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Extracellular calcium differentially affects intracellular responses to nanosecond pulsed electric fields

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ABSTRACT

Nanosecond pulsed electric fields (nsPEFs) have received considerable attention because of their unique effects on living cells. Exposure of cultured cells to nsPEFs is known to induce Ca^{2+} influx by forming small pores in the plasma membrane. Furthermore, nsPEFs have been reported to induce activation of multiple cellular signaling pathways. To examine the relationship between Ca^{2+} influx and intracellular signaling events after nsPEF exposure, we applied nsPEFs to HeLa S3 cells suspended in either Ca^{2+}-free or Ca^{2+}-containing medium and analyzed the activation of signaling pathways. We observed that the presence of extracellular Ca^{2+} was required for AMPK activation but dispensable for the initiation of stress responses in nsPEF-exposed HeLa S3 cells. JNK and p38, which are major regulatory factors in MAPK pathways, were activated by nsPEFs irrespective of the presence or absence of extracellular Ca^{2+}. The absence of extracellular Ca^{2+}, however, led to augmented phosphorylation of JNK and p38, suggesting positive effects of Ca^{2+} influx on negative feedback controls in MAPK pathways. Taken together, these observations demonstrate that Ca^{2+} influx induced by nsPEFs differentially participates in intracellular signaling events.

Keywords: Nanosecond pulsed electric field, calcium, signal transduction.

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Abbreviations: AMPK, AMP-activated protein kinase; **CaMKK,** Calcium/calmodulin-dependent protein kinase kinase; **MAPK,** mitogen-activated protein kinase; **nsPEF:** nanosecond pulsed electric field.

INTRODUCTION

Electrical pulses are widely utilized in life sciences for broad applications, since distinct effects on living cells can be achieved by changing pulse conditions, such as pulse width. Electrical pulses for milli-to-microseconds are well-suited to electroporation of the plasma membrane, and thus, are broadly used to transfer macromolecules into living cells, such as transfection of plasmid DNA and electrochemotherapy with anti-tumor drugs (Mir, 2006; Sersa et al., 2008; Teissie et al., 2005). In contrast to the milli-to-microsecond electrical pulses, nanosecond pulsed electric fields (nsPEFs) do not generate membrane pores useful for macromolecule transfer, but intense nsPEFs are known to effectively induce cell death *in vitro* as well as *in vivo*, indicating the significant potential of nsPEFs as a novel means for cancer therapy (Beebe et al., 2013; Morotomi-Yano et al., 2013; Morotomi-Yano et al., 2014; Nuccitelli et al., 2014).

Biological actions of nsPEFs are accounted for, at least in part, by the activation of intracellular responses. Previous studies have clearly demonstrated that intracellular apoptotic and necrotic programs are activated after exposure to intense nsPEFs in a cell-type dependent manner (Morotomi-Yano et al., 2013; Morotomi-Yano et al., 2014; Ren and Beebe, 2011; Ren et al., 2012). Under mild nsPEF conditions that do not cause massive cell death, activation of multiple intracellular signaling pathways is readily detectable using biochemical methods, such as western blotting. Mitogen-activated protein kinases (MAPKs) play important roles in the control of cellular proliferation and differentiation, and their activation is primarily mediated by protein phosphorylation(Kyriakis and Avruch, 2001). Our previous studies demonstrated that nsPEFs induce transient phosphorylation of multiple protein components in the three major MAPK pathways (Morotomi-Yano et al., 2011a; Morotomi-Yano et al., 2011b). Additionally, nsPEFs were shown to induce stress

responses, during which intracellular signals were transduced by protein phosphorylation (Morotomi-Yano et al., 2012b). Human and mammalian cells have four stress responsive protein kinases that differentially activate intracellular signaling pathways in response to harmful stimuli and adverse conditions (Holcik and Sonenberg, 2005). Our previous study has shown that, among four stress-responsive protein kinases, PERK and GCN2 are activated by nsPEFs (Morotomi-Yano et al., 2012b). Taken together, these previous studies suggest the physiological significance of intracellular responses to nsPEFs.

Another important and unique aspect of the effects of nsPEFs on living cells is the rapid mobilization of intracellular Ca^{2+}. Several critical studies have described a transient increase in cytoplasmic Ca^{2+} after nsPEF exposure (Beier et al., 2012; Vernier et al., 2003; White et al., 2004). Although Ca^{2+} is known to be stored in the endoplasmic reticulum and mitochondria, the primary source of increased cytoplasmic Ca^{2+} by nsPEFs appears to be Ca^{2+} influx through the plasma membrane, since the absence of extracellular Ca^{2+} markedly reduced the duration and extent of Ca^{2+} mobilization after nsPEF exposure (Beier et al., 2012; White et al., 2004). A substantial portion of Ca^{2+} influx is thought to occur through so-called nanopores, which are small pores formed in the plasma membrane by nsPEFs and allow permeation of small molecules, such as ions and water (Pakhomov et al., 2009; Vernier et al., 2006). In addition to nanopore-mediated Ca^{2+} influx, the actions of nsPEFs on various Ca^{2+} channels in the plasma membrane appear to contribute to elevated cytoplasmic Ca^{2+} (Craviso et al., 2010; Vernier et al., 2008).

Although the activation of intracellular signaling and Ca^{2+} mobilization are two important features of how nsPEFs affect on human cells, limited information is available regarding the physiological relationship between these nsPEF-induced events. Recently, we showed one example of extracellular Ca^{2+}-dependent signal activation after nsPEF exposure (Morotomi-Yano et al., 2012a). AMP-activated protein kinase (AMPK) is a critical regulatory factor for cellular homeostasis, and its activation is mediated by protein phosphorylation (Hardie, 2011). In normal cells, AMPK is phosphorylated by LKB1, which can function in both the presence and absence of elevated intracellular Ca^{2+}. Because LKB1 acts as a tumor suppressor (Vaahtomeri and Makela, 2011), the LKB1 gene is often deleted in tumor cells, including HeLa S3 cells (Tiainen et al., 1999). In LKB1-deficient cells, calcium/calmodulin-dependent protein kinase kinase (CaMKK) compensates for LKB1 deficiency and functions in AMPK phosphorylation (Hawley et al., 2005). In contrast to LKB1, CaMKK requires elevated levels of cytoplasmic Ca^{2+} for its catalytic activity (Hawley et al., 2005). We demonstrated that extracellular Ca^{2+} is required for nsPEF-induced AMPK phosphorylation in HeLa S3 cells but is dispensable for that in LKB-proficient Jurkat cells (Morotomi-Yano et al., 2012a). This observation indicates a functional connection between nsPEF-induced Ca^{2+} influx and intracellular signaling events.

In this study, we extended our experimental approach used in the previous study to further understand the biological significance of extracellular Ca^{2+} in the control of intracellular signaling induced by nsPEFs. We found that the initiation of stress responses and MAPK activation were extracellular Ca^{2+}-independent. Interestingly, the absence of extracellular Ca^{2+} resulted in augmented MAPK phosphorylation, suggesting the involvement of elevated cytoplasmic Ca^{2+} in the negative feedback control of nsPEF-induced MAPK signaling. Together with our previous observation regarding the Ca^{2+}-dependency of AMPK activation, these results highlight the complicated involvement of Ca^{2+} influx in intracellular responses to nsPEFs.

MATERIALS AND METHODS

Cell culture

HeLa S3 cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C and cultured in αMEM (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS, Equitech Bio, Kerrville, TX, USA) and penicillin/streptomycin (Wako Pure Chemical Industries, Osaka, Japan).

For the exposure of cells to nsPEFs, Ca^{2+}-free DMEM (Thermo Fisher Scientific, Waltham, MA, USA) and dialyzed FBS (Sigma-Aldrich, St. Louis, MO, USA) were used. Low molecular weight materials, such as Ca^{2+}, were removed from this FBS by dialysis with a cut-off value of 10,000 Da. Ca^{2+}-free DMEM was supplemented with 10% dialyzed FBS (Ca^{2+}-free medium). $CaCl_2$ was added to aliquots of Ca^{2+}-free medium at 1 mM to give Ca^{2+}-containing medium. For the exposure to nsPEFs, cells were washed twice with Dulbecco's phosphate buffered saline (D-PBS) and detached from dishes with 1 mM EDTA dissolved in D-PBS. Detached cells were suspended in the Ca^{2+}-free medium and collected by centrifugation at 120 × *g* for 3 min. Pelleted cells were suspended in Ca^{2+}-free or Ca^{2+}-containing medium at 2×10^6 cells/ml and immediately used for experiments. For the analysis of GCN2 phosphorylation, Ca^{2+}-free DMEM was replaced by Ca^{2+}-free Spinner-Modified MEM (Wako Pure Chemical Industries, Osaka, Japan) supplemented with nonessential amino acids, because GCN2 phosphorylation was constitutively elevated in DMEM for unknown reasons.

Generation of nsPEFs and exposure of cells to nsPEFs

The pulsed power modulator system used in this study is shown in Figure 1A (Li et al., 2009). This system consisted of a control unit, a charger unit, and a pulsed power unit equipped with a magnetic pulse compression circuit. The cell suspension (400 μl) was placed in an electroporation cuvette that had a 4-mm gapped pair of aluminum electrodes (#5540; Thermo Fisher Scientific, Waltham, MA, USA) and exposed to 20 shots of 20 kV/cm nsPEFs at 1 Hz. Voltage waveforms of nsPEFs were monitored with a high-voltage probe (Type P6015A, Tektronix, Beaverton, OR, USA) and a digital oscilloscope (Type TDS2012S, Tektronix, Beaverton, OR, USA). A typical example of the voltage wave forms observed in our experiments is shown in Figure 1B. After exposure to nsPEFs, 300 μl of the cell suspension was removed from the cuvette and diluted with

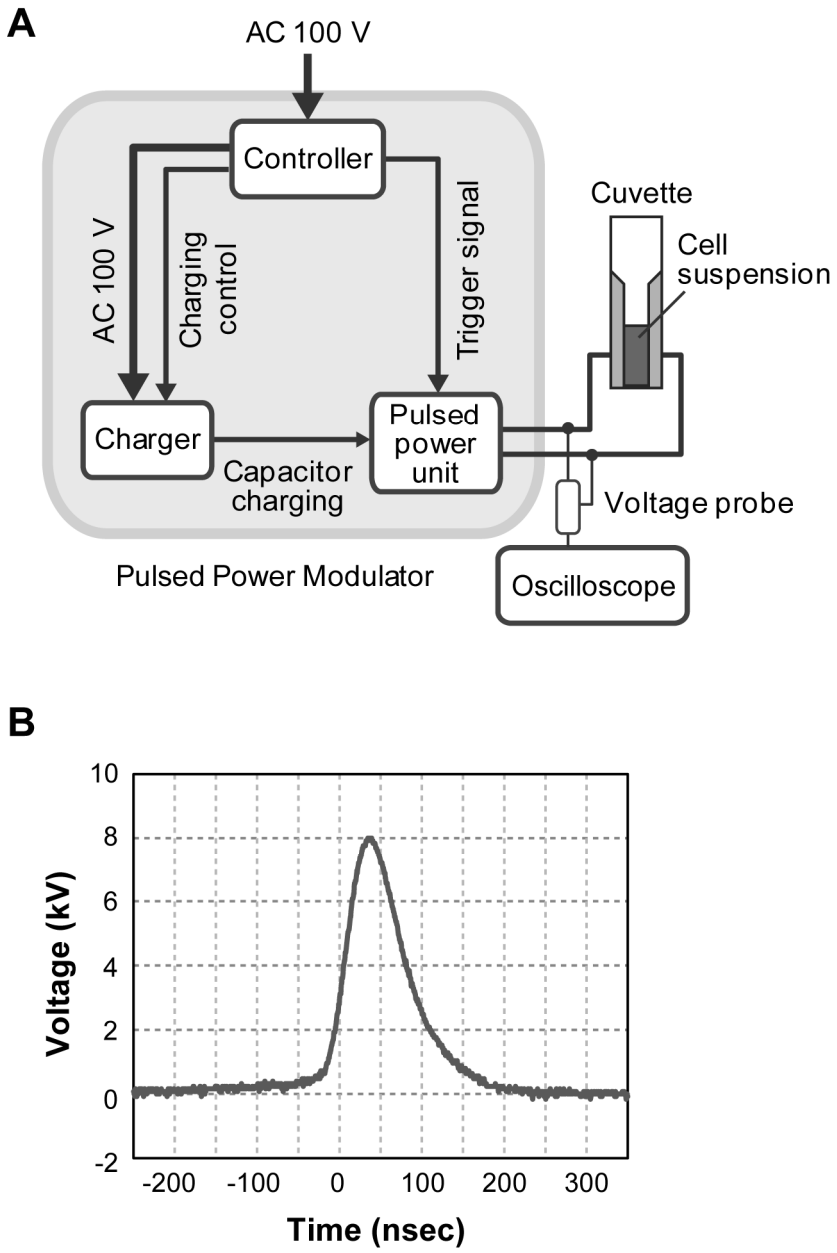

Figure 1. Generation and application of nsPEFs. (A): Schematic representation of the electronic system used for the generation of nsPEFs and the exposure of cultured cells to nsPEFs. (B): A typical example of voltage waveforms of nsPEFs used in this study. The maximum voltage of this pulse was 8 kV, resulting in 20 kV/cm electric fields in an electroporation cuvette with a pair of 4-mm gapped electrodes.

1.2 ml Ca^{2+}-free or Ca^{2+}-containing medium. Following incubation at 37°C, the cells were collected and snap-frozen in liquid nitrogen.

Western blotting

Frozen cells were lysed in a buffer containing 20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Igepal, protease inhibitors (Complete EDTA-free, Roche Applied Science, Mannheim, Germany), and phosphatase inhibitors (PhosStop, Roche Applied Science, Mannheim, Germany). Cell lysates were centrifuged at 20,000 × *g* for 10 min, and the protein concentration of each lysate was measured using the Bradford method (Bradford, 1976) with a Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). SDS polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting was performed according to standard procedures (Green and Sambrook,

2012). Equal amounts of total proteins (30 µg/lane) were used for all SDS-PAGE/western blotting in this study. Following antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA) and used at the indicated dilution; AMPK (#5831, 1:2000), phosphorylated AMPK (#2535, 1:2000), PERK (#3192, 1:2000), GCN2 (#3302, 1:1000), and phosphorylated JNK (#4668, 1:4000). An antibody against phosphorylated GCN2 (#ab75836, 1:1000) was obtained from Abcam (Cambridge, UK). Antibodies for p38 (#612168, 1:10000), phosphorylated p38 (#612280, 1:10000), and JNK (#610627, 1:5000) were obtained from BD Biosciences (San Jose, CA, USA). An antibody for β-actin (#A1978, 1:10000) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antigen-antibody complexes were detected by a chemiluminescence method using secondary antibodies conjugated to horse radish peroxidase (Santa Cruz Biotechnologies, Dallas, TX, USA) and a Super Signal West Pico reagent (Thermo Fisher Scientific, Waltham, MA, USA). Chemiluminescence was detected using a ChemiDoc XRS+ Molecular Imager (Bio-Rad Laboratories, Hercules, CA, USA), and images were acquired using a Quantity One image analyzing software (Bio-Rad Laboratories, Hercules, CA, USA). Quantification of chemiluminescence intensities in western blots was performed using an Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA).

RESULTS

Exposure of HeLa S3 cells to nsPEFs induces AMPK phosphorylation in an extracellular-Ca^{2+}-dependent manner

In this study, nsPEFs were generated by a pulsed power modular that was designed and manufactured in Kumamoto University (Figure 1A) (Li et al., 2009). A suspension of HeLa S3 cells in Ca^{2+}-free or Ca^{2+}-containing medium was placed in an electroporation cuvette with a pair of aluminum electrodes and exposed to 20 kV/cm nsPEFs at 1 Hz. A typical voltage waveform of nsPEFs in this study is shown in Figure 1B. The pulse width at half maximum was estimated to be approximately 80 ns.

Using these settings, we analyzed AMPK phosphorylation after nsPEF exposure by western blotting. As shown in Figure 2, AMPK in unexposed cells was constitutively phosphorylated at a low level in the presence or absence of extracellular Ca^{2+}. When nsPEFs were applied to cells in Ca^{2+}-containing medium, AMPK phosphorylation quickly increased within 1 min and returned to the basal level by 15 min after nsPEFs. When cells were suspended in Ca^{2+}-free medium, exposure to nsPEFs showed marginal effects on AMPK phosphorylation. These observations confirmed that extracellular Ca^{2+} is required for activated AMPK phosphorylation in HeLa S3 cells.

Initiation of stress responses induced by nsPEFs is Ca^{2+}-independent

Next, we examined whether extracellular Ca^{2+} affects the initiation of stress responses induced by nsPEFs. PERK and GCN2 are stress-responsive protein kinases that are activated by autophosphorylation upon stress induction (Holcik and Sonenberg, 2005). These two factors are thought to function in stress sensing because no cellular factors are located upstream of these proteins in their signaling pathways. We applied nsPEFs to HeLa S3 cells suspended in Ca^{2+}-free or Ca^{2+}-containing medium and analyzed the phosphorylation of these proteins by western blotting. In the analysis of PERK, phosphorylated and unphosphorylated forms can be readily distinguished by electrophoretic mobilities (Harding et al., 1999), because of the heavy phosphorylation of PERK after stress induction. As shown in Figures 3A and 3B, the band shifts of PERK after nsPEF exposure occurred to a similar extent in the presence or absence of extracellular Ca^{2+}, indicating that PERK activation by nsPEFs is independent of extracellular Ca^{2+}. Next, we analyzed the effects of extracellular Ca^{2+} on GCN2 phosphorylation by western blotting using an antibody that specifically recognizes the phosphorylated form of GCN2. We found that GCN2 phosphorylation induced by nsPEFs was largely indistinguishable, regardless of whether extracellular Ca^{2+} was present (Figure 3C). Taken together, these results indicate that the stress responses are initiated by nsPEFs independently of the presence or absence of extracellular Ca^{2+}.

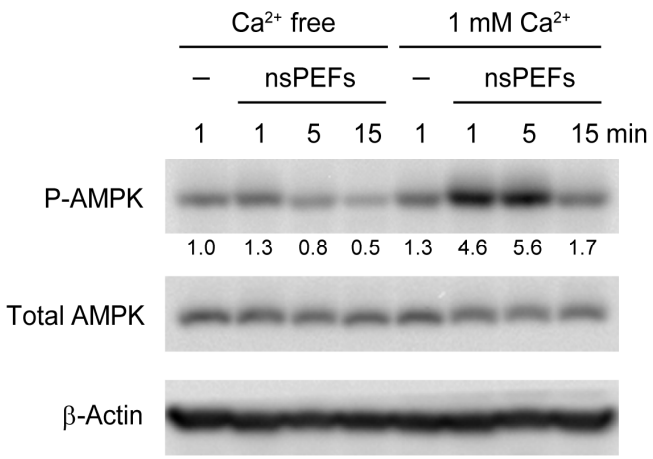

Figure 2. Extracellular Ca^{2+}-dependent induction of transient AMPK phosphorylation by nsPEFs in HeLa S3 cells. HeLa S3 cells were suspended in either Ca^{2+}-free or Ca^{2+}-containing medium and exposed to 20 shots of 20 kV/cm nsPEFs at 1 Hz. Unexposed control samples (-) were included in the experiment. After incubation at 37°C for the indicated times, total cell lysates were prepared. Equal protein amounts (30 µg protein/lane) were subjected to SDS-PAGE followed by western blotting in duplicate, and AMPK phosphorylation was analyzed using antibodies against phosphorylated AMPK (Upper panel) and total AMPK (Middle panel). Signals of phosphorylated AMPK in the western blot were quantified and normalized by those of total AMPK to give relative intensities. The value of the Ca^{2+}-free unexposed sample was set to 1.0. As a loading control, β-actin was visualized (Lower panel).

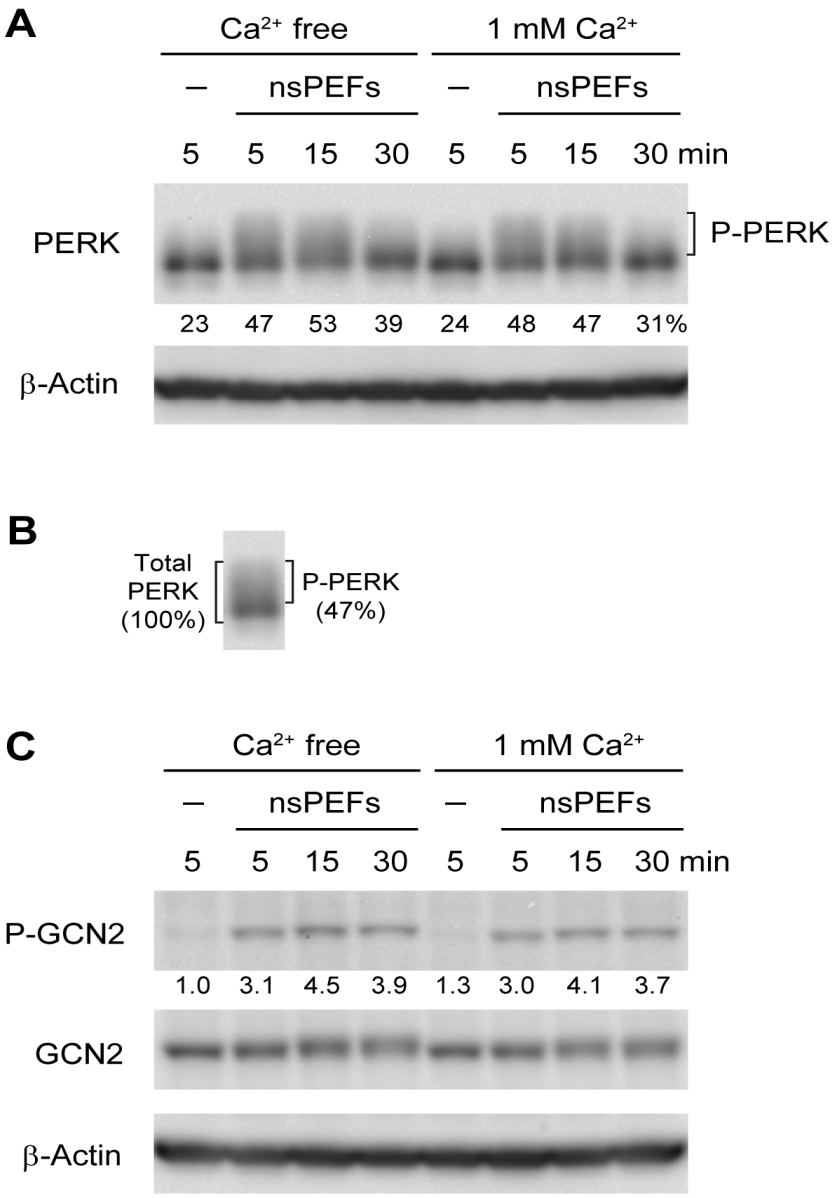

Figure 3. Phosphorylation of stress-responsive protein kinases by nsPEFs independently of the presence or absence of extracellular Ca^{2+}.
(A): Western blot analysis of PERK. HeLa S3 cells suspended in either Ca^{2+}-free or Ca^{2+}-containing medium were exposed to 20 shots of 20 kV/cm nsPEFs, and the status of PERK was analyzed by western blotting using an anti-PERK antibody. Phosphorylated species of PERK (P-PERK) are known to migrate more slowly than unphosphorylated PERK in gel electrophoresis (Harding et al., 1999) (Upper panel). A ratio of phosphorylated PERK to total PERK in each lane was quantified (see below) and is shown under the upper panel. As a loading control, β-actin was detected (Lower panel).
(B): An example of quantification of total and phosphorylated PERK in the western blot. A portion of the western blot shown in (A) is highlighted (Ca^{2+}-free, 5 min after nsPEF exposure). First, the signal intensity of total PERK was measured and set to 100%. Then, phosphorylated PERK in the blot was measured, and a ratio of phosphorylated PERK to total PERK was calculated.
(C): Western blot analysis of GCN2 phosphorylation. SDS-PAGE/western blotting was performed in duplicate for phosphorylated GCN2 (P-GCN2, Upper panel) and total GCN2 (Middle panel). In the lower panel, β-actin was detected as a loading control.

Absence of extracellular Ca^{2+} leads to augmented phosphorylation of MAPKs after nsPEF exposure

MAPKs play important roles in multiple cellular processes, including proliferation and apoptosis (Kyriakis and Avruch, 2001). Along with other cellular factors, MAPKs constitute signaling pathways that are primarily transduced by protein phosphorylation. In this study, we analyzed the phosphorylation status of two major MAPKs, JNK and p38, which are representative and well-characterized members of human MAPKs (Wagner and Nebreda, 2009). Because of alternative splicing, multiple bands were detected in western blotting for JNK (Gupta et al., 1996; Pulverer et al., 1991). Western blot analysis showed that phosphorylation of JNK and p38 was activated by nsPEFs regardless of whether extracellular Ca^{2+} was present (Figures 4 and 5), suggesting that Ca^{2+} influx is dispensable for initiating MAPK activation by nsPEFs. However, the absence of extracellular Ca^{2+} led to augmented phosphorylation of JNK and p38. In Figure 4A, the phosphorylation of JNK in the absence of extracellular Ca^{2+} was stronger than that in the presence of extracellular Ca^{2+} after nsPEF exposure. At 45 min, JNK phosphorylation disappeared in the presence of Ca^{2+} but slightly remained in the absence of Ca^{2+}. Figure 4B shows the quantification of signal intensities observed on western blots, which highlights a difference in JNK phosphorylation in the presence and the absence of extracellular Ca^{2+}.

Phosphorylation of p38 was also augmented in the absence of extracellular Ca^{2+} (Figure 5). In the presence of Ca^{2+}, p38 phosphorylation reached a maximum at 5 min and markedly declined at 15 min. In the absence of Ca^{2+}, a high level of p38 phosphorylation was sustained at 15 min (Figures 5A and 5B). These observations demonstrate that extracellular Ca^{2+} has suppressive effects on MAPK phosphorylation after nsPEF exposure.

DISCUSSION

Exposure of human cells to nsPEFs is known to induce rapid Ca^{2+} mobilization, a large part of which is accounted for by the influx of extracellular Ca^{2+} through the plasma membrane (Beier et al., 2012; Craviso et al., 2010; Vernier et al., 2008; Vernier et al., 2003; White et al., 2004). Although the physiological importance of Ca^{2+} influx induced by nsPEFs has been proposed in previous studies, its detailed information at the molecular levels has been largely obscure. In this study, we observed differential involvement of extracellular Ca^{2+} in intracellular signaling events induced by nsPEFs. First, nsPEFs induced the elevated AMPK phosphorylation in an extracellular Ca^{2+}-dependent manner in HeLa S3 cells. It has been previously shown that CaMKK is responsible for nsPEF-induced AMPK phosphorylation in HeLa S3 cells and requires elevated cytoplasmic Ca^{2+} for its enzymatic activity (Morotomi-Yano et al., 2012a). Thus, extracellular Ca^{2+}-dependent AMPK phosphorylation after nsPEF exposure indicates a direct link between nsPEF-induced Ca^{2+} influx and activation of intracellular signaling (Figure 6).

In contrast to the AMPK phosphorylation, stress responses (Figure 3) and MAPK activation (Figures 4 and 5) were initiated by nsPEFs without extracellular Ca^{2+}, suggesting that activation of these pathways arises for other reasons. One possible explanation for the triggering of these pathways may be the direct action of nsPEFs on proteins in these signaling pathways. In particular, to initiate stress responses, nsPEFs may act directly on PERK and GCN2 molecules, because both PERK and GCN2 are located in the most upstream positions of the signaling cascades of stress responses and are thought to directly sense adverse stimuli. Recently, a theoretical prediction of direct effects of nsPEFs on protein structures has been proposed (Beebe, 2015). Our results on nsPEF-induced activation of PERK and GCN2 provide an important clue for understanding the molecular details of nsPEF actions on proteins.

Another possible mechanism for the activation of intracellular signaling by nsPEFs may involve the physical impacts of nsPEFs on the plasma membrane. Although nsPEFs do not generate large membrane pores suited for DNA transfer by electroporation, it has been clearly shown that nsPEFs cause structural perturbations of the plasma membrane, such as nanopore formation (Pakhomov et al., 2009; Vernier et al., 2006). The plasma membrane functions as a platform for assembly of multiprotein complexes, and many intracellular signal cascades, including several MAPK pathways, start from these membrane-bound and membrane-associated proteins (Katz et al., 2007; Kyriakis and Avruch, 2001). Previous studies have shown that fine structural changes in the plasma membrane often lead to the activation of signal transduction. For example, alterations in membrane fluidity by physical and chemical means result in signal activation (Jean-Louis et al., 2006; Nagy et al., 2007). The effects of nsPEFs on the plasma membrane may be involved in triggering the observed signal transduction, although further studies are needed to identify the initial events in the nsPEF-induced signal transduction.

Contrary to our expectations, extracellular Ca^{2+} had negative effects on MAPK phosphorylation by nsPEFs, and the absence of extracellular Ca^{2+} led to the augmentation of nsPEF-induced MAPK phosphorylation (Figures 4 and 5). MAPK pathways involve many cellular proteins, some of which function as negative regulators to prevent excessive activation (Kyriakis and Avruch, 2001). A representative example of negative regulators in MAPK pathways is the MAPK phosphatases, which dephosphorylate activated MAPKs and consequently down-regulate MAPK pathways (Dickinson and Keyse, 2006; Junttila et al., 2008). Thus, it seems conceivable that nsPEF-induced Ca^{2+} influx could positively affect catalytic activities or protein-protein interactions of negative

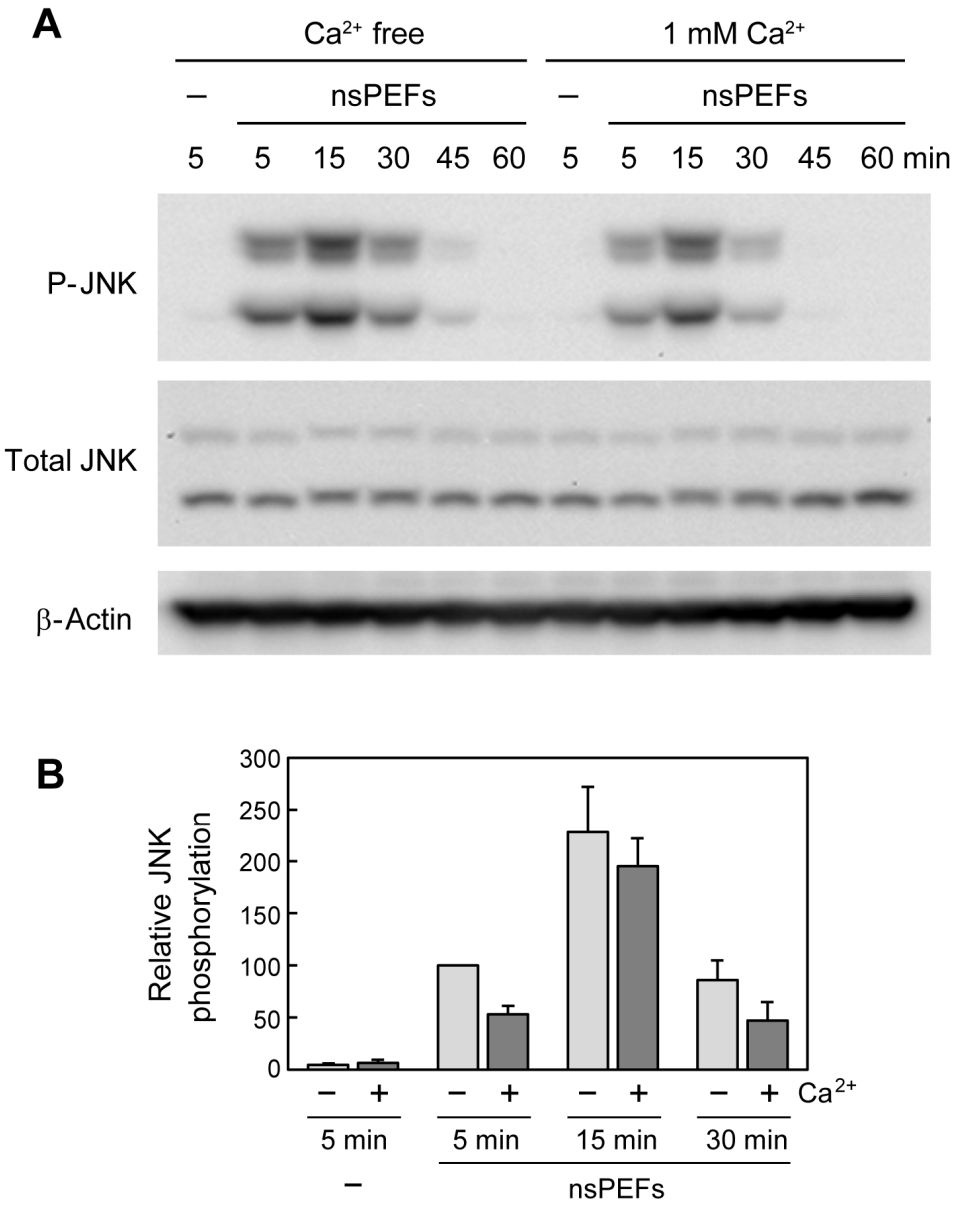

Figure 4. Augmented JNK phosphorylation by nsPEFs in the absence of extracellular Ca^{2+}.
(A). HeLa S3 cells were suspended in either Ca^{2+}-free or Ca^{2+}-containing medium and exposed to 20 shots of 20 kV/cm nsPEFs. Western blot analysis of JNK was performed in duplicate using antibodies against phosphorylated JNK (Upper panel) and total JNK (Middle panel). Due to alternative splicing of JNK mRNA, two major bands for JNK were detected in western blotting (Gupta et al., 1996; Pulverer et al., 1991). As a loading control, β-actin was detected (Lower panel).
(B). Quantification of western blot signals for JNK phosphorylation. Signals of phosphorylated JNK in the western blot were quantified and normalized by those of total JNK to give relative intensities. The value for phosphorylated JNK at 5 min in the absence of Ca^{2+} was set to 100. The experiments from nsPEF exposure to the western blotting were repeated four times, and the average values of the quantified signals with standard errors are shown.

regulators in the MAPK pathways (Figure 6). This may account for the observed negative effects of Ca^{2+} on nsPEF-induced MAPK activation, but further investigation is necessary to test this idea.

Previous studies have shown that intense nsPEFs efficiently induce cell death *in vitro* as well as *in vivo*

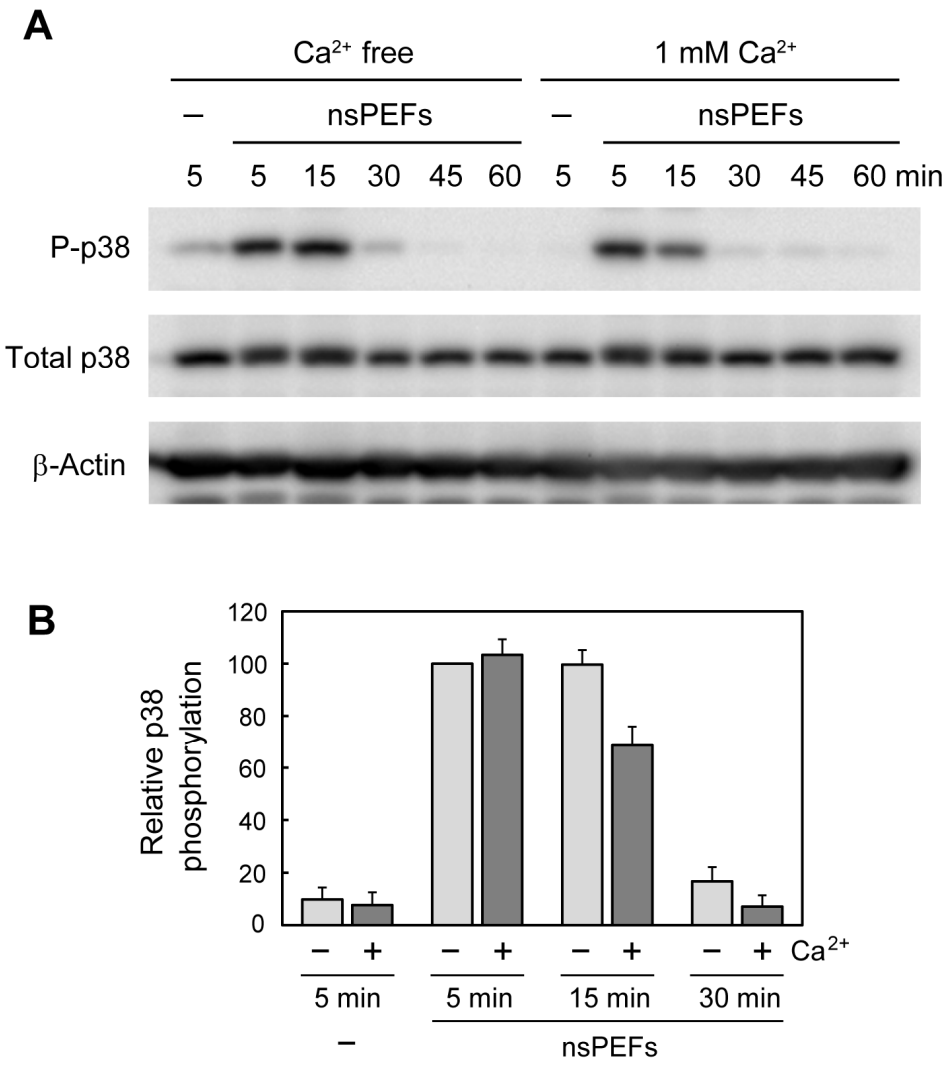

Figure 5. Augmented p38 phosphorylation by nsPEFs in the absence of extracellular Ca^{2+}. (A): Western blot analysis using antibodies against phosphorylated (Upper panel) and total (Middle panel) p38 was performed as described in Figure 4A. (B): Quantification of western blot signals for p38 phosphorylation was carried out as described in Figure 4B.

(Beebe et al., 2013; Nuccitelli et al., 2014) and that Ca^{2+} affects cellular sensitivity to nsPEFs (Morotomi-Yano et al., 2013; Morotomi-Yano et al., 2014). In this study, we demonstrated that Ca^{2+} is differently involved in the activation of AMPK, MAPK, and stress response pathways, and these nsPEF-induced signaling events may be involved in the modulation of cell death and survival in a complicated manner. For example, stress responses are generally considered to contribute to cell survival, but their prolonged activation promotes cell death. MAPK pathways are known to differently participate in cell death induction in a cell type-dependent manner. Further studies on the physiological relationship between signaling events and cell death in a nsPEF-exposed cell will provide mechanistic insights in the nsPEF actions and contribute to the clinical applications of nsPEFs.

CONCLUSION

This study demonstrates complicated participation of extracellular Ca^{2+} in modulation of nsPEF-induced intracellular signaling. Further studies to functionally dissect nsPEF-induced intracellular events with respect to Ca^{2+} dependency will offer a comprehensive understanding of the biological actions of nsPEFs and will provide an important basis for future clinical application of nsPEFs.

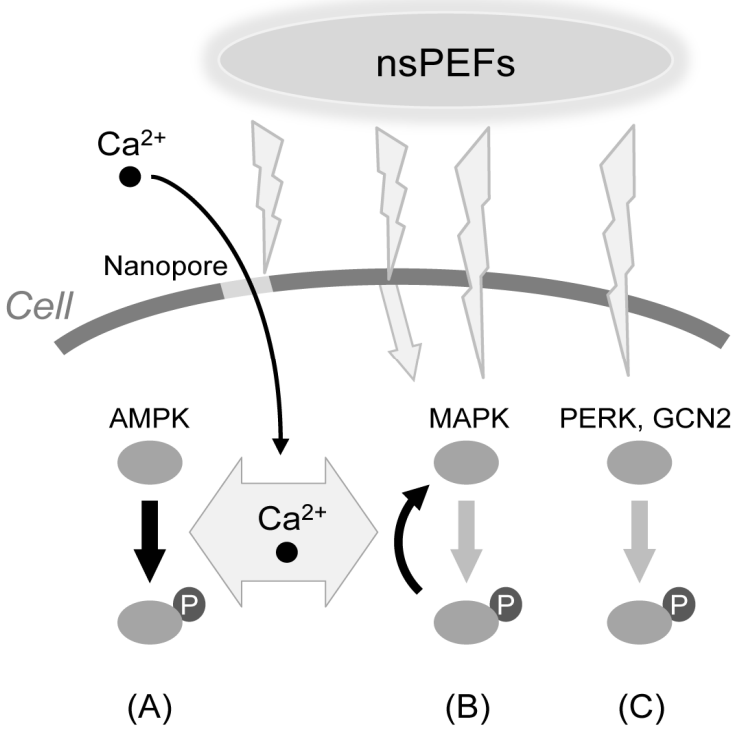

Figure 6. Model for the involvement of extracellular Ca^{2+} in nsPEF-induced intracellular signaling.
nsPEFs appear to act at multiple sites in a cell. The plasma membrane is one of the major sites of nsPEF actions, and small pores, known as nanopores, are generated by nsPEFs. The formation of nanopores on the plasma membrane leads to the influx of extracellular Ca^{2+}. In the model, black arrows indicate cellular events, in which Ca^{2+} is directly involved.
(A): Requirement of extracellular Ca^{2+} for nsPEF-induced AMPK phosphorylation. AMPK is activated via phosphorylation by the protein kinase CaMKK, which requires elevated cytoplasmic Ca^{2+} for its catalytic activity.
(B): Negative effects of extracellular Ca^{2+} on MAPK activation. MAPK pathways are activated by nsPEFs in the presence or absence of extracellular Ca^{2+}. The absence of extracellular Ca^{2+} leads to augmented MAPK phosphorylation, which may be attributed to the possible involvement of Ca^{2+} influx in the negative feedback regulation of MAPK pathways.
(C): Extracellular Ca^{2+}-independent activation of stress-responsive protein kinases by nsPEFs. PERK and GCN2 are activated by nsPEFs irrespective of the presence or absence of extracellular Ca^{2+}. Because PERK and GCN2 are located in the most upstream positions in the stress response signaling cascades, nsPEFs may directly act on these proteins.

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