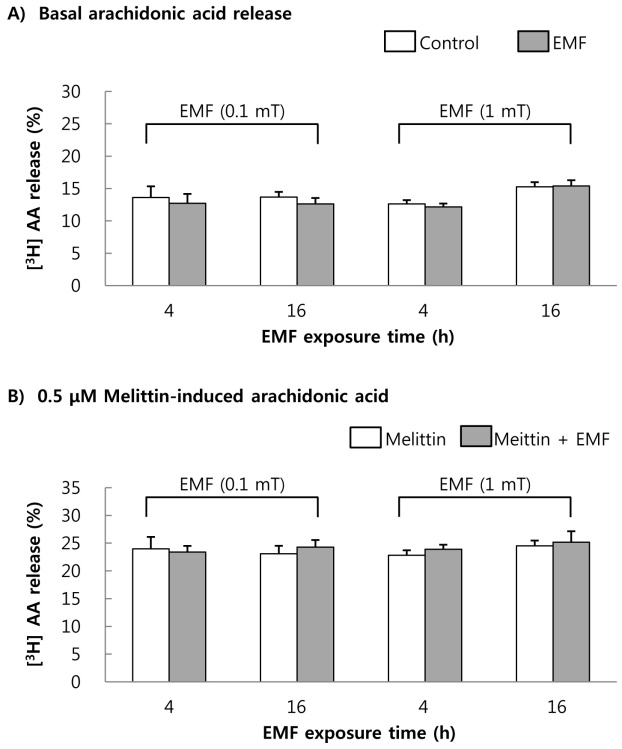

Fig. 1. The changes of basal (A) and 0.5 μM melittin-induced [^{3}H]AA release (B) in RAW 264.7 cells. The cells were exposed to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h and were labeled with [^{3}H]AA for 2 h. The radioactivity of released [^{3}H]AA was measured in the presence or absence of 0.5 μM melittin. Results are indicated in mean±S.D. from four separate experiments.

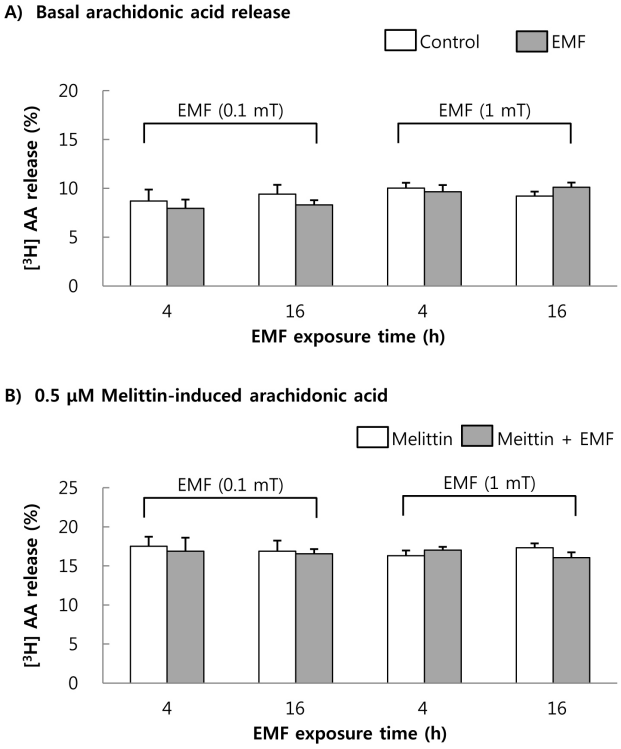

Fig. 2. The changes of basal (A) and 0.5 μM melittin-induced [^{3}H]AA release (B) in RBL 2H3 cells. The cells were exposed to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h and were labeled with [^{3}H]AA for 2 h. The radioactivity of released [^{3}H]AA was measured in the presence or absence of 0.5 μM melittin. Results indicate mean±S.D. from four separate experiments.

1 mM DTT and did not show any PLA_2 activity in the absence of Ca^{2+} (Fig. 3).

The specific activity of RAW 264.7 cell-derived PLA_2 was 143 pmol/min/mg protein, which was not affected by exposure to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h (Fig. 4A). The specific activity of RBL 2H3 cell-derived PLA_2 was 293 pmol/min/mg protein, which was 2 fold higher than that of RAW 264.7 cell-derived PLA_2. In RBL 2H3 cells PLA_2 activity was not affected by exposure to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h (Fig. 4B).

To confirm the effect of EMF on $sPLA_2$ activity of the cells, we used $sPLA_2$-specific substrate, 10-pyrene PC [23]. $sPLA_2$ obtained from honey bee venom dose-dependently hydrolyzed 10-pyrene PC, whereas the change of $sPLA_2$ activity was not observed in both cells exposed to EMF (Fig. 5).

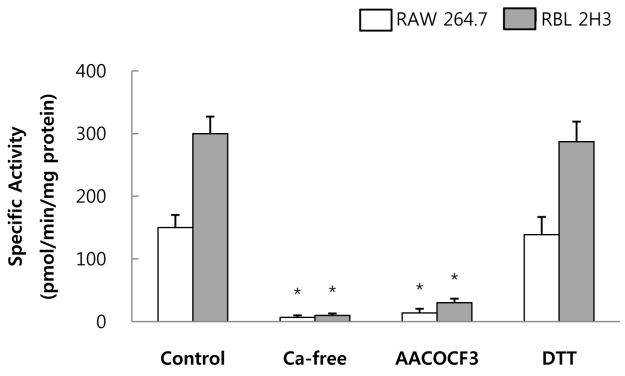

Fig. 3. Cell-derived PLA_2 activity in the presence of 5 mM $CaCl_2$. PLA_2 (25 μg protein) derived from RAW 264.7 cells and RBL 2H3 cells was incubated with 1-palmitoyl-2-[^{14}C]arachidonyl phosphatidylcholine in the presence of 10 μM $AACOCF_3$ ($cPLA_2$ inhibitor) or 1 mM DTT ($sPLA_2$ inhibitor) and in the absence of $CaCl_2$. Results indicate mean±S.D. from four separate experiments. *Significantly different from Control ($p < 0.05$).

The change of PLC activity derived from the cells exposed to EMF

To investigate the change of PLC activity derived from the cells exposed to EMF, the hydrolysis of [^{3}H]phosphatidylinositol was measured. The specific activity of RAW 264.7 cell-derived PLC was 920 fmol/min/mg protein, which was not affected by exposure to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h (Fig. 6A). The specific activity of RBL 2H3 cell-derived PLC was 711 fmol/min/mg protein, which was lower than that of RAW 264.7 cell-derived PLC. In RBL 2H3 cells, PLC was not affected by exposure to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h (Fig. 6B).

The change of PLD activity in the cells exposed to EMF

To investigate the change of PLD activity in the cells exposed to EMF, we measured the synthesis of [^{3}H]phosphatidylethanol in [^{3}H]oleic acid-labeled cells. PMA (1 μM) in-

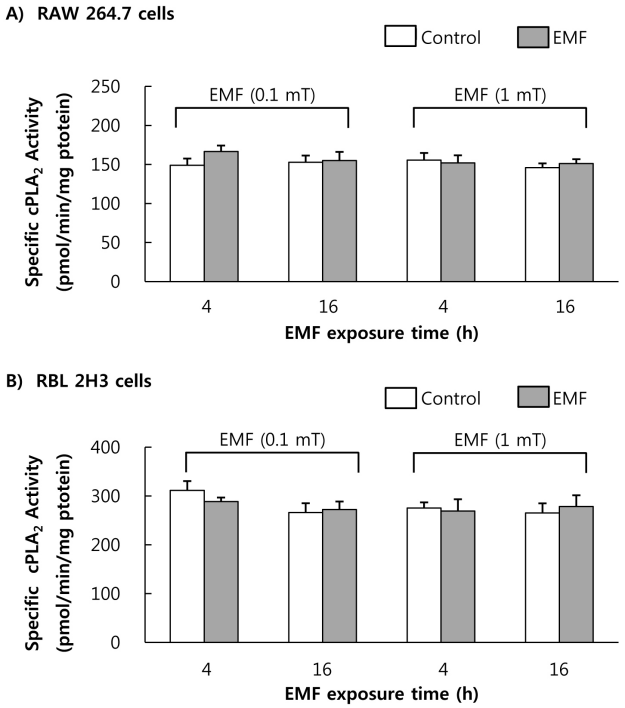

Fig. 4. Effect of EMF on cell-derived $cPLA_2$ activity. Cell-derived $cPLA_2$ was obtained from the RAW 264.7 cells (A) and RBL 2H3 cells (B) exposed to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h. $cPLA_2$ (25 μg protein) was incubated with 1-palmitoyl-2-[^{14}C]arachidonyl phosphatidylcholine in the presence of 5 mM $CaCl_2$ and 1 mM DTT. Results indicate mean±S.D. from four separate experiments.

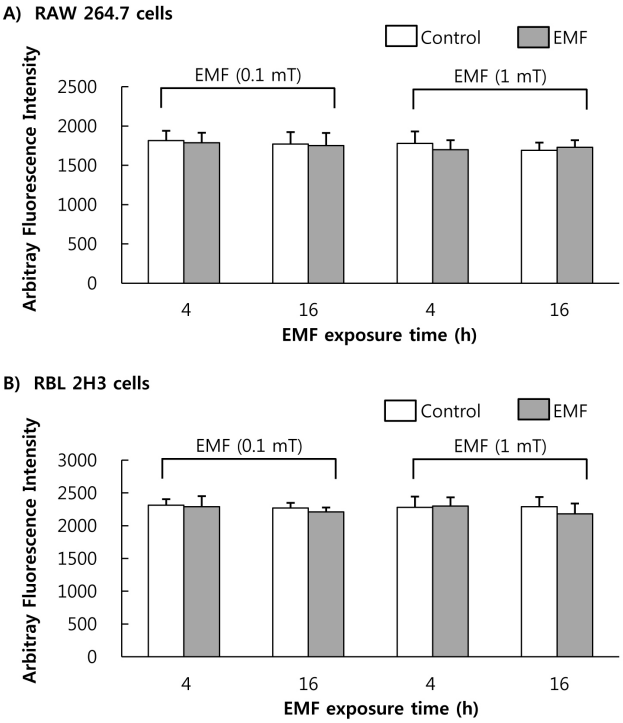

Fig. 5. Effect of EMF on cell-derived $sPLA_2$ activity. Cell-derived $sPLA_2$ was obtained from the RAW 264.7 cells (A) and RBL 2H3 cells (B) exposed to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h. $sPLA_2$ (100 μg protein) was incubated with 10-pyren phosphatidylcholine. Results indicate mean±S.D. from four separate experiments.

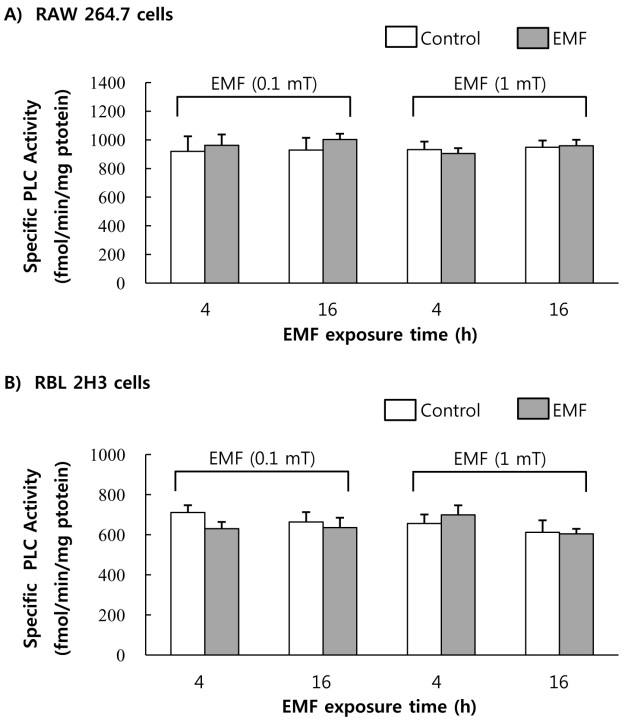

Fig. 6. Effect of EMF on cell-derived PLC activity. Cell-derived PLC was obtained from the RAW 264.7 cells (A) and RBL 2H3 cells (B) exposed to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h. PLC (25 μg protein) was incubated with [^{3}H]phosphatidylinositol. Results indicate mean±S.D. from four separate experiments.

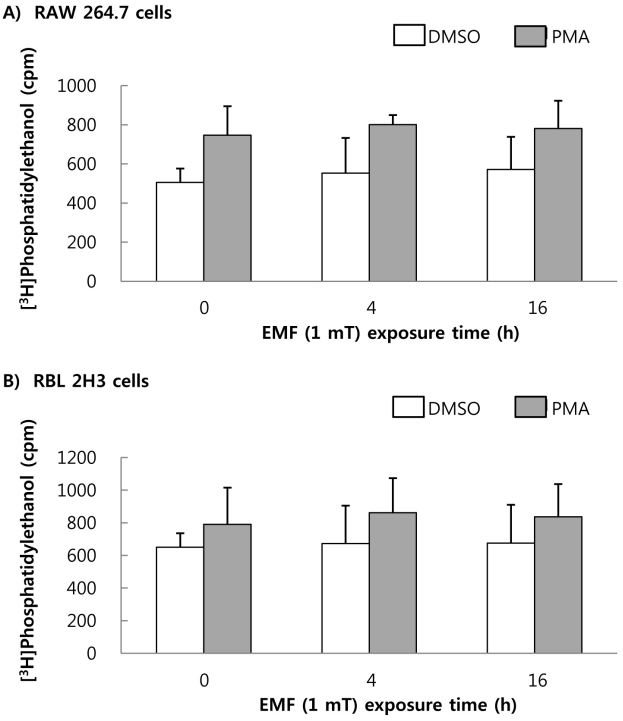

Fig. 7. Effect of EMF on PLD activity. RAW 264.7 cells (A) and RBL 2H3 cells (B) were exposed to EMF (60 Hz, 1 mT) for 4 or 16 hand were labeled with [^{3}H]oleic acid, for 3 h. The radioactivity of [^{3}H]phosphatidylethanol produced by PLD was measured in the presence or absence of 1 μM PMA. Results indicate mean±S.D. from four separate experiment.

creased the production of [^{3}H]phosphatidylethanol in RAW 264.7 cells and RBL 2H3 cells by 1.5 and 1.2 fold, respectively (Fig. 7). EMF (60 Hz, 1 mT) for 4 or 16 h did not influence on the basal and 1 μM PMA-induced [^{3}H]phosphatidylethanol formation in both cells (Fig. 7).

DISCUSSION

Guidelines on high-frequency and 50/60 Hz electromagnetic fields were issued by IRPA/INIRC in 1988 and 1990, respectively. The basic hypothesis that emerged from the original study was that the contribution of ambient residential 50/60 Hz magnetic fields from external sources such as power lines could be linked to an increased risk of childhood leukemia [1]. However, epidemiologic studies suggested a correlation between leukemia and EMF exposure in electrical workers [2], while others have showed that no such correlation exists [3]. The mechanism underlying the interaction between EMF and cellular systems is still remains elusive. It is suspected that hypothetical changes induced by EMF could be initiated at the cell surface, affecting the surface constituents associated with signaling pathway, such as membrane-receptor complexes. Phospholipases such as PLA_2, PLC and PLD cleave phospholipids in cell membrane and play an important role in modulation of cellular function. It is necessary to see the changes of phospholipase activity by EMF understanding the possible adverse effect of EMF in leukocytes. In this study, we used two leukocytes, RAW 264.7 cells (murine macrophage) and 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 were 0.0833 mT and 0.4167 mT, respectively [24]. The intensity of EMF used in this experiment is 2.4 fold higher than the limited that of occupational exposure.

To investigate the effect of EMF on PLA_2 activity in cellular system, we measured basal and melittin-induced AA release in [^{3}H]AA-labeled RAW 264.7 cells and RBL 2H3 cells [25]. Melittin, an endogenous PLA_2 activator [22], significantly increased AA release in RAW 264.7 cells and RBL 2H3 cells by 1.8 and 2.0 fold, respectively. EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h did not demonstrate any influence on the basal and 0.5 μM melittin-induced [^{3}H]AA release in both cells. AA release is an indirect measure of PLA_2 activity in cellular system because other enzymes, such as arachidonyl-CoA synthetase, CoA-dependent acyltransferase, and CoA-independent transacylase are involved in free AA production [26].

To confirm the direct effect of EMF on PLA_2 activity, we measured PLA_2 activity using [^{14}C]AA-PC and 10-pyren PC. RAW 264.7 and RBL 2H3 cell derived-PLA_2 were significantly inhibited in the presence of 10 μM $AACOCF_3$ by 90.7% and 90%, but was not influenced by 1 mM DTT and did not show any PLA_2 activity in the absence of Ca^{2+} and this data had been previously reported where in the cell-derived PLA_2 mainly appears to be $cPLA_2$ [27]. Under this assay condition, specific activity of cell-derived $cPLA_2$ was

not affected by exposure to EMF (60 Hz, 0.1 or 1 mT) for 4 or 16 h. Also, in the $sPLA_2$ assay using an $sPLA_2$-specific substrate, 10-pyrene PC [23], the change of $sPLA_2$ activity was not observed in both cell groups exposed to EMF. These data suggest that EMF (60 Hz, 0.1 or 1 mT) did not affect PLA_2 activity in RAW 264.7 cells and RBL 2H3 cells. It has been reported that EMF decreased the PGE_2 production by down-regulating COX-2 in human keratinocyte cell line (HaCat) stimulated with lipopolysaccharide [28], but till date there are no reports published describing the effects of EMF on PLA_2 activity.

The changes of PLC and PLD activities in the cells exposed to EMF were measured using [^{3}H]phosphatidylinositol and formation of [^{3}H]phosphatidylethanol. These data suggest that EMF (60 Hz, 0.1 or 1 mT) did not affect PLC and PLD activity in RAW 264.7 cells and RBL 2H3 cells. Clejan et al (1996) have reported that EMF (2 T) temporarily inactivate phosphatidylcholine-PLC but activate phosphatidylcholine-PLD in human hematopoietic cell line, TF-1 cells [29]. The discrepancy between the data of the present study and the above information may be due to the intensity of EMF. The intensity of EMF used in TF-1 cells is 2000 fold higher than that used in this experiment and is 4800 fold higher than that the limit of occupational exposure. In conclusion, the outcome of this study data suggest that the exposure of EMF (60 Hz, 0.1 or 1 mT) which is 2.4 fold higher than the limit of occupational exposure does not induce phospholipases-associated signal pathway in RAW 264.7 cells and RBL 2H3 cells.

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