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The Effect of Pulsed Electromagnetic Field Stimulation of Live Cells on Intracellular Ca^{2+} Dynamics Changes Notably Involving Ion Channels

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Abstract

Pulsed electromagnetic field (PEMF) therapy is a non-invasive treatment with promising therapeutic efficacy for bone and cartilage-related pathologies, as well as the attenuation of osteoarthritis pain. Despite extensive research on certain PEMF applications, the underlying treatment mechanism remains unclear. The aim of this study is to investigate the impact of PEMF stimulation on intracellular calcium ions changes and related gene expression were measured using KG-1 cells (a human myelomonocytic cell line) and HUVECs (human umbilical vein endothelial cells). In both the KG-1 and HUVECs, PEMF stimulation resulted in enhanced Ca^{2+} influx via purinergic receptor channels, Ca^{2+} release from intracellular store, and store-operated Ca^{2+} entry into the cells. In the KG-1 cells, PEMF stimulation at 50 Hz for 40 minutes increased the ATP-sensitive $[Ca^{2+}]_i$ increment by about 30%, as well as the CPA-induced $[Ca^{2+}]_i$ increment increased by about 18%. The response of $[Ca^{2+}]_i$ due to PEMF stimulation appeared in the opposite direction in HUVECs. Our study revealed that PEMF controls $[Ca^{2+}]_i$ regulation through specific ion channels, depending on the cell type. We established that it is necessary to precisely control the intensity and duration of PEMF, depending on the condition being treated and the type of tissue.

Keywords: Pulsed electromagnetic field (PEMF); Intracellular Ca^{2+}; Ion Channels; Purinergic receptors; Store-operated Ca^{2+} entry

Abbreviations: PEMF: Pulsed Electromagnetic Field; SD: Standard Deviation; PTM: Post-Translational Modification; CRAC: Ca^{2+} Release-Activated Ca^{2+}; PWM: Pulse Width Modulation; ER: Endoplasmic Reticulum; CPA: Cyclopiazonic Acid; SOCE: Store-Operated Ca^{2+} Entry; SERCA: Sarco-Endoplasmic Reticulum Ca^{2+} ATPase

Introduction

PEMF therapy has been used on patients with bone fracture and non-union of the bones [1-3]. In recent decades, a considerable number of researchers have studied the application of PEMF therapy for other conditions such as osteotomy gap healing, post-surgical healing, wound healing, and arthritis amelioration, with effectiveness demonstrated in human and mouse clinical studies [4-6]. More recently, there are an increasing number of research groups studying the mechanism of the clinical effectiveness of PEMF therapy using cell-based in vitro research methodologies. Enhanced osteoblastogenesis-related gene expression was observed in osteoblast-like cells upon PEMF treatment, [7] and PEMF therapy has a potential in treating it by modulating inflammation and prompting tissue repair through mesenchymal stromal cells. was shown to help increase the osteogenic effect of BMP-2 in mesenchymal stem cells [8]. In addition to its osteogenic effect, PEMF also effected a decrease in the inflammatory cytokines TNF-alpha and NFkB in macrophage-like cells [9]. Although these studies suggest that PEMF is effective in wound healing and at attenuating inflammation, the specific mechanisms at the cellular level that are induced by PEMF stimulation of cells remain unresolved. A study by Petecchia and colleagues reported that an electromagnetic field applied over 27 days promoted osteogenic differentiation of BM-MSCs (bone-marrow mesenchymal stem cells) through a Ca^{2+}-related mechanism [10].

According to another study, weak electro-magnetic stimulation may effect conformational changes in the charge

distribution of membrane proteins such as ion channels and may well account for protein synthesis initiation via DNA stimulation [11,12]. Based on these findings, we considered it meaningful to observe the initial effect of PEMF by focusing on the ion channels that are central to cellular ionic homeostasis. In particular, this study focused on the regulation of $[Ca^{2+}]_i$, which is an prominent second messenger that plays an important role in signal transduction cascades, cytoskeletal reorganization, cell migration, angiogenesis, differentiation, and proliferation. The regulation of cytosolic Ca^{2+} concentration at the nanomolar level is very important for the maintaining of homeostasis for various essential functions of cell survival. Usually, there are two pathways in the regulation of $[Ca^{2+}]_i$; influx through the plasma cell membrane, and release from the organellar store. As a pathway for Ca^{2+} influx from outside the cell, we focused on changes in ATP-sensitive purinergic receptors, which play an important role in several cell types including immune cells. For organellar Ca^{2+} release to the cytosol, we analyzed two key molecules, ORAI and STIM proteins. These two proteins interplay to regulate $[Ca^{2+}]_i$ levels, which are important for the Ca^{2+} release-activated Ca^{2+} (CRAC) channels and angiogenesis of endothelial cells, as well as various other functions of immune cells including phagocytosis.

Although it has been reported that persistent, weak PEMF stimulation affects the differentiation of stem cells and the $[Ca^{2+}]_i$ homeostasis of various cells, there have been few reports on how PEMF directly affects ion channels during the initial stage of PEMF stimulation. Studies on the direct effect of PEMF stimulation on purinergic receptors that directly respond to ATP (which acts globally on cells as a paracrine or autocrine signal molecule), and to Ca^{2+} (which regulates cytosol through intracellular stores) might provide vital clues about cellular functions such as cell differentiation, cell proliferation, and immune activity. In this study, using KG-1 and HUVECs, we investigated how under a one-hour duration of weak PEMF stimulation applied to live cells could induce changes in $[Ca^{2+}]_i$ concentration-related ion channel function and genetic expression regulation.

Materials and methods

Cell culture and PEMF exposure

KG-1 (Korean Cell Line Bank, Seoul, Korea) cells were cultured in RPMI1640 medium (Croning, New York, USA) containing 10 % heat inactivated fetal bovine serum (Cytiva, Marlborough, USA) and 50 U/ml Penicillin, 50 ug/ml streptomycin (Thermo Fisher, Waltham, USA). HUVECs (ATCC) were kindly donated by Wide River Institute of Immunology and cultured in Vascular cell basal medim (ATCC, Manassas, USA) containing Endothelial cell growth kit (ATCC, Manassas, USA). Both cell types were cultured in a humidified incubator (37 °C, 5 % CO_2). KG-1 cell density was maintained in the range 2x105 to 1x106 viable cells/mL. HUVECs were subculture when 70~80 % confluence. The medium was changed twice a week. Glasses cover slips (25 mm) coated with Poly-L-Lysine (Sigma-Aldrich, Saint Louis, USA) were prepared, onto which the cells were attached. After overnight, cells were stimulated with or without (control) and treated with the indicated drugs. The PEMF duration and frequency varied according to the experimental design.

PEMF stimulation system design & PEMF parameters

A system with 6-channel magnetic flux cores was designed to evaluate the effects of PEMF stimulation. The PEMF cores had outer and inner diameters of 60 and 35 mm, respectively, and a height of 30 mm. The PEMF stimulation system was designed to allow adjustment of stimulation intensity and frequency. The voltage and current applied to the cores could be controlled in multiple stages by adjusting the PEMF stimulation intensity in the range 10–50 mT. The PEMF generation frequencies were adjustable in the range 1-1000 hz. In particular, the design allowed the duty ratio to be varied from 10-50% through pulse width modulation (PWM), to solve the exothermic problem of magnetic field generation cores. Moreover, mono-phasic, bi-phasic, and time-interval bi-phasic stimulation generation modes could be selected. The pulse frequencies used in the experiments were 10 Hz [pulse duty ratio: 30%, intensity: mono-phasic stimulation of 26.2 mT], 30 Hz [pulse duty ratio: 30%, intensity: mono-phasic stimulation of 20.3 mT], and 50 Hz [pulse duty ratio: 30%, intensity: mono-phasic stimulation of 15 mT].

RNA purification, cDNA synthesis and PCR

1x107cells were prepared for RNA purification by washing once with Dulbecco's Phosphate-Buffered Saline (Welgene, Gyeongsan, S.Korea), whereafter a pellet was created from the cells in suspension via centrifugation (1200 rpm, 5 min). Total RNA was extracted using RNeasy Mini Kit (Qiagen, 74106) following the manufacturer's instructions, and thereafter stored at -20°C. cDNA synthesis was performed on 1 μg of RNA using RT Premix Kit (Intronbio, Seongnam, S.Korea). DNA was amplified using the 2x TOPsimple PCR PreMIX–HOT (Enzynomics, Daejeon, S. Korea) and loaded onto a 2% Agarose Gel (Lonza, Basel, Switzerland). 100 bp DNA ladder (Enzynomics, Daejeon, S.Korea) used as size marker. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control for quantitative analysis (Table 1).

Live cell [Ca2+]i Measurement

Cells attached to Poly-L-Lysine (Sigma-Aldrich, Saint Louis, USA) coated glass coverslips were overnight culture before PEMF stimulation. Control and PEMF-stimulated cells were loaded with 2 ug/ml Flou-4 AM (Thermo Fisher Scientific, Berkeley, USA) for 40 min at 37°C in a physiological external solution consisting of (in mM) NaCl 125, KCl 5, HEPES 10, glucose 10, $MgCl_2$ 1, $CaCl_2$ 1.8 pH 7.4. After loading, cells were transferred to an open perfusion

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chamber for fluorescence microscopy measurements (Nikon, Tokyo, Japan) at 488 nm. Live cell imaging occurred every 1s using a cooled EM-CCD camera (Imag EM X2, Hamamatsu, Japan). The camera and shutter were controlled by Meta Fluor Software (Molecular Devices, Foster City, CA). The $[Ca^{2+}]_i$ amplitude was quantified by measuring the average intensity of the Fluo-4 AM-positive cells across all of the time-lapse fluorescence images. Single cells were defined as regions of interest (ROIs) (Figure 1E, upper, left). The fluorescence intensity from the time-lapse fluorescence image, acquired using MetaFluor Software, was processed by converting it into a file compatible with the Origin program (SPSS Inc., Chicago, USA). ROI signals were calculated by subtracting the background noise signals and analyzing with MetaFluor Software.

Table 1: List of primers used for PCR.

Gene	Acc. No.	Forward Primer (5'-3')	Reverse Primer (5'-3')	bp
GAPDH	NM_001289746.2	GCCAAGGTCATCCATGACAACT	GAGGGGCCATCCACAGTCTT	95 bp
P2Y14	NM_014879.4	AATCTAGCCGCAACATATTCAGC	GTCTGACTCTTTGTGTAGGGGAT	94 bp
P2Y13	NM_176894.3	ATCGTGCTGTTAGGGCTCATA	CAAGATCGTATTTGGCAGGGAG	153 bp
P2Y12	NM_022788.5	CACTGCTCTACACTGTCCTGT	AGTGGTCCTGTTCCCAGTTTG	190 bp
P2Y11	NM_002566.5	AGCTCCTATGTGCCCTACCA	GCGGCCATGTAGAGTAGAGG	197 bp
P2Y6	NM_176796.3	GTGTCTACCGCGAGAACTTCA	CCAGAGCAAGGTTTAGGGTGTA	159 bp
P2Y4	NM_002565.4	TGGCATTGTCAGACACCTTGT	AAAGAAAGCGGACGAACTTGC	114 bp
P2Y2	NM_176072.3	GGTGTCTGGGCGTCTTACG	TGGTGGTGACAAAGTAGAGCA	132 bp
P2X7	NM_002562.6	TATGAGACGAACAAAGTCACTCG	GCAAAGCAAACGTAGGAAAAGAT	95 bp
P2X6	NM_005446.5	GAACCCCAGTTTTCCATCATCA	GGCGTCACAAGGAAGTTGGT	149 bp
P2X5	NM_175080.3	CTGTCGCTGTTCGACTACAAG	CCCATACGACCAGGTACGC	112 bp
P2X4	NM_001256796.2	TGGCGGATTATGTGATACCAGC	GTCGCATCTGGAATCTCGGG	112 bp
P2X3	NM_002559.5	ACGCCAACAGAGTCATGGATG	CGCACTGGCTGTCTGATACA	159 bp
P2X2	NM_001282165.2	AGCTGGGCTTTATCGTGGAGA	TTGGGGTTGCACTCCGATG	127 bp
P2X1	NM_002558.4	CAACGACACTGTGAAGACGTG	CGTGGAAAGCTGATGCTGTTC	135 bp
Stim1	NM_003156.4	TGT GGA GCT GCC TCA GTA TG	AAG AGA GGA GGC CCA AAG AG	183 bp
Stim2	NM_020860.4	CAC GCC CAC CTC ATA ACT GG	TCA AGC CTC TCC TGT AAG TCC A	187 bp
Orai1	NM_032790.3	GAC TGG ATC GGC CAG AGT TAC	GTC CGG CTG GAG GCT TTA AG	116 bp
Orai2	NM_001271818.2	GAG GCC GTG AGC AAC ATC C	GGA GGA ACT TGA TCC AGC AGA	157 bp
Orai3	NM_152288.3	CCTGGTTGGTTGGGTCAAGT	CAGAGGACCGTGGGAGATTG	143 bp

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). All experiments were repeated at least three times, and the results are presented as the mean ± standard deviation (SD). Significance between group means was determined using Student's t-test, and statistical significance was defined as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

PEMF stimulation in vitro increased ATP-sensitive Ca^{2+} influx in KG-1 cells

In the first series of experiments, we investigated whether purinergic receptors are genetically and functionally present in KG-1 cells and how the activity of these receptors is altered by PEMF stimulation of live cells. We identified genes and measured $[Ca^{2+}]_i$ in the cells with use of ATP, a representative agonist of purinergic receptors. We performed RT-PCR to analyze the mRNA expression of seven P2X subtypes and seven P2Y subtypes in KG-1 cells; P2X1, P2X4, P2X5, P2X7 and all the tested P2Y subtypes were positively expressed (Figure 1D). From these results, we could predict the presence of purinergic receptors showing their functional performance and the regulating of $[Ca^{2+}]_i$ in KG-1 cells.

Cells loaded with Fluo-4 AM were divided into ROIs for each cell (Figure 1E, left) and the average intensity (488 nm) fluctuating in real time was measured to evaluate changes in single-cell $[Ca^{2+}]_i$. As we expected, $[Ca^{2+}]_i$ increased drastically upon addition of ATP (20 μM), which demonstrated that purinergic receptors in KG-1 cells are not only expressed genetically, but also function as membrane channel proteins (Figure 1A). Since such a reaction was not observed in the absence of extracellular Ca^{2+} (data not

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shown), it was concluded that increased Ca^{2+} in the cytosol upon ATP addition was caused by Ca^{2+}influx from outside of the cells. Next, in order to establish how the purinergic receptor activity is affected by PEMF stimulation of the live cells, we stimulated the cells at 10, 30, and 50 Hz intensities over a 40 min period using a stimulator developed in-house (Figure 1E, right) to observe changes in intracellular $[Ca^{2+}]_i$ upon ATP treatment. Prior experiments were conducted at three time points within 1 hour. 40 minutes showed the most stable results which was selected as the time point (data not shown). Compared to the control, the rise in $[Ca^{2+}]_i$ resulting from ATP addition was shown to increase in the cells that were subjected to PEMF stimulation, and furthermore there was a significant difference in the 50 Hz stimulation group (Figures 1B &1C). We therefore selected 50 Hz intensity for all subsequent PEMP stimulation experiments.

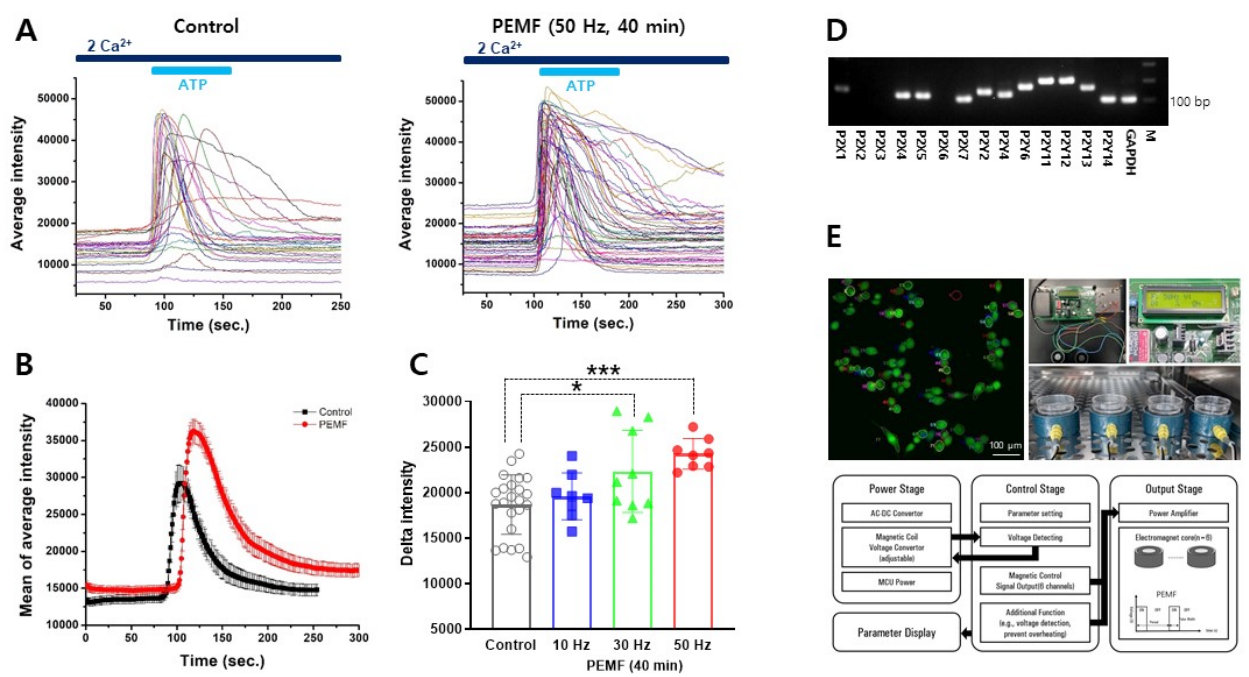

Figure 1: PEMF Stimulation promotes ATP-induced intracellular Ca^{2+} ($[Ca^{2+}]$ i) increase in KG-1 cells. (A) Representative traces showing combined each ATP-induced $[Ca^{2+}]$ i transients from individual single cells in control (without PEMF stimulation) and PEMF stimulated cells. (B) Representative traces showing that averaged (Mean ± SEM.) $[Ca^{2+}]$i response induced by ATP. (C) Graph illustrating the mean net increases in $[Ca^{2+}]$i revealed by the averaged intensity obtained from three different PEMF stimulation conditions versus control. (D) mRNA expression of P2 type of purinergic receptors were amplified by reverse transcription-polymerase chain reaction (RT-PCR) using the control KG-1 cells. Original gel showing expression levels of seven P2X subtypes and six P2Y subtypes. (E) Images showing morphology of adherent KG-1 cells and regions of interest for analysis (upper, left). PEMF-generating device and control system with 6-channels magnetic coils (upper, right). Schematic design of the PEMF system (lower). The coils were placed in the CO_2 incubator for cell stimulation. Experiments were performed more than three times in each condition. Asterisks indicate *P < 0.05, ***P < 0.001.

Endoplasmic reticulum (ER) Ca^{2+} release and store-operated Ca^{2+} entry (SOCE) affected by PEMF stimulation in KG-1 cells

We also studied the signaling pathways associated with store-operated Ca^{2+} entry (SOCE), to investigate if Ca^{2+} release from intracellular store was also affected by PEMF stimulation in KG-1 cells. For this we used the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor cyclopiazonic acid (CPA; 10 μM). CPA increased $[Ca^{2+}]_i$ under conditions of absence of extracellular Ca^{2+}, indicating the release of Ca^{2+} from intracellular stores into the cytosol. The sequential exchange of extracellular solution contained Ca^{2+} (2 mM) showed definitively the surge of $[Ca^{2+}]_i$ (Figure 2A). When extracellular Ca^{2+} is increased after intracellular store depletion, Ca^{2+} rapidly flows into the cytosol, which is a Ca^{2+} dynamics pattern that typically reveals SOCE activity. In accordance with the results of the purinergic receptors, the mean net incremental change in $[Ca^{2+}]_i$ by both CPA and the subsequent large change in extracellular Ca^{2+}, were also augmented by PEMF stimulation (Figures 2B & 2C). KG-1 cells expressed all subtypes of Stim and Orai genes (Figure 2D).

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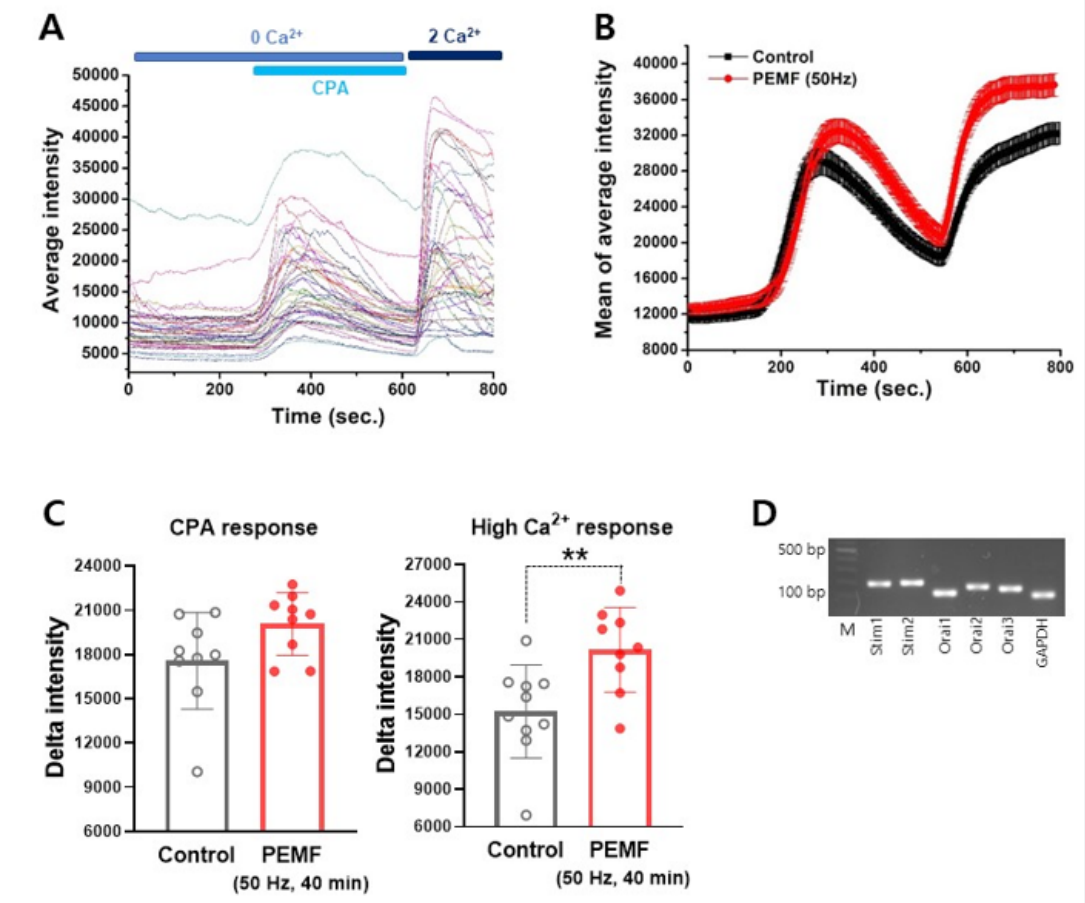

Figure 2: PEMF stimulation promotes cytoplasmic store Ca^{2+} release and store-operated Ca^{2+} entry in KG-1 cells. (A) Representative traces showing $[Ca^{2+}]$ i response induced by store depletion using CPA in the absence of extracellular Ca^{2+} in control cells, followed Ca^{2+} influx into the cells by increasing extracellular Ca^{2+}. (B) Traces showing comparison of averaged (Mean ± SEM.) $[Ca^{2+}]$ i response in control and PEMF stimulated cells. (C) Graph illustrating the mean net increases in $[Ca^{2+}]$ i obtained by CPA and extracellular high Ca^{2+} change. (D) Original gel showing significant levels of Orai1, Orai2, Orai3, Stim1, and Stim2. Asterisks indicate **P < 0.01.

ATP-sensitive Ca^{2+} influx and ER Ca^{2+} release of HUVECs were decreased by PEMP stimulation.

To further investigate whether PEMF stimulation affects Ca^{2+} signaling in other cell types, HUVECs were used, with the hypothesis that weak PEMF stimulation might affect blood vessels and/or blood flow. Using the same protocols as for the KG-1 cells, the gene expression levels and $[Ca^{2+}]_i$ dynamics related to purinergic receptors and SOCE were investigated in HUVECs. It was confirmed that P2X1, P2X4, P2X5, P2X7 and all of the P2Y subtypes were positively expressed in HUVECs (Figure 3D). In single-cell $[Ca^{2+}]_i$ experiments of HUVECs, two differences were noted in comparison with KG-1 cells; Firstly, the pattern of elevated $[Ca^{2+}]_i$ upon the HUVECs' treatment with ATP showed an oscillatory profile in comparison with that of the KG-1 cells. Secondly, unlike KG-1 cells, incremental changes in $[Ca^{2+}]_i$ upon addition of ATP were noticeably reduced by PEMF stimulation (Figure 3A, 3B & 3C). As shown in the fluorescence images (Figure 3E), the fluorescence intensity of Ca^{2+} by ATP was significantly decreased after PEMF stimulation.

We next examined signaling pathways associated with SOCE in HUVECs under condition of PEMF stimulation compared with the control. All subtypes of Stim and Orai genes were expressed in HUVECs as they were in KG-1 cells (Figure 4D), however, SOCE activity was not measured in the single-cell $[Ca^{2+}]_i$ experiment (data not shown). So, we measured only the Ca^{2+} released into the cytosol by addition of CPA. Interestingly, the CPA response was also remarkably reduced by PEMF stimulation, like the effects of ATP on the HUVECs (Figures 4A, 4B & 4C). These results suggest that PEMF stimulation can affect intracellular Ca^{2+} movement through specific ion channels, and that even the same type of ion channels can have different responses depending on the cell type.

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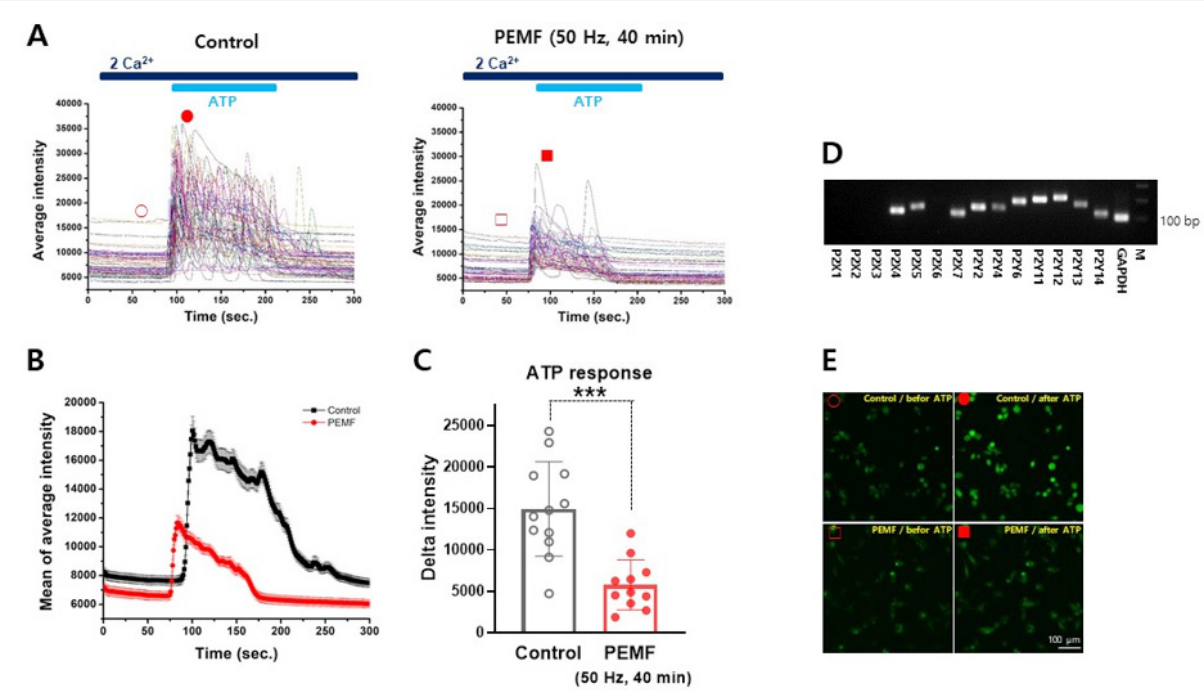

Figure 3: PEMF Stimulation represses ATP-induced [Ca^{2+}] i increase in HUVECs. (A) Representative traces showing combined each ATP-induced [Ca^{2+}] i transients from individual single cells in control and PEMF stimulated cells. (B) Representative traces showing that averaged (Mean ± SEM.) [Ca^{2+}] i response induced by ATP. (C) Graph illustrating the mean net increases of ATP-induced [Ca^{2+}] i from PEMF stimulation condition versus control. (D) Original gel showing expression level of genes which seven subtypes of P2X and six subtypes of P2Y. (E) Showing the morphologies of HUVECs at 100X magnification and comparing the ATP effects between control (upper) and PEMF treated (lower) cells (○, □; before ATP, / ●, ■; after ATP). Asterisks indicate ***P < 0.001.

Discussion

The general aim of this work was to investigate the effects of the in vitro PEMF stimulation of two different cell systems, in terms of changes in $[Ca^{2+}]_i$. There are several biochemical studies and experiments reported in the literature verifying the effect of PEMF regarding cellular differentiation, cell proliferation, osteogenesis, angiogenesis, and wound healing, however few of these focused on intracellular signaling. When we looked at the various responses of cells that were known to be affected by weak PEMF stimulation, we had hypothesized that changes in [Ca^{2+}]i might be a common factor regarding the various cellular responses observed. The intracellular free Ca^{2+}, capable of prompting cellular signaling pathways, is related to Ca^{2+} ions entering specialized transporters in the plasma membrane and their release from internal stores, primarily the ER. To obtain an unambiguous quantitative characterization of the effects of PEMF in relation to $[Ca^{2+}]_i$ changes, we used a live cell Ca^{2+} imaging system, and performed analysis of genes related to ion channels using RT-PCR, in two different cell systems.

A purinergic receptor is a transmembrane receptor protein that is activated by the extracellular ligands of nucleotides and nucleosides [13]. Not only are these proteins broadly expressed in the many organs and cells including immune cells, but they are also involved in various molecular functions and cell signaling [13,14]. From the RT-PCR results, it was confirmed that P2X1,4,5 genes were present in KG-1 cells and P2X4 gene present in HUVECs, and the six subtypes (P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, and P2Y13) of P2Y we tested were all present in KG-1 cells. The P2 type receptor is classified further into two subtypes, P2X and P2Y receptors, according to their mechanisms of action. The P2X family is an ATP-gated cation channel which makes cation-permeable pores on the surface of the cells that allow influx of the Ca^{2+} ion [15]. The P2X family has seven different subtypes (P2X1-P2X7) and are broadly expressed in various cell types including excitable neurons, smooth muscle cells, and non-excitable epithelial or immune cells. P2Y receptors functionally belong to a G-protein coupled receptor family and have eight subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) according to its agonists and coupling G-proteins [16]. Since it is signaling cascade upon ligand stimulation couples with various intracellular signaling proteins and pathways to induce physiological alterations, they show slower response compared with P2X receptors which act as the ion-channels themselves [17].

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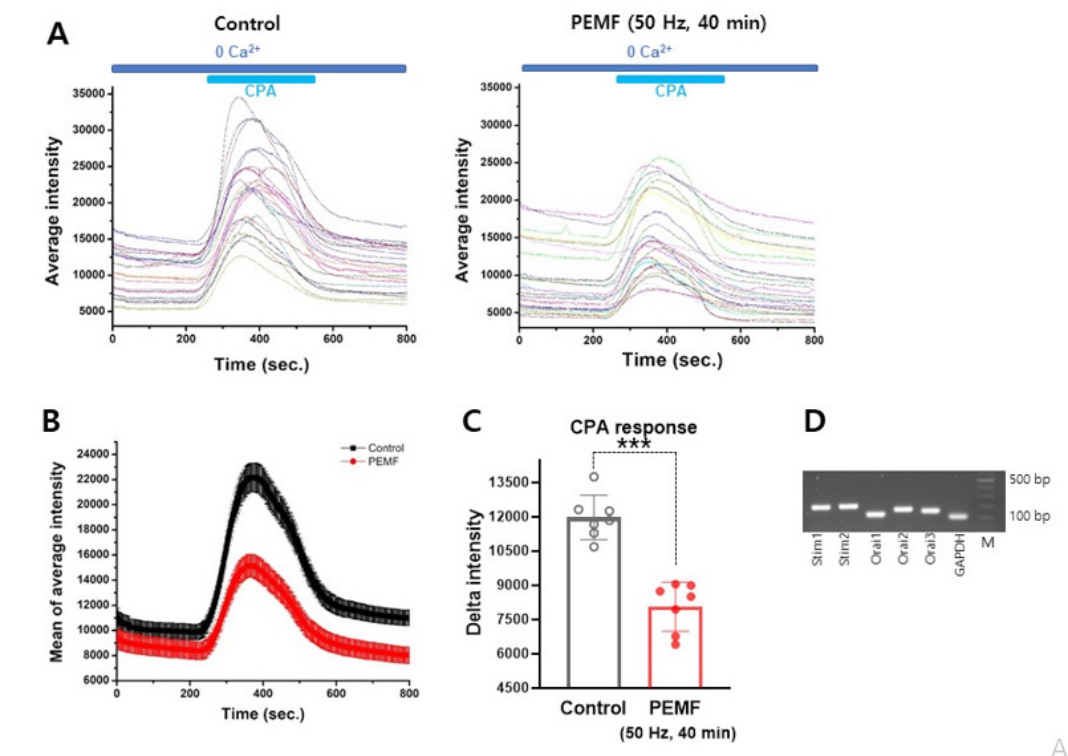

Figure 4: PEMF stimulation represses cytoplasmic store Ca2+ release in HUVECs. (A) Representative traces showing [Ca2+] i response induced by store depletion using CPA in the absence of extracellular Ca2+ in control and PEMF stimulated cells. (B) Representative traces showing that averaged (Mean ± SEM.) [Ca2+] i response induced by CPA. (C) Graph illustrating the mean net increases in [Ca2+] i obtained by CPA from PEMF stimulation condition versus control. (D) Original gel showing significant levels of Orai1, Orai2, Orai3, Stim1, and Stim2. Asterisks indicate ***P < 0.001.

In immune cells, P2Y receptors are involved in several types of signaling; P2Y6 mediates release of IL-8, and P2Y11 controls ATP-induced activation of dendritic cells [18,19]. The P2X family of proteins are expressed in both the myeloid and lymphoid lineage cells, such as neutrophils, eosinophils, macrophage, dendritic cells, T and B lymphocytes, and natural killer cells [20]. In accordance with their broad expression in immune cells, the P2X family plays an important role in the immune system and in the controlling of angiogenesis and wound repair [21]. The present study demonstrated that PEMF stimulation affected two different $[Ca^{2+}]_i$ regulatory systems, but the expression of genes and proteins was not changed (data not shown), though it is difficult to expect a change in gene or protein expression during a 40 min period only. This suggests that further studies on the effects of PEMF on post-translational modification (PTM) such as phosphorylation, methylation, glycosylation, and ubiquitination of ion channels are needed. It is well known that post-translational processes are essential for proper functioning of many membrane proteins and the modulation of their expression levels. In particular, since SOCE regulates cytosolic calcium by the interaction of ER's Ca^{2+} sensor interstitial interacting molecules (STIM1 and STIM2) and the plasma membrane Orai (Orai1, Orai2, Orai3) Ca^{2+} channels, numerous research has demonstrated the functional regulation of SOCE by PTMs such as myristoylation, s-glutathionylation, and s-nitrosylation [22-24].

We also found that another feature of PEMF stimulation when looking at the 'Mean of average intensity' in the figures. In the same manner that an increase or decrease of $[Ca^{2+}]_i$ was caused by PEMF stimulation, basal Ca^{2+}(start point of $[Ca^{2+}]$i) was also affected in a similar pattern. These results suggest that PEMF may act on other types of ion channels related to the regulation of $[Ca^{2+}]_i$ besides purinergic receptors and SOCE. The rapid increase in $[Ca^{2+}]_i$ following ATP (20 μM) treatment over a 100 sec period is mostly caused by the P2X receptors, and the changes in $[Ca^{2+}]$ i is probably responsible for various immune functions and endothelial cells activities. Also, since it was confirmed that the P2Y receptor genes were significantly expressed in both cells, if PEMF stimulation is given long-term and/or intensively, the effect of the changes in $[Ca^{2+}]_i$, manifested through P2Y receptor gene expression, can be expected.

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The paracrine effects of ATP are important in chemotaxis. Autocrine and paracrine modes of ATP also regulate the migration of various cell types such as neutrophils, dendritic cells and macrophages [25,26]. ATP along with UTP released by apoptotic cells acts as a chemo-attractive molecule for phagocytes vis the P2 receptors [27,28]. Ca^{2+} is also a crucial second messenger in endothelial cells and plays a key role in regulating a number of physiologic processes, including cell migration, angiogenesis, and blockade function. During inflammation, Ca^{2+}-related signaling in endothelial cell promotes several leukocyte-endothelial cell interactions, mediating leukocyte rolling, adhesion, and trans-endothelial migration [29]. These observations, together with our findings, indicate that purinergic signaling and $[Ca^{2+}]_i$ regulation must be finely studied to ensure the efficient application of PEMF stimulation.

Conclusion

PEMF therapy has been shown to be effective in treating a variety of conditions, such as fractures, wound healing, and arthritis, and ongoing research is underway to investigate its mechanisms, including its potential to treat etiology. This study examined PEMF's impact on cells, finding enhanced calcium influx and release in KG-1 and HUVECs, but with opposite effects on calcium responses in these cell types. These findings suggest that PEMF can influence calcium regulation through specific channels, highlighting the importance of tailoring treatment parameters to specific conditions and tissue types.

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