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Stimulation of Capacitative Calcium Entry in HL-60 Cells by Nanosecond Pulsed Electric Fields*

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Nanosecond pulsed electric fields (nsPEFs) are hypothesized to affect intracellular structures in living cells providing a new means to modulate cell signal transduction mechanisms. The effects of nsPEFs on the release of internal calcium and activation of calcium influx in HL-60 cells were investigated by using real time fluorescent microscopy with Frua-2 and fluorometry with Fura-2. nsPEFs induced an increase in intracellular calcium levels that was seen in all cells. With pulses of 60 ns duration and electric fields between 4 and 15 kV/cm, intracellular calcium increased 200–700 nM, respectively, above basal levels (~100 nM), while the uptake of propidium iodide was absent. This suggests that increases in intracellular calcium were not because of plasma membrane electroporation. nsPEF and the purinergic agonist UTP induced calcium mobilization in the presence and absence of extracellular calcium with similar kinetics and appeared to target the same inositol 1,4,5-trisphosphate- and thapsigargin-sensitive calcium pools in the endoplasmic reticulum. For cells exposed to either nsPEF or UTP in the absence of extracellular calcium, there was an electric field-dependent or UTP dose-dependent increase in capacitative calcium entry when calcium was added to the extracellular media. These findings suggest that nsPEFs, like ligand-mediated responses, release calcium from similar internal calcium pools and thus activate plasma membrane calcium influx channels or capacitative calcium entry.

The release of internally stored calcium in mammalian cells can stimulate responses to agonists, activate growth, and initiate release of key factors in the apoptosis pathway (1). This calcium mobilization also triggers the influx of calcium from the external medium into the cell as a means of further propagating calcium signals and to replenish depleted pools of calcium and is termed capacitative calcium entry. The continuing effort to manipulate cell signaling pathways for therapeutic benefit has led to the exploration of electric field effects on cells (2–5). Current electric field applications include electroporation of the plasma membrane for introduction of drugs, genes, or other macromolecules into cells (6–12). The pulse lengths for this kind of electric field treatment are in the range of 0.1 to 20 ms. With advances in pulsed power technology, it has been proposed that pulsed electric fields of ultrashort duration (<1 µs) may manipulate specific intracellular functions (13, 14). Current modeling of how these pulsed electric fields affect cells depict a cell as an excitable circuit (15). In this model, the electric fields administered to a cell charge the plasma and internal membranes which act as dielectric layers, and between these the cytoplasm acts as a conductive medium. With these ultrashort pulses the plasma membrane is not exposed to an electric field of sufficient duration to charge it to such a voltage that significant electroporation occurs (15). Based on molecular modeling of nanosecond duration pulsed electric fields (nsPEFs) the effects seen should be largely targeted on internal organelle membranes. To study calcium signaling, applied electric fields could be used to manipulate the movement of ions, such as calcium, by interaction with intracellular membranes. Therefore, because of the increasing interest in electric field effects on biological systems, nsPEFs were used to provoke calcium mobilization in mammalian cells.

Previous studies showed that nsPEF treatment increased apoptosis signaling mechanisms in both Jurkat and HL-60 cells through assays for caspase activation, annexin binding, and cytochrome c localization (16). Other studies using electric fields weaker than those that induce apoptosis have been shown to cause a proliferative effect on cells. Therefore, with these pulses it may be possible, depending on the duration and intensity of the electric field applied, to induce varied cell signaling mechanisms resulting in non-apoptotic cellular responses in the same cell type.

In order to enact such responses to nsPEF treatment, the cell may use physical and/or chemical signals to initiate signal transduction cascades within the cell. Calcium signaling is ubiquitous in all cell types and plays a key role in several cellular events (17). Because many cellular functions are dependent on calcium signaling to achieve an effect, the response of internal calcium levels to nsPEF stimulation was examined. To compare nsPEF-induced effects on intracellular free calcium ([Ca²⁺]), we used the purinergic agonist UTP (18–21), which has been shown previously to induce calcium fluxes and

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1 The abbreviations used are: nsPEF, nanosecond pulsed electric field; PI, propidium iodide; IP₃, inositol 1,4,5-trisphosphate; CCE, capacitative calcium entry; [Ca²⁺], intracellular free calcium; PLC, phospholipase C; ER, endoplasmic reticulum; TRP, transient receptor potential; TRPC, TRP canonical; TRPV, TRP (vanilloid); TRPM, TRP melastatin.


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capacitative calcium entry in HL-60 cells. These agonists induce calcium signaling by binding to a specific purinergic receptor of the P2Y<sub>2</sub> (previously called P<sub>A1U</sub>) subtype (18, 19). P2Y<sub>2</sub> receptors were shown previously to be present in HL-60 cells (22). This receptor signaling involves G-protein activation of phospholipase C (PLC) with a subsequent increase in the second messenger inositol 1,4,5-trisphosphate (IP<sub>3</sub>) (for review see Ref. 17). IP<sub>3</sub> then initiates the release of calcium from IP<sub>3</sub>-sensitive calcium stores into the cytoplasm. This increase in calcium stimulates the opening of store-operated channels in the plasma membrane allowing influx of calcium into the cell for replenishment of the internal stores. This influx is believed to be via a CCE mechanism. The precise molecular nature of the CCE pathway in many cells is unknown. However, there is much evidence suggesting that members of the transient receptor potential (TRP) family of proteins may act as the plasma membrane calcium channel. The TRP proteins are classified as TRPC (canonical), TRPV (vanilloid), and TRPM (melastatin) (23). The transmembrane architecture of the TRPs is similar to voltage-gated and cyclic nucleotide-gated channels. HL-60 cells are positive for TRPC1, TRPC3, TRPV1, TRPV2, TRPV5, TRPV6, and TRPM2 (24). From this collection of channel proteins, TRPC1, TRPC3, TRPC4, and TRPV6 have been shown to participate in CCE (25). Therefore, HL-60 cells possess several candidates that could act as CCE channels.

How the internal calcium store depletion communicates to the CCE process in the plasma membrane is still being studied. Two main theories exist for this coupling as follows: the first suggests physical interaction between the IP<sub>3</sub> receptor in the ER and the CCE channel, and the second suggests the existence of a secondary messenger molecule or calcium-inducing factor (26, 27).

Preliminary reports showing that nsPEF stimulation of HL-60 cells increased [Ca<sup>2+</sup>]<sub>i</sub> were presented at ElectroMed (28) and SPRBM (29) conferences. The purpose of this study was to investigate the mechanism by which nsPEF increased [Ca<sup>2+</sup>]<sub>i</sub>. The data show that nsPEF can induce calcium transients similar to those seen following purinergic receptor stimulation and thapsigargin. This response was observed in the absence of classical plasma membrane electroporation. Previous studies in HeLa cells showed that classical electroporation pulses (100 μs, 500 V/cm) also stimulated calcium responses similar to those seen with hormones (30). The results presented here further support the hypothesis that when nsPEFs are used below the threshold for plasma membrane electroporation and apoptosis induction are applied, intracellular signal transduction cascades can be triggered, resulting in signaling events that are common with normal ligands. The application of nsPEFs to cells and tissues provides a new tool to investigate signal transduction mechanisms, including calcium signaling, by modulating intracellular release, and capacitative calcium entry through receptors in the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Non-transformed HL-60 cells were used for this study and were obtained from American Type Culture Collection (ATCC, Manassas, VA). They were cultured in 75-cm<sup>2</sup> flasks in phenol red HFM1 1640 medium (Medimatch Cellgro) supplemented with 20% fetal bovine serum (Atlanta Biologicals, Norcross, GA); 1% l-glutamine, and 1% penicillin/streptomycin (Mediatech Cellgro) and incubated at 37 °C with 5% CO<sub>2</sub>. HL-60 cells in log-phase were removed from the culture and resuspended in a physiological buffer containing 145 mM NaCl, 5 mM KCl, 0.4 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM MgSO<sub>4</sub>/TH<sub>2</sub>O, 6 mM glucose, 5 mM HEPES (pH 7.4) prior to experimentation. 1.5 mM CaCl<sub>2</sub> was added to this buffer when loading cells with fluorescent indicators unless otherwise stated. In some experiments extracellular calcium was omitted by washing the cells at least once in the same physiological buffer without added calcium. The addition of 1.5 mM EGTA prior to nsPEF treatment was made in the buffer that was measured by atomic absorption spectrophotometry and was found to be 1.5 ± 0.05 mM in the presence of calcium and 3.08 ± 0.35 μM in buffer without added calcium (n = 3).

**Administration of nsPEF—**Cell suspension (7.7 × 10<sup>6</sup> cells/ml) was added into the Bio-Rad gene pulser cuvettes prior to nsPEF treatment. nsPEF was delivered by means of a cable pulse generator to a cuvette with two parallel plate electrodes, separated by 0.4 cm, containing cell suspensions. The electric fields delivered were 4, 6.5, 10, and 15 kV/cm. The generator consisted of a 10-ohm pulse-forming network (five 5-μm cables in parallel) and a spark gap in atmospheric air into a nanosecond closing switch (16). Post-pulse, the cell suspension was removed from the nsPEF cuvette and assayed. Cell viability was >95% indicating that these nsPEF conditions were below the threshold for apoptosis or necrosis.

**Microscopic Analysis of Internal Calcium Response**—An Olympus photomicroscope with a Kodak DC-120 digital camera was used with the fluorescent indicator Fluo-3 (Molecular Probes, Eugene, OR) to assess changes in intracellular free calcium. HL-60 cells were suspended in the above-described physiological buffer containing 2 mM calcium. Fluo-3/AM (2 μM) was added to the cells, and the cells were then incubated at 37 °C for 45 min. The cells were then washed and resuspended in the physiological buffer with or without 2 mM calcium. An aliquot of HL-60 cell suspension was loaded into a space between metal electrodes affixed to a slide for real time monitoring of calcium transients. A description of this nsPEF apparatus can be found in the article by Deng et al. (31). The Merlon software (PerkinElmer Life Sciences) was used to detect changes in gray scale of the selected cell areas, and we compared these changes to a background region.

**Assessment of Membrane Integrity**—Propidium iodide (PI) (Molecular Probes, Eugene, OR), at a final concentration of 10 μg/ml, was added to HL-60 cell suspension (7.7 × 10<sup>6</sup> cells/ml) in a 0.4-cm BioSmith cuvette. For each of the four electric field settings, the cells were given two sets of 50-ns pulses. Immediately following nsPEF treatment, cells were then removed from the cuvettes and transferred to flow cytometry vials for analysis (CellQuest software) on a FACS Calibur flow cytometer (BD Biosciences). As a positive control, 0.1% Triton was added to the cells to induce membrane disruption in the presence of PI. The cell suspensions were analyzed within 2 min of treatment.

**Fluorometric Analysis of Internal Calcium Response**—To analyze the response of a greater number of cells, the fluorescent indicator Fura-2 (Molecular Probes, Eugene, OR) was used with a fluorometer. HL-60 cells were incubated with Fura-2/AM (2 μM) in the physiological buffer described above containing 2 mM calcium for 45 min at 37 °C. The cells were then washed and resuspended in calcium-free or calcium-containing buffer at a concentration of 7.7 × 10<sup>6</sup> cells/ml. Calcium measurements were performed in a SPEX ARCM spectrofluorometer, similar to that described previously (32). HL-60 cells were first placed in the fluorometer cuvette, to obtain a base-line reading, and cells were then removed from the fluorometer cuvette with a Pasteur pipette and added to the BioSmith cuvette. Cells were treated with nsPEF (13) and then immediately removed from the BioSmith cuvette and added back to the fluorometer cuvette (located in close proximity to the pulse generator, which took between 5 and 10 s), and the fluorescence measurements were continued. For those experiments that included the addition of UTP, an aliquot of UTP was pipetted into the BioSmith cuvette and mixed by pipetting up and down gently. For Fig. 3, A and B, BioSmith electroporation cuvettes were used to perform calcium measurements in the fluorometer by using a fiber optic light guide. The cells were excited at 340 and 380 nm through the clear side of the cuvette between the aluminum plates. The light emitted from the top of the cuvette was transmitted to the photomultiplier by using a fiber optic light guide. HL-60 cells were placed in the cuvette to which 1.5 mM EGTA was added and mixed into the cell suspension, and a base-line fluorescence reading was acquired. The cuvette was then removed from the fluorometer and treated with nsPEF (13) and then immediately replaced into the fluorometer (located in close proximity to the pulse generator, which took between 5 and 10 s), and the fluorescence measurements were continued. Although this method produced reliable results, the fluorescence signals contained noise. This was reduced significantly by removing the cell suspension from the BioSmith cuvette after treatment and transferring it to the fluorometer cuvette. Figs. 1 and 4–8 were prepared by using this method.
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RESULTS

Single Cell Analysis of the Effect of nsPEF on Intracellular Free Calcium—In order to assess how nsPEF stimulation affects internal events in cells, real time microscopic analysis of cells with the fluorescent calcium indicator Fluo-3 was utilized. HL-60 cell suspension was loaded into the nsPEF slide (31), and a group of cells in the field of vision between the electrodes was chosen to analyze. Typically 10–15 cells could be viewed at one time, and their response to nsPEF was recorded. All cells that were present in the field of view of the microscope were analyzed for changes in internal calcium. In Fig. 1, each cell in the field of view of the microscope is represented as an individual line, and the average response was presented as a heavy line. The fluorescence of each cell was monitored over the time of the experiment and was compared with background. Following the nsPEF pulse (Fig. 1, black arrow), all of the cells responded with an increase in [Ca\(^{2+}\)]. This coordinated increase in [Ca\(^{2+}\)] was not seen in untreated cells. This increase in [Ca\(^{2+}\)], following nsPEF could result from intracellular calcium mobilization followed by CCE.

nsPEF Does Not Induce Electroporation of the Plasma Membrane at Low Electric Fields—From Fig. 1 a rise in intracellular calcium is seen when cells are treated with nsPEF. The next step was to determine whether this increase was because of influx from the extracellular media or from internal store release. Our hypothesis states that nsPEF should affect internal membranes; therefore, the increase in [Ca\(^{2+}\)], because of nsPEF stimulation would result from internal store release. To test this theory, evidence of plasma membrane electroporation at the electric fields used in this study was investigated. Classical electroporation experiments applied electric fields of sufficient duration to induce dielectric breakdown in the plasma membrane (33, 34). To assess plasma membrane integrity under our experimental conditions, the indicator PI was used with flow cytometry. The data revealed no increase in PI uptake when compared with control (control averaged 1.5 ± 0.5% and the highest electric field used for treatment averaged 0.9 ± 0.3% fluorescence gated for PI). In all subsequent experiments in this study, cells were treated within this electric field range to ensure plasma membrane integrity.

Effect of UTP on HL-60 Cells in the Presence and Absence of Extracellular Calcium—Because evidence of plasma membrane electroporation was not found, the increase in [Ca\(^{2+}\)], due to nsPEF stimulation, in the absence of extracellular calcium, is likely to be coming from the release of internal stores. A traditional agonist, UTP, which induces internal store release, was used as a stimulus, and the response of the cell was compared with the response to nsPEF. Fig. 2 shows a representative experiment of HL-60 cells that were treated with 10 \(\mu\)M UTP in the presence of 1.5 mM calcium and in the absence of calcium, by chelation with 1.5 mM EGTA. If an increase in calcium is seen in the absence of extracellular calcium, then the source of the increase must be internal stores. The 1.5 mM EGTA, however, caused an unexpected response of “draining” the cell of internal calcium. When EGTA was added to HL-60 cell suspension, the calcium concentration, as measured with Fura-2, began to decrease shortly after addition as seen in Fig. 2. This is because of the EGTA chelating the extracellular calcium and then creating an unfavorable calcium sink that causes the cell to extrude its internal calcium stores into the extracellular media. Therefore, HL-60 cells are unable to maintain their internal calcium stores for very long (~100 s) when placed in a calcium-deficient environment. A dose-response experiment of EGTA (1.0, 1.5, 2.0, and 3.0 mM) to HL-60 cells allowed us to choose an amount of EGTA that chelates most of the extracellular calcium but does not cause a draining of internal stores as rapidly as higher concentrations (data not shown). In order to create an environment void of extracellular calcium and still see an increase in calcium because of the addition of UTP or nsPEF, the stimulus had to be given quickly after the EGTA-induced calcium decrease was seen. The initial increase in calcium to UTP in the absence of calcium (presence of EGTA) was similar to that seen in the presence of extracellular calcium; however, the response was considerably more transient. The [Ca\(^{2+}\)], decreased to basal values at 50 s and to ~10 ns after 150 s when extracellular calcium was low. According to our measurements, the concentration of extracellular calcium in the calcium-deficient media was 3 mM; therefore, in an environment where there exists a gradient of calcium,
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Fig. 3. Fiber optic light guide allows “capture” of HL-60 response to nsPEF stimulation in the presence of equimolar amounts of EGTA to calcium. HL-60 cells were treated with 10 μM UTP (A) and nsPEF (B) in the presence 1.5 mM of calcium. 1.5 mM EGTA was added to the cuvette prior to stimulation to chelate calcium in those experimental traces marked as such. In the presence of EGTA, a response to UTP and to nsPEF is still seen representing calcium release from internal stores. Traces are a representative experiment (n = 4) and are the average of four replicates within one experiment.

higher in the extracellular medium, calcium entry into the cell was still not occurring after stimulation, rather efflux was evident. In Fig. 2 HL-60 cells were incubated with 1.5 mM EGTA until the [Ca^{2+}]_{i} began to fall below basal levels, such that by 200 s the [Ca^{2+}]_{i} was ~50% of that seen at zero time. The cells then were stimulated with 10 μM UTP at 200 s, and a modest yet transient response of about 50 nM was still seen, confirming that the increase in calcium was because of internal store release.

**Fiber Optic Light Guide Measurement in Conjunction with EGTA Shows That the Increase in Internal Calcium Was Not Because of Influx from the Extracellular Media**—In order to capture the rapid response of intracellular calcium to UTP and nsPEF in the absence of extracellular calcium, fiber optic light guides were used to monitor fluorescence in the BioSmith cuvettes after stimulation. Fig. 3A shows the effect of 10 μM UTP on [Ca^{2+}]_{i}. Each trace represents the mean of four replicates, and Fig. 3A represents 1 of 4 separate experiments performed on different days. Consistent with what we observed previously, UTP produced a rapid and prolonged increase in [Ca^{2+}]_{i}, when calcium was present in the medium. When 1.5 mM EGTA was added at zero time, there was an immediate and gradual decline in [Ca^{2+}]_{i}, consistent with previous results (Fig. 2). The addition of UTP at 15 s to cells treated with EGTA at zero time induced a rapid and transient rise in [Ca^{2+}]_{i}, such that by 90 s post-treatment the [Ca^{2+}]_{i} was back to the pre-stimulated base line. These experiments show that in HL-60 cells there is a very small window of time (about 1 min) in which to observe purinergic stimulation of [Ca^{2+}]_{i}, when the extracellular calcium was chelated with EGTA. The data in Fig. 3B shows the effect of a 60-ns, 15 kV/cm pulse on [Ca^{2+}]_{i}, in similar calcium-deficient conditions. When calcium was present in the extracellular medium, there was a rapid and sustained increase in [Ca^{2+}]_{i}. When EGTA was added to the medium, there was an immediate and steady decline in [Ca^{2+}]_{i}, similar to that seen in Fig. 3A. When cells were pulsed at 15 kV/cm in the presence of 1.5 mM EGTA, there was an immediate increase in [Ca^{2+}]_{i}, that began to decrease after ~10 s, and after ~60 s the [Ca^{2+}]_{i} was back to base line and continued to decline below base line. This difference in peak heights between the presence of calcium and when EGTA was present was not significant. These data show that nsPEF treatment produces a rapid mobilization of intracellular calcium similar to that observed with UTP (Fig. 3A). This method of acquiring fluorescent signals by using a light guide resulted in reliable data; however, it also generated much noise. To reduce this noise level, we transferred the HL-60 cell suspension from the BioSmith cuvette to a fluorometry cuvette after treatment, and we continued to measure fluorescence readings. Because this transition took longer than 20 s when we used cell suspension with added EGTA, the calcium response was often missed. Therefore, we continued our experiments in media that contained no added calcium so that the calcium concentration in the media was nominal. This allowed us to capture the increase in internal calcium released from internal stores in response to UTP or nsPEF treatment. Also the data were represented as a 340/380 ratio rather than nM concentrations because mixing of reagents for Fura-2 calibration was difficult with the fiber optic light guide and BioSmith cuvettes.

**Effect of Single nsPEF Pulses and Traditional Agonists on HL-60 Cells in Calcium-deficient Media**—The amount of calcium released from internal stores and that which entered from the extracellular media was evaluated using the fluorescent indicator Fura-2, which permits [Ca^{2+}]_{i} to be quantified. These measurements of the response to nsPEF and other agonists could then be compared. The data in Fig. 4A show a typical result when cells were pulsed once at 15 kV/cm for 60 ns. In the presence of extracellular calcium, there was a rapid and sustained increase in [Ca^{2+}]_{i}, of ~600 nM. In the absence of extracellular calcium, the basal [Ca^{2+}]_{i}, was lower than when extracellular calcium was present, suggesting that HL-60 cells are not able to completely maintain internal calcium levels in calcium-deficient environments. One 15 kV/cm pulse for 60 ns in the absence of extracellular calcium gradually increased [Ca^{2+}]_{i}, by ~140 nM, which then slowly declined after ~10 s. Because this result is similar to agonist-induced increases in [Ca^{2+}]_{i}, in many different cell types, HL-60 cells were challenged with a known agonist that increased [Ca^{2+}]_{i}, UTP was chosen because this has been used previously to elevate [Ca^{2+}]_{i}, in HL-60 cells (33). The data in Fig. 4B shows that 10 μM UTP increased [Ca^{2+}]_{i}, by ~300 nM when extracellular calcium was present. This agonist, at 10 μM, produced a rapid and sustained increase in [Ca^{2+}]_{i}, that was of a similar magnitude and duration to that observed with the one 60-ns 10 kV/cm pulse. A 100
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Evidence of Capacitative Calcium Entry Activation in HL-60 Cells—In order to evaluate the activity of CCE, HL-60 cells were treated with nsPEF in calcium-deficient media, and the response of the cells to calcium re-introduction was monitored. The data in Fig. 5A shows that when cells were pulsed for 60 ns with an amplitude of 6.4 or 15 kV/cm in calcium-deficient medium, there was a small and transient increase in [Ca\(^{2+}\)], consistent with the data shown in Fig. 4A. The 15 kV/cm pulse produced a larger increase in [Ca\(^{2+}\)], than that seen with the 6.4 kV/cm pulse. When calcium was added to the cells that had been pulsed, there was a rapid rise in [Ca\(^{2+}\)], consistent with calcium influx channels being activated. The increase in [Ca\(^{2+}\)] was dependent on the electric field intensity of the pulse because the 15 kV/cm pulse produced a larger increase in [Ca\(^{2+}\)], than the 6.4 kV/cm pulse (see Fig. 6 for more complete electric field-dependent cell responses), and these increases in [Ca\(^{2+}\)] were greater than the increase in [Ca\(^{2+}\)], seen in control (untreated) cells. The data in Fig. 5B show that the effect of UTP on [Ca\(^{2+}\)], is small and transient in low calcium-containing buffer, consistent with the data shown in Fig. 4B. The addition of 2 mM calcium to these UTP-treated cells produced a rapid rise in [Ca\(^{2+}\)], that was comparable with that observed when cells were treated with UTP in calcium-containing media (Fig. 4B). Because UTP has been shown previously to promote capacitative calcium entry in HL-60 cells, the data shown in Fig. 5B was entirely consistent with this process. Fig. 5C represents the response of HL-60 cells to 0.1 μM thapsigargin incubated in calcium-containing medium and calcium-deficient medium. This figure also illustrates capacitative calcium entry response when calcium (1.5 mM) is re-introduced to thapsigargin-treated cells in a calcium-deficient environment. The results in Fig. 5A show that nsPEF treatment of HL-60 cells promotes a stimulation of calcium entry similar to that seen following UTP and thapsigargin stimulation implying that nsPEF treatment was also promoting capacitative calcium entry.

Dose-dependent Increase in [Ca\(^{2+}\)], and CCE to nsPEF—In order to evaluate the electric field dependence of increasing [Ca\(^{2+}\)], and CCE with nsPEF, HL-60 cells were treated with nsPEF at varying electric fields. The data in Fig. 6 show the effect of one 60-ns pulse at various electric field settings in the presence and absence of extracellular calcium. This figure also shows the nsPEF effect on [Ca\(^{2+}\)], of adding extracellular calcium to the media after stimulation as a measure of CCE (see

Fig. 4. Effect of one 60-ns pulse at 15 kV/cm, 10 μM UTP, and 0.1 μM thapsigargin on [Ca\(^{2+}\)], in Fura-2-loaded HL-60 cells incubated with and without extracellular calcium. HL-60 cells (incubated with and without extracellular calcium) were placed in the fluorometer cuvette to obtain a baseline [Ca\(^{2+}\)], value and then were moved into the BioSmith cuvette. The cells were pulsed for 60 ns at 15 kV/cm and then moved back into the fluorometer cuvette, and [Ca\(^{2+}\)], was again measured (A). The effect of 10 μM UTP on [Ca\(^{2+}\)], was measured in HL-60 cells in the presence and absence of extracellular calcium (B). Shown in B is a dose-response curve of UTP to increase [Ca\(^{2+}\)], in HL-60 cells in the presence of extracellular calcium. Various concentrations of UTP were added to Fura-2-loaded cells, and at 15 s the [Ca\(^{2+}\)], was measured. The effect of 0.1 μM thapsigargin on [Ca\(^{2+}\)], was measured in HL-60 cells in the presence and absence of extracellular calcium (C). Representative traces of at least three experiments are shown.
Fig. 5. Capacitative calcium entry into HL-60 cells treated with one 60-ns pulse each of 6.4 and 15 kV/cm and cells treated with UTP. HL-60 cells were incubated in the absence of extracellular calcium. Cells, incubated in calcium-deficient media, were treated with one 60-ns pulse at 6.4 and 15 kV/cm. After [Ca^{2+}], had returned to near basal values, 2.0 mM calcium was added to the cells (A). Untreated cells acted as a control and show that some basal calcium influx occurred in this calcium-deficient situation. Cells were treated with 10 \mu M UTP, and when [Ca^{2+}], had returned to basal values 2 mM calcium was added (B). The increase in [Ca^{2+}], was similar to that observed when cells were given UTP in the presence of extracellular calcium (Fig. 4B). 0.1 \mu M thapsigargin was also used for comparison to show capacitative calcium influx (C). Representative traces of at least three experiments are shown.

Fig. 6. Effect of one 60-ns pulse each of 4.0, 6.4, 10.0, and 15.0 kV/cm on [Ca^{2+}], in HL-60 cells. The three conditions shown are as follows: \( \Box \), when 1.5 mM extracellular calcium was present; \( \bigcirc \), in the absence of extracellular calcium; \( \square \), when 2 mM calcium was added to cells after stimulation in the absence of extracellular calcium (see Fig. 4A for representative experiment). This represents the entry through the CCE. Data represent the mean \( \pm \) S.E. from three separate experiments.

Fig. 5A for typical experiment). With calcium present in the extracellular medium, nsPEF induced an electric field-dependent increase in [Ca^{2+}],. At 15 kV/cm, the effect on [Ca^{2+}], was \(~800 \) nM with one pulse. Because of the high voltage equipment necessary to generate our nsPEF pulses, an increase in electric fields above 15 kV/cm with the 0.4-cm cuvettes risked mechanical breakdown. To counter this, multiple pulses at the 15 kV/cm electric field were administered in attempt to achieve a saturation effect with nsPEF comparable with that seen with 100 \mu M UTP. Increasing the number of pulses to 3, 5, and 10, separated by approximately 1-s intervals, did not produce any larger effect on [Ca^{2+}], than a single 15 kV/cm pulse. However, at lower electric fields increasing the pulse number caused additional increases in intracellular calcium, but not above levels induced by 15 kV/cm (data not shown). An effect of nsPEF on intracellular mobilization was observed (Fig. 6, solid circles). However, the maximum effect on [Ca^{2+}], was \(~50 \) nM, and this effect was much less than that observed when calcium was present in the medium. However, when calcium was added back to the media of these stimulated cells (Fig. 6, open circles), there was an electric field-dependent rapid rise in [Ca^{2+}], that approached values similar to those seen when calcium was present during stimulation (Fig. 6, open squares). Now that it has been determined that HL-60 cells respond to nsPEF in an electric field-dependent manner, it is important to assess whether or not the nsPEF treatment was depleting the same intracellular stores as UTP.

Effect on [Ca^{2+}], in HL-60 Cells Following Sequential Stimulation with UTP and nsPEF—The data in Fig. 7 show the pulse-dependent nature of the increase in [Ca^{2+}],, with the 15 kV/cm pulse producing an effect \( \sim 4 \) times larger than the effect seen with 4 kV/cm. The data in Fig. 6 indicated that the nsPEF treatment of HL-60 cells was promoting intracellular calcium mobilization and calcium influx via a capacitative influx mech-
anism similar to the effect of UTP, an agent that also promotes capacitative calcium influx. Therefore, it seemed logical that the increase in [Ca$^{2+}$], induced by UTP, would be influenced by prior nsPEF treatment if nsPEFs and UTP were sharing the same capacitative mechanism. When cells were pulsed with 15 kV/cm, the subsequent effect of UTP to increase [Ca$^{2+}$], was greatly reduced, and although there was a very rapid increase in [Ca$^{2+}$], the effect on [Ca$^{2+}$], was very transient. When cells were pulsed at 4 kV/cm, which produced a smaller increase in [Ca$^{2+}$], the subsequent UTP challenge produced a rapid increase in [Ca$^{2+}$], that was more prolonged in nature. This is similar to the effect seen without prior nsPEF treatment (Fig. 4B). Thus the effect of UTP to increase [Ca$^{2+}$], was inversely proportional to the electric field intensity of the pulse. These data suggest that nsPEF treatment and UTP target the same intracellular calcium pools.

In order to more firmly support this hypothesis, the responses of these same type of experiments in the presence of calcium were compared with those in media deficient of calcium. The data in Fig. 8A show the effect of one 15 kV/cm pulse on [Ca$^{2+}$], followed by stimulation with UTP, and the effect of UTP stimulation on [Ca$^{2+}$], followed by one 15 kV/cm pulse, when extracellular calcium was deficient. One 15 kV/cm pulse produced an increase of ~80 nM, which rapidly declined to a value just above the pre-stimulated level, and at this point (180 s) 10 μM UTP was added. The effect of UTP to increase [Ca$^{2+}$], was much smaller (peak effect reduced by ~66 ± 12% S.E., n = 3) than that observed when UTP was added alone. Thus one pulse appeared to partially deplete the same pool of intracellular calcium that UTP was mobilizing, and this pool was the ER, based on the known signaling pathway for UTP in HL-60 cells. The reverse protocol in which cells were challenged first with UTP and then pulsed was also employed. UTP produced a transient increase in [Ca$^{2+}$], consistent with UTP mobilizing calcium from the ER. Following this mobilization by UTP, cells were stimulated with one 15 kV/cm pulse. The increase in [Ca$^{2+}$], caused by the pulse was reduced by ~68 ± 6% S.E. (n = 3) that produced when cells were pulsed before UTP addition. This experiment was therefore consistent with the notion that nsPEFs and UTP were mobilizing calcium from the same intracellular pool, which is most likely the ER.

The data in Fig. 8B show the effect of one 15 kV/cm pulse on [Ca$^{2+}$], followed by stimulation with UTP and the effect of UTP stimulation on [Ca$^{2+}$], followed by one 15 kV/cm pulse, when extracellular calcium was present. In this experiment the elevation of [Ca$^{2+}$], induced by nsPEF treatment and UTP was because of both intracellular calcium mobilization (Fig. 8A) and calcium influx. The addition of UTP produced a rapid rise in [Ca$^{2+}$], that was much larger than when there was no extra-
cellular calcium present (Fig. 8A), and then it began to gradually decline. Cells were then pulsed at 15 kV/cm, which produced a rapid increase in \([\text{Ca}^{2+}]\), which was reduced by \(-71 \pm 9\%\) S.E. \((n = 3)\) when compared with the effect observed before UTP stimulation. When cells were initially pulsed with 15 kV/cm, a rapid rise in \([\text{Ca}^{2+}]\), was observed that was comparable with that observed with UTP stimulation by itself. Following this pulse, cells were stimulated with UTP. This effect of UTP was reduced by \(-69 \pm 6\%\) S.E. \((n = 3)\) when compared with the effect of UTP by itself. Therefore, the fact that each previous stimulus reduced the effect of the subsequent stimulus is consistent with the theory that the same signaling pathway was being targeted. This is likely because of release of calcium from the ER.

The data in Fig. 5A and Fig. 6 support the concept that 60-ns nsPEF treatment initially mobilized intracellular calcium that then induced CCE. An agent that is commonly used to investigate CCE is thapsigargin (36), which is a potent inhibitor of the sarco-ER \(\text{Ca}^{2+}\)-ATPases. Treatment of cells with thapsigargin promoted an emptying of the sarco-ER calcium stores with subsequent stimulation of CCE. The data in Fig. 9 show that thapsigargin produced a gradual and sustained increase in \([\text{Ca}^{2+}]\). If nsPEF treatment depletes the sarco-ER of calcium, then it would be expected that nsPEF treatment after thapsigargin treatment would show a reduced release of calcium when compared with nsPEF treatment alone. The data in Fig. 9 show that the effect of one 15 kV/cm pulse to increase \([\text{Ca}^{2+}]\), after thapsigargin treatment was reduced by \(-63 \pm 20\%\) S.E. \((n = 3)\). This result supports the notion that nsPEF treatment and thapsigargin are depleting the same calcium pool, which is believed to be the sarco-ER. The data in Fig. 9 also show the converse experiment in which cells were first pulsed at 15 kV/cm and then challenged with thapsigargin. The one 15 kV/cm pulse increased \([\text{Ca}^{2+}]\), to \(-400\) nM which then began to gradually decline. At \(-50\) s electrically pulsed cells were challenged with thapsigargin (Fig. 9, arrow); however, \([\text{Ca}^{2+}]\), did not increase and in fact still gradually declined, thus indicating that nsPEF treatment had depleted the thapsigargin-sensitive store of calcium by 100% \((n = 3)\), which is the ER.

**DISCUSSION**

Recently, investigations of the effects of ultrashort, high intensity pulsed electric fields or nsPEF on mammalian cells have demonstrated distinct differences on cell structure and function compared with classical plasma membrane electroporation. It was demonstrated previously that nsPEF invokes signal transduction mechanisms that initiate apoptosis cascades in several human cell lines (39) including HL-60 cells (14, 16). In the studies reported here, we show that at pulse durations and electric fields that are below the threshold for apoptosis and plasma membrane electroporation, nsPEF recruits signal transduction mechanisms that are similar to those utilized by natural ligands and chemical stimuli. Calcium mobilization induced by nsPEF, purinergic agonists, and thapsigargin exhibit similar kinetics and appear to utilize the same calcium channels present in intracellular and plasma membranes. Specifically, HL-60 cells exposed to nsPEF and UTP exhibited a rapid and transient increase in \([\text{Ca}^{2+}]\), in the absence of extracellular calcium and a rapid and more sustained increase in \([\text{Ca}^{2+}]\), in the presence of external calcium. Applications of nsPEF followed by UTP and UTP followed by nsPEF elicited less robust calcium mobilization compared with either stimulus alone, suggesting common sources for calcium mobilization. Based on UTP-stimulated calcium mobilization through PLC and IP3, the nsPEF-stimulated intracellular calcium channel is expected to be in the ER, and the plasma membrane channel is expected to be a capacitative calcium channel. These CCE channels are likely to be one or more of the TRP channels that are present in HL-60 cells (24). These observations indicate that HL-60 cells do in fact sense and respond to nsPEF in a fashion that, at least in part, mimics the response seen with naturally occurring stimuli.

Based on a simple electric model of the cell and from observations with human cells (13–16, 31, 39) as the pulse duration is decreased into the submicrosecond range (time domain), effects are less likely to occur on the plasma membrane and more likely to occur on subcellular membranes. Data reported here are consistent with the hypothesis that nsPEFs bypass the plasma membrane and exert effects primarily on intracellular structures. The absence of PI uptake by cells exposed to nsPEF strongly suggests that calcium does not enter the cells through pores formed by classical plasma membrane electroporation. It could be argued that plasma membrane pores smaller than PI are present, but increases in intracellular calcium were observed in cells in the absence of calcium-containing media and in the presence of EGTA, indicating the release of calcium from intracellular structures. Additional support that nsPEFs affect intracellular structures and functions is provided by the observations that they induced an electric field-dependent increase in capacitative calcium entry when calcium was added to cells in calcium-free media after exposure to the pulse. These findings provide additional evidence that nsPEFs alter intracellular structures without causing plasma membrane electroporation.

The increase in \([\text{Ca}^{2+}]\), in response to nsPEF demonstrates that this method of stimulation bypasses the plasma membrane and targets intracellular structures and functions. The mechanism(s) for HL-60 cell responses to nsPEF stimulation is still unknown, but the data presented suggest this intracellular electromanipulation does increase \([\text{Ca}^{2+}]\), reaching concentrations that are similar to a natural stimulus such as UTP. Furthermore, calcium elevations appear to reflect classical signaling kinetics. Based on the findings presented here, we pro-
pose several possible hypotheses that may explain nsPEF effects on intracellular calcium levels.

One possibility is that nsPEF could form transient pores in ER and/or mitochondrial membranes. This would explain the increase in internal calcium levels because calcium would leak out of these organelles down its concentration gradient into the cytoplasm. This theory is supported by the theoretical calculations of Gowrishankar and Weaver (37). The cell could interpret this intracellular calcium mobilization as a naturally occurring signal that then activates CCE in the plasma membrane. Propagation of this calcium signal could then be translated into calcium-mediated cell signaling events including increased gene expression through calcium-dependent transcription promoter mechanisms. The calcium signal could also increase protein translation events that are generally involved in regulation of signal transduction through post-translational modification of proteins. Although ER electroporation remains a possibility, the relatively slow kinetics of calcium mobilization induced by nsPEF and UTP, as opposed to a much more rapid electroporation-induced calcium mobilization, suggests that intracellular membrane electroporation events may not be triggered by nsPEF.

A second possibility is that the nsPEF pulse could be gating channels directly. Voltage-gated calcium channels could be likely candidates, but they have not been identified in HL-60 cells (38). Furthermore, verapamil and diltiazem, which inhibit voltage-gated calcium channels, had no effect on nsPEF-induced increases in \([\text{Ca}^{2+}]_i\) (data not shown). Based on the potential source of the intracellular calcium, IP$_3$ receptors present in internal membranes are more possible candidates. IP$_3$ receptors and/or other calcium channels could be triggered by electric field-induced conformational changes that cause the channels to open. Even though the pulse duration is so short and the electric fields are relatively low (below the threshold for apoptosis), effects on conformational changes in proteins are possible.

The third possibility is that the nsPEF pulse could mimic a ligand signal that could trigger receptors on internal membranes thus causing calcium to be released from the internal stores into the cytoplasm. If the cell interpreted nsPEF as a ligand-binding event, then releasing pooled internal calcium would generate the CCE events that were observed. Although effects on intracellular structures and functions are highly likely, additional effects may occur at the plasma membrane that are not measured. For example, nsPEF could trigger activation of the purinergic receptors or G-proteins in the plasma membrane. Alternatively, plasma membrane perturbations could activate PLC or otherwise trigger IP$_3$ release from membrane, causing calcium release from the ER. Additional studies are required to determine the mechanism(s) for nsPEF-induced calcium mobilization in HL-60 and other cells.

Because nsPEFs do not exist in nature, cells have evolved in the absence of these intense electric fields and/or these high frequencies. Nevertheless, the data reported here clearly indicate that cells have sensors that can respond to nsPEF. The data indicate that calcium mobilization does not occur by mechanisms that are related to classical plasma membrane electroporation, but more likely occurs by nsPEF-triggered effects on intracellular structures and functions as we have reported previously (13–16). The nature of the cell sensor(s) remains to be determined, but it is clear that this sensor(s) is coupled to signal transduction mechanisms that mobilize intracellular calcium in ways that mimic natural ligands in HL-60 cells. nsPEF-induced calcium mobilization is not specific to HL-60 cells but also has been observed in human Jurkat cells and neutrophils, (29, 39). These observations suggest that nsPEFs can be used as stimuli to modulate signal transduction mechanisms that alter cell structure and function and, as shown here, to probe cellular mechanisms for calcium mobilization through intracellular calcium channels and CCE through the plasma membrane.

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REFERENCES

Stimulation of Capacitative Calcium Entry in HL-60 Cells by Nanosecond Pulsed Electric Fields

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