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Nanosecond pulsed electric fields activate AMP-activated protein kinase: Implications for calcium-mediated activation of cellular signaling

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ABSTRACT

Nanosecond pulsed electric fields (nsPEFs) are increasingly being recognized as a potential tool for use in the life sciences. Exposure of human cells to nsPEFs elicits the formation of small membrane pores, intracellular Ca^{2+} mobilization, signaling pathway activation, and apoptosis. Here we report the activation of AMP-activated protein kinase (AMPK) by nsPEFs. AMPK activation is generally achieved by the phosphorylation of AMPK in response to changes in cellular energy status and is mediated by two protein kinases, LKB1 and CaMKK. Exposure to nsPEFs rapidly induced phosphorylation of AMPK and its downstream target ACC in both LKB1-proficient and LKB1-deficient cells. In LKB1-deficient cells, AMPK activation by nsPEFs was mediated by CaMKK and required extracellular Ca^{2+}, which suggested the occurrence of Ca^{2+} mobilization and its participation in AMPK activation by nsPEFs. Our results provide experimental evidence for a direct link between activated cellular signaling and Ca^{2+} mobilization in nsPEF-exposed cells.

1. Introduction

Ultrashort pulsed electric fields of nanosecond duration (nanosecond pulsed electric fields, nsPEFs) have received increasing attention owing to their unique actions on human cells. Exposure of cultured human cells to nsPEFs elicits the activation of several intracellular signaling pathways, including MAP kinase pathways [1,2] and a stress response pathway [3]. Notably, intense nsPEFs efficiently induce apoptosis in human and mouse tumor cells and are considered a promising new approach for cancer therapy [4–7]. Unlike conventional electroporation, which employs milli-to-microsecond electric pulses, exposure of human cells to nsPEFs causes the formation of small membrane pores called nanopores, which can allow permeation of ion, water, and low-molecular-weight compounds, but not macromolecules such as plasmid DNA [8,9]. In accordance with nanopore formation in the cell membrane, increased conductance of the cell membrane is detectable for minutes after nsPEF exposure [10,11], and enhanced sensitivity of nsPEF-exposed cells to the non-permeable anti-tumor drug bleomycin can be achieved [12]. Furthermore, nsPEFs provoke a remarkable increase in cytoplasmic Ca^{2+}, which presumably arises from the cumulative effects of nanopore formation and direct actions of nsPEFs on intracellular Ca^{2+} stores and various Ca^{2+} channels in the cell membrane [13–16]. Although many studies have documented Ca^{2+} mobilization after nsPEF exposure and have assumed its implication in biological responses to nsPEFs, little experimental evidence is currently available to permit understanding of the relationship between Ca^{2+} mobilization and specific intracellular responses after nsPEF exposure.

Human cells have elaborate signaling pathways that are turned on in response to internal and external perturbations and that induce homeostatic mechanisms. AMP-activated protein kinase (AMPK) is an important regulatory factor in such homeostatic mechanisms and functions as a sensor for cellular energy status [17]. AMPK plays a critical role in the coordination between energy-producing and energy-consuming cellular processes including metabolism and cell growth [17]. Cellular energy status can be represented by the ratio of AMP to ATP, which is kept very low under normal physiological conditions [18]. As its name suggests, AMPK is activated by elevated levels of AMP; AMP directly binds to the AMPK protein and provokes conformational changes that allow AMPK phosphorylation at threonine 172 [19–21]. AMPK phosphorylation is mainly catalyzed by two protein kinases in human cells. LKB1 is the major kinase for AMPK phosphorylation [22], and calcium/calmodulin-dependent protein kinase kinase (CaMKK) is the other AMPK kinase [23,24]. Germline mutations in the

Abbreviations: ACC, acetyl-CoA carboxylase-2; AMPK, AMP-activated protein kinase; CaMKK, calcium/calmodulin-dependent protein kinase kinase; nsPEF, nanosecond pulsed electric field.

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human *LKB1* gene cause cancer-prone Peutz-Jeghers syndrome [25], indicating that LKB1 functions in tumor suppression. Accordingly, LKB1 is often deficient in cell lines derived from human malignant tumors such as HeLa S3 cells [26], in which CaMKK plays a critical role in AMPK phosphorylation. Although LKB1 and CaMKK can function in a single cell, the physiological role of CaMKK in AMPK phosphorylation is often obscure in the presence of functional LKB1 and is more evident in LKB1-deficient cells [23,24]. The enzymatic activity of CaMKK requires elevated levels of intracellular Ca^{2+} [27]. AMPK phosphorylation by CaMKK is achieved in the presence of increased levels of cytoplasmic Ca^{2+}, whereas LKB1 functions in AMPK phosphorylation independently of Ca^{2+}.

In this study, we demonstrated that nsPEFs activate AMPK in human cells. In Jurkat cells, nsPEFs elicited the rapid phosphorylation of AMPK as well as acetyl-CoA carboxylase-2 (ACC), which is a downstream target of activated AMPK [28]. In HeLa S3 cells, which are deficient in LKB1, the activation of AMPK by nsPEFs was remarkably suppressed by the CaMKK inhibitor STO-609 [29], indicating the phosphorylation of AMPK by CaMKK in HeLa S3 cells. Furthermore, extracellular Ca^{2+} was required for CaMKK-mediated AMPK activation by nsPEFs, which suggested the involvement of Ca^{2+} mobilization in nsPEF-induced AMPK phosphorylation. Taken together, these results provide experimental evidence that nsPEF-induced Ca^{2+} mobilization directly affects specific cellular signaling events in human cells.

2. Materials and methods

2.1. Cell culture and application of nsPEFs

Cells were maintained in a humidified atmosphere of 5% CO_2. Jurkat cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. HeLa S3 cells were grown in αMEM supplemented with 10% FBS and penicillin/streptomycin.

For the application of nsPEFs, cells were suspended in media lacking antibiotics at 1×10^7 ml^{-1} for the Jurkat cells and 2×10^6 ml^{-1} for the HeLa S3 cells. For analysis of the effects of extracellular Ca^{2+}, cells were suspended in a Ca^{2+}-free medium (#21068, GIBCO Life Technologies) that was supplemented with 10% dialyzed FBS (Sigma–Aldrich), from which Ca^{2+} was eliminated by dialysis against 0.15 M NaCl by using a cut-off cartridge of molecular weight 10,000 Da. The cell suspension (400 μl) was placed in an electroporation cuvette that contained a pair of parallel aluminum electrodes with a 4 mm-gap (#5540, MβP, Thermo Fisher Scientific). A train of nsPEFs was generated by a pulsed power modulator [30] and applied to the cell suspension in the cuvette at 1 Hz. Voltage waveforms of the electric pulses were monitored by a high-voltage probe (P6015A, Tektronix) and a digital phosphor oscilloscope (DPO4054, Tektronix). A typical waveform of nsPEFs under our standard experimental conditions was reported in our previous study [1], and the average pulse width at 50% maximum was estimated to be approximately 80 ns. After exposure to nsPEFs, the cells were immediately diluted 5-fold into pre-warmed media and incubated at 37 °C.

For the inhibition of CaMKK catalytic activity, cells were preincubated in media containing 20 μM STO-609 (Santa Cruz Biotechnology) at 37 °C for 30 min, exposed to nsPEFs in the presence of 20 μM STO-609 and diluted into prewarmed media containing 20 μM STO-609 as described above.

2.2. Western blotting

Cells were lysed in an extraction buffer that contained 20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Igepal, a protease inhibitor cocktail (Complete EDTA-free, Roche), and a phosphatase inhibitor cocktail (PhosStop, Roche) on ice for 20 min. Cell lysates were centrifuged at 20,000*g* for 10 min, and protein concentrations of the cleared lysates were measured using a Protein Assay reagent (Bio-Rad). Equal amounts of proteins in the cleared lysates were resolved by SDS–PAGE and electrotransferred to a PVDF membrane. Proteins of interest were reacted with specific primary antibodies and detected by a chemiluminescence method using secondary antibodies conjugated with horse radish peroxidase (Santa Cruz Biotechnology) and a Super Signal West Pico chemiluminescent substrate reagent (Thermo Scientific). Following primary antibodies were obtained from Cell Signaling Technology; anti-phosphorylated AMPKα (Thr172), anti-phosphorylated ACC (Ser79), anti-AMPK, anti-ACC, and anti-LKB1. Antibodies for CaMKKβ and β-actin were purchased from GeneTex and Sigma–Aldrich, respectively.

3. Results

3.1. Activation of AMPK in Jurkat cells by nsPEFs

Previous studies reported that Jurkat cells are relatively sensitive to nsPEFs and prone to apoptosis after nsPEF exposure [31,32]. In this study, we therefore employed relatively mild nsPEF conditions for the Jurkat cells as compared to those for the HeLa S3 cells that were employed in our previous study [1–3]. We applied nsPEFs at different intensities and shot numbers to Jurkat cells and analyzed AMPK phosphorylation by western blotting (Fig. 1A). In the untreated cells, weak constitutive phosphorylation of AMPK was detectable, and the signals for phosphorylated AMPK in the western blot increased with the shot numbers of nsPEFs (Fig. 1A). The total amounts of AMPK were also examined using antibody that reacts with AMPK irrespective of its phosphorylation status (Fig. 1A). The signal intensities for the total AMPK in the western blot were virtually constant among the nsPEF-exposed and untreated samples. However, for the nsPEF-exposed cells, the signals for AMPK became broader, which reflected the increased phosphorylation of AMPK after nsPEF exposure. Time-course analysis demonstrated the rapid induction of AMPK phosphorylation by nsPEFs (Fig. 1B). AMPK phosphorylation reached a maximum at 1 min, persisted for 15 min, and then gradually decreased (Fig. 1B).

Phosphorylated AMPK is the enzymatically active form of AMPK that catalyzes the phosphorylation of multiple downstream target proteins [20,21]. ACC is one of these proteins that are phosphorylated by activated AMPK [28], and ACC phosphorylation is thus considered to be indicative of AMPK activation. We analyzed ACC phosphorylation in nsPEF-exposed cells by western blotting using an antibody against phosphorylated ACC. In parallel with increased AMPK phosphorylation, ACC phosphorylation was elevated by nsPEFs (Fig. 1A, B). Taken together, our results indicate that nsPEFs activated AMPK via its phosphorylation in Jurkat cells.

3.2. CaMKK-mediated AMPK activation by nsPEFs in LKB1-deficient HeLa S3 cells

AMPK phosphorylation is mainly mediated by two upstream protein kinases, LKB1 and CaMKK. The catalytic activity of CaMKK requires increased concentrations of cytoplasmic Ca^{2+} [27], and is inhibited by STO-609 [29] (Fig. 2A). LKB1 is a major protein kinase for AMPK phosphorylation [22], and its enzymatic activity is not affected by Ca^{2+} or STO-609 (Fig. 2A). LKB1 plays an important role in tumor suppression, and several tumor-derived cell lines lack functional LKB1 [26]. One of these LKB1-deficient cell lines is HeLa S3 [26], and we thus utilized HeLa S3 cells to analyze the involvement of CaMKK in nsPEF-induced AMPK phosphorylation. First, we

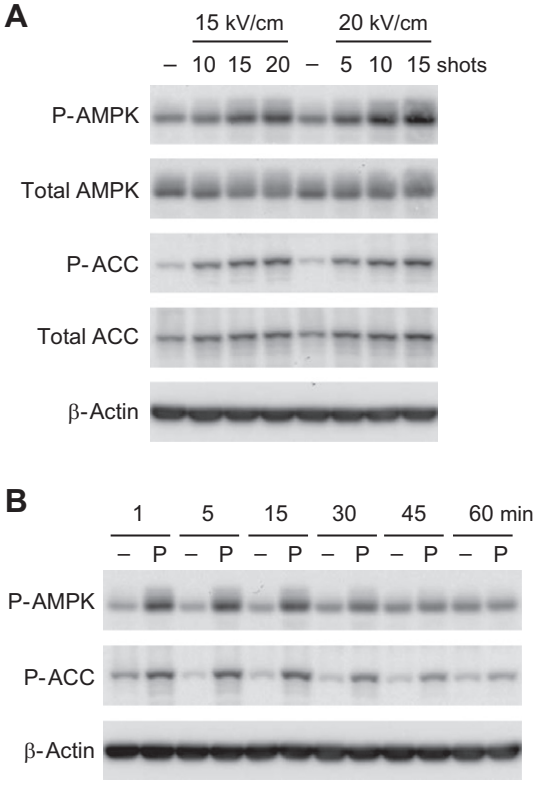

Fig. 1. Activation of AMPK in Jurkat cells by nsPEFs. (A) AMPK activation by nsPEFs. Jurkat cells were exposed to the indicated numbers of shots of either 15 kV/cm or 20 kV/cm nsPEFs. After incubation for 15 min at 37 °C, the cells were collected and subjected to western blot analysis. As a negative control, untreated cells were incubated at 37 °C for 15 min (−). Phosphorylated forms of AMPK (P-AMPK) and ACC (P-ACC) were detected using their specific antibodies. Total amounts of AMPK and ACC were shown using antibodies that react with AMPK or ACC irrespective of their phosphorylation status. As a loading control, β-actin was visualized. (B) Time-course of AMPK activation after nsPEF exposure. Jurkat cells were exposed to 10 shots of 20 kV/cm nsPEFs and incubated at 37 °C for the indicated times. Untreated cells (−) were included in the analysis as a negative control. Western blotting was performed using antibodies against phosphorylated AMPK (P-AMPK) and phosphorylated ACC (P-ACC). As a loading control, β-actin was detected.

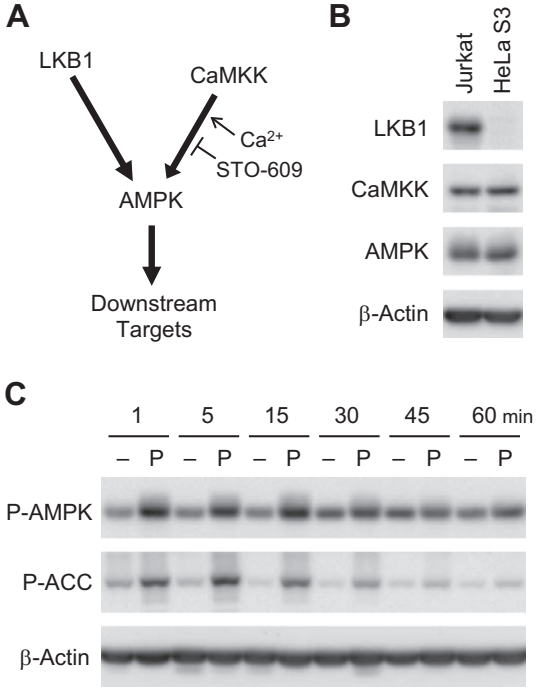

Fig. 2. Activation of AMPK in HeLa S3 cells by nsPEFs. (A) A schematic diagram of the AMPK signaling pathway. Human cells have two major upstream kinases for AMPK phosphorylation. The catalytic activity of CaMKK requires the presence of intracellular Ca^{2+}, and is inhibited by STO-609. LKB1 activity is affected by neither Ca^{2+} nor STO-609. (B) HeLa S3 cells lack LKB1. Total cell lysates were prepared from Jurkat and HeLa S3 cells that were not exposed to nsPEFs. The levels of LKB1, CaMKK, AMPK and β-actin proteins were analyzed by western blotting using their specific antibodies. (C) Time-course of AMPK after nsPEF exposure in HeLa S3 cells. HeLa S3 cells were exposed to 20 shots of 20 kV/cm nsPEFs and incubated at 37 °C for the indicated times. Western blot analysis of phosphorylated AMPK (P-AMPK) and phosphorylated ACC (P-ACC) was performed as described in Fig. 1B.

confirmed that Jurkat cells express both LKB1 and CaMKK proteins and that HeLa S3 cells have CaMKK but lack LKB1 (Fig. 2B). Next, HeLa S3 cells were exposed to 20 shots of 20 kV/cm nsPEFs and subjected to western blot analysis of AMPK phosphorylation. As shown in Fig. 2C, AMPK phosphorylation rapidly increased after nsPEF exposure, and ACC phosphorylation also increased in parallel. These results indicate that nsPEFs activate AMPK in the absence of LKB1.

AMPK activation by nsPEFs in HeLa S3 cells suggested that CaMKK directly participates in AMPK activation in this cell line. To test this hypothesis, we treated HeLa S3 cells with the CaMKK inhibitor STO-609 and analyzed the phosphorylation of AMPK and ACC. As shown in Fig. 3A, STO-609 remarkably suppressed the nsPEF-induced phosphorylation of AMPK and ACC, which indicated the crucial role of CaMKK in nsPEF-induced AMPK activation in HeLa S3 cells. Next, we performed the same experiments using Jurkat cells and observed that STO-609 treatment exerted a small inhibitory effect on the nsPEF-induced phosphorylation of AMPK and ACC in Jurkat cells (Fig. 3B). This observation was in good agreement with the previous reports that the involvement of CaMKK in AMPK phosphorylation is obscure in the presence of functional LKB1 [23,24]. Taken together, the above observations showed that nsPEF-induced AMPK phosphorylation is mediated by CaMKK in HeLa S3 cells.

3.3. Extracellular Ca^{2+} is involved in CaMKK-mediated AMPK activation by nsPEFs

CaMKK requires elevated levels of cytoplasmic Ca^{2+} for its activated enzymatic function [27]. Because it has been well documented that nsPEFs increase the concentration of cytoplasmic Ca^{2+} [13–16], we next examined the possible involvement of Ca^{2+} influx in CaMKK-mediated AMPK activation. We applied nsPEFs to HeLa S3 cells in the presence and absence of Ca^{2+} and performed western blot analysis. We observed that the phosphorylation of AMPK and ACC in the HeLa S3 cells was induced by nsPEFs in the presence of 1 mM Ca^{2+} but considerably abrogated in Ca^{2+}-free media (Fig. 4A). When nsPEFs were applied to the Jurkat cells, in which LKB1 plays a major role in AMPK activation in a Ca^{2+}-independent manner, the phosphorylation of AMPK and ACC was induced in the presence or absence of Ca^{2+} (Fig. 4B). These observations indicate that CaMKK-mediated AMPK activation in HeLa S3 cells requires the presence of extracellular Ca^{2+}, which further suggests that the influx of Ca^{2+} is induced by nsPEFs and directly affects the intracellular signal pathway involving CaMKK and AMPK.

4. Discussion

In this study, we demonstrated rapid AMPK activation by nsPEFs and the differential contributions of LKB1 and CaMKK to this process in relation to extracellular Ca^{2+}. In Jurkat cells, which express both LKB1 and CaMKK, the CaMKK inhibitor and Ca^{2+} media had marginal effects on nsPEF-induced AMPK activation, which

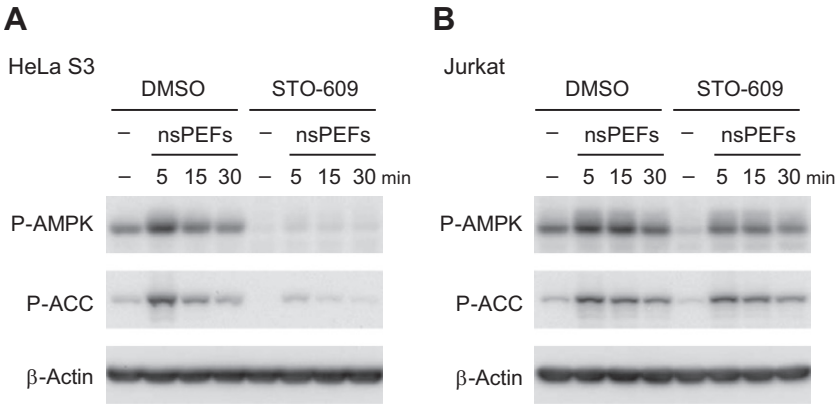

Fig. 3. AMPK activation by nsPEFs is suppressed by the CaMKK inhibitor STO-609 in HeLa S3 cells, but not in Jurkat cells. (A) Phosphorylation of AMPK and ACC in HeLa S3 cells treated with STO-609. HeLa S3 cells were pretreated with 20 μM STO-609 at 37 °C for 30 min, exposed to 20 shots of 20 kV/cm nsPEFs, and incubated at 37 °C for the indicated times in the presence of 20 μM STO-609. As a negative control, the cells were treated with DMSO, exposed to nsPEFs, and incubated at 37 °C for the indicated times in the presence of DMSO. Phosphorylation of AMPK and ACC was analyzed by western blotting as described in Fig. 1B. (B) Phosphorylation of AMPK and ACC in Jurkat cells treated with STO-609. Jurkat cells were pretreated with 20 μM STO-609 or DMSO, exposed to 10 shots of 20 kV/cm nsPEFs, and incubated at 37 °C for the indicated times in the presence of either 20 μM STO-609 or DMSO. Phosphorylation of AMPK and ACC was analyzed by western blotting as described in Fig. 1B.

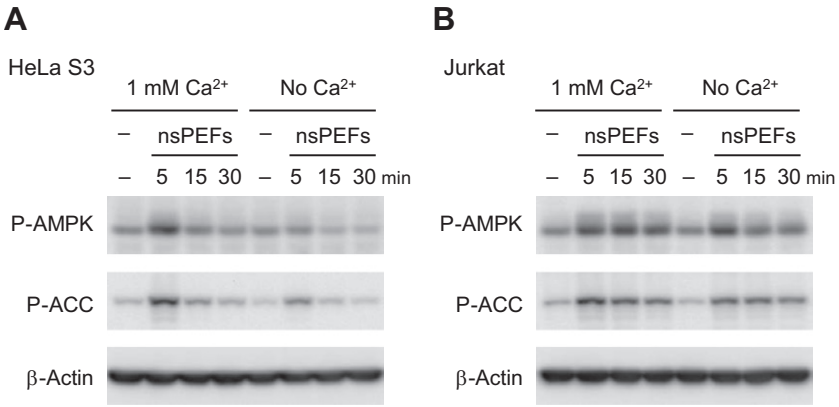

Fig. 4. Presence of extracellular Ca^{2+} is essential for nsPEF-induced AMPK activation in HeLa S3 cells, but not in Jurkat cells. (A) AMPK activation in HeLa S3 cells in the presence and absence of extracellular Ca^{2+}. HeLa S3 cells in a culture medium either with or without 1 mM Ca^{2+} were exposed to 20 shots of 20 kV/cm nsPEFs and incubated at 37 °C for the indicated times. Phosphorylation of AMPK and ACC was analyzed by western blotting as described in Fig. 1B. (B) AMPK activation in Jurkat cells in the presence and absence of extracellular Ca^{2+}. Jurkat cells in a culture medium either with or without 1 mM Ca^{2+} were exposed to 10 shots of 20 kV/cm nsPEFs and processed as described in (A).

indicated the predominant role of LKB1 in AMPK activation in Jurkat cells. In HeLa S3 cells, which lack functional LKB1, nsPEF-induced AMPK activation was mediated by CaMKK and required extracellular Ca^{2+}. Because elevated levels of intracellular Ca^{2+} are prerequisite for CaMKK-mediated AMPK activation, our observation raises a question of how extracellular Ca^{2+} participates in the nsPEF-induced, CaMKK-mediated AMPK phosphorylation.

Many previous studies have shown that nsPEF exposure causes increased levels of cytoplasmic Ca^{2+} [13–16]. In the presence of extracellular Ca^{2+}, the exposure of cells to nsPEFs provokes a relatively persistent elevation of the cytoplasmic Ca^{2+} [14]. In Ca^{2+}-free media, nsPEFs elicit a transient increase in the cytoplasmic Ca^{2+}, which probably means the release of Ca^{2+} from internal Ca^{2+} stores, most likely from the ER [14,16]. Addition of Ca^{2+} to the Ca^{2+}-free media after nsPEF exposure restores the duration and magnitude of the nsPEF-induced Ca^{2+} elevation [14], which clearly indicates the occurrence of Ca^{2+} influx after nsPEF exposure. Previous studies suggested that multiple mechanisms account for the nsPEF-induced Ca^{2+} mobilization. One of these mechanisms is the formation of nanopores in the cell membrane. Nanopores are relatively stable [10,11] and allow ions and low-molecular-weight compounds such as YO-PRO-1, which is much larger than Ca^{2+}, to permeate the cell membrane [8,9,12]. In addition to the cell membrane, nsPEFs can directly act on intracellular Ca^{2+} stores such as the ER [13,14,16]. The release of Ca^{2+} from intracellular stores may drive store-operated Ca^{2+} channels and may allow Ca^{2+} influx to occur through the cell membrane. Furthermore, in excitable cells such as chromaffin cells, voltage-gated Ca^{2+} channels in the cell membrane have been reported to participate in the Ca^{2+} influx induced by nsPEFs [15]. Currently, nsPEF-induced Ca^{2+} mobilization seems to reflect the cumulative effect of these multiple nsPEF actions on cells. Because a release of Ca^{2+} from the ER in the cytoplasm is transient due to the limited amount of Ca^{2+} storage in the ER, Ca^{2+}-dependent intracellular events generally require Ca^{2+} influx through the cell membrane [33]. Our observations of the requirement of extracellular Ca^{2+} in HeLa S3 cells indicate the occurrence of Ca^{2+} influx ensures the CaMKK-mediated AMPK activation by nsPEFs. Although nsPEF-induced Ca^{2+} mobilization has been well documented and is believed to be important for nsPEF-induced biological responses, specific signaling events provoked in response to Ca^{2+} mobilization by nsPEFs have not been identified. Our data obtained in this study provide an important clue for understanding the molecular basis for Ca^{2+}-dependent intracellular reactions. In addition, the biochemical approach employed in this study may be useful for

analyzing other nsPEF-induced intracellular events that are associated with Ca^{2+} mobilization.

Finally, the current study raises an additional important question of why AMPK is activated after nsPEF exposure. The most plausible explanation is that nsPEFs disturb the energy status of a cell. AMPK functions as a sensor for cellular energy levels and plays critical roles in the coordinated control of multiple cellular processes that are responsible for energy homeostasis. Low cellular energy status is reflected by an increased ratio of AMP to ATP [18], and AMP directly binds to AMPK and induces allosteric changes in the AMPK protein, which are essential for AMPK phosphorylation and its catalytic activity [19,20]. Thus, nsPEF-induced AMPK activation implies low energy status in nsPEF-exposed cells. In accordance with this hypothesis, the previous study demonstrated reduced ATP levels after nsPEF exposure [7]. Of note, mitochondria have been proposed to be the site of nsPEF actions, because nsPEFs permeabilize the mitochondrial membrane [34] and decrease the mitochondrial membrane potential [7]. These observations are consistent with the possible nsPEF-induced disturbance in the energy status, although further detailed analysis is required to test this hypothesis. Because AMPK is involved in multiple cellular processes, elucidation of the molecular mechanism underlying nsPEF-induced AMPK activation will provide a foundation for understanding the actions of nsPEFs on human cells.

Acknowledgments

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