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CONTROL OF CALCIUM ENTRY IN HUMAN FIBROBLASTS BY FREQUENCY-DEPENDENT ELECTRICAL STIMULATION

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1. ABSTRACT

Modulation of intracellular calcium ion concentration ($(Ca^{2+})_i$) could be used to control cellular and molecular responses that are important in cell and tissue engineering. Electrical stimulation (ES) has been used to activate plasma membrane ion channels including Ca^{2+}channels, and to induce changes in $(Ca^{2+})_i$. Strong direct current (dc) ES depolarizes the membrane electrical potential (MEP) and, thereby, causes rapid increases in $(Ca^{2+})_i$. Electrocoupling mechanisms that could control $(Ca^{2+})_i$ increases induced by modes of ES other than dc have not been elucidated, however. Here we report that 30 min of continuous exposure to a 1 or 10 Hz, 2 V/cm ES induces an $(Ca^{2+})_i$ increase by ~ 6-fold (baseline 25 nM) in human fibroblasts in culture. In contrast, a 100 Hz, 2 V/cm ES causes no significant $(Ca^{2+})_i$ increase. Either depletion of $Ca^{2+}$from the extracellular medium or incubation of cells with verapamil inhibits the $(Ca^{2+})_i$ increase, indicating that Ca^{2+} influx through verapamil-sensitive Ca^{2+}channels is required for the $(Ca^{2+})_i$ increase induced by oscillatory ES. More intense ES by a 1 Hz or a dc 10 V/cm electric field causes a rapid 20 to 25-fold $(Ca^{2+})_i$ increase. We hypothesize that selective, partial activation of Ca^{2+}channels is likely to mediate Ca^{2+}influx. These results suggest that optimal ES could be used to control Ca^{2+}entry and, thereby, regulate cellular calcium homeostasis without adversely affecting cell viability.

2. INTRODUCTION

Intracellular calcium ion concentration ($(Ca^{2+})_i$) is known to regulate important biological processes including signal transduction (1-4), cytoskeletal reorganization (5-7), cell differentiation and proliferation

(8-11), cell adhesion (12, 13), and cell migration (14, 15). Because intracellular Ca^{2+}plays a critical role in mediating these cellular and molecular effects, the molecular mechanisms that regulate $(Ca^{2+})_i$ have been extensively studied. At least three known Ca^{2+}pathways are thought to be involved in regulation of $(Ca^{2+})_i$. First, $(Ca^{2+})_i$ can be increased by activation of voltage-gated Ca^{2+}channels (VGCCs) in the plasma membrane. These Ca^{2+}-selective, voltage-operated channels can be activated by changes in the membrane electrical potential (MEP) induced by exogenous electrical stimulation (ES) (16, 17). Second, $(Ca^{2+})_i$ can be increased by activation of stretch-activated cation channels (SACCs) in the plasma membrane. Although the SACC is not specific for Ca^{2+}, both mechanical (18) and electrical (19) stimuli have been shown to induce $(Ca^{2+})_i$ increases mediated by SACC activation. Third, $(Ca^{2+})_i$ can be increased by activation of internal Ca^{2+}stores. The latter mechanism is typically mediated by signal transduction cascades initiated at the plasma membrane level (1, 2).

Because the cell membrane is highly resistive and the cytosol is conductive (20), the cell can be considered to represent an electrically non-conducting object. Application of an exogenous ES is likely to cause changes in MEP. The MEP in resting cells is typically -40 to -100 mV (21). To activate VGCCs, the strength of an exogenous electrical stimulus must be sufficiently large to alter the MEP. For example, cells 50 μm in diameter exposed to a 10 V/cm ES would experience a maximum potential difference of ~ 40 mV. An induced potential difference of this magnitude would likely depolarize the cathode-facing side and hyperpolarize the anode-facing side of the cell (22, 23), thereby initiating Ca^{2+}influx at the cathode-facing side of the cell. Application of a dc ES (10 V/cm) to fibroblasts in culture was shown to cause a > 20-fold increase in $(Ca^{2+})_i$ by activating VGCCs (17). Uncontrolled increases in $(Ca^{2+})_i$ could, however, lead to cell death (24, 25) by damaging membranes and proteins (26) and by promoting free radical synthesis (27, 28). Theoretically, ES of lower strength could cause partial or selective activation of one or more VGCC subtypes. For example, application of a 2 V/cm ES would be expected to induce an ~ 8 mV change in MEP. MEP changes of magnitude < 10 mV may not activate all Ca^{2+}channels. In sensory neurons, L- and T-type VGCCs are activated at MEPs of -10 mV and -70 mV, respectively (21). Assuming that the resting MEP in neurons is about -70 mV (29), then L-type VGCC activation would require a MEP change of ~ 60 mV, while T-type VGCCs could be activated by a MEP change of less than 10 mV. It is interesting to note that the 2 V/cm strength also represents the physiological upper limit for natural electrical activities that are thought to be important for wound healing (20, 30, 31).

While strong dc ES (10 V/cm) has been shown to activate VGCCs rapidly and induce more than 20-fold increases in $(Ca^{2+})_i$, effects of the application of non-dc ES modes on $(Ca^{2+})_i$ have not been documented. Oscillatory ES represents such mode that has been postulated to affect $(Ca^{2+})_i$. We have demonstrated that oscillatory ES can induce cell surface receptor (CSR) redistribution (32), changes in cytoskeletal organization (33), SACC activation (19), and integrin-mediated cell migration (34), all without adversely affecting cell viability. Based on these findings, changes in $(Ca^{2+})_i$ in response to oscillatory ES could be expected, but quantitative measurements have not been performed. Moreover, the mechanisms by which oscillatory ES could mediate $(Ca^{2+})_i$ increases remain to be elucidated. Unlike the sustained MEP alterations caused by strong dc ES, application of oscillatory ES is expected to alternately depolarize opposite sides of the cell with the same frequency as the oscillatory stimulus. It is not clear how periodic MEP depolarization and repolarization could be coupled to VGCC activation, and there is currently no satisfactory theoretical treatment for VGCC activation in response to modulated MEP. Conformational changes in VGCCs that are required to mediate Ca^{2+}influx in response to oscillatory ES could theoretically depend on the mode, strength, and duration of ES. Effects of modulated MEP on Ca^{2+} flux across the plasma membrane can also be experimentally characterized. In the present study, digitized fluorescence video microscopy is used to examine quantitatively the $(Ca^{2+})_i$ increases in human fibroblasts induced by strong (10 V/cm) or weak (2 V/cm) oscillatory ES, and to elucidate the biophysical mechanisms responsible for $(Ca^{2+})_i$ increases. While strong ES is found to cause rapid $(Ca^{2+})_i$ increases (> 20-fold), weak oscillatory ES induces $(Ca^{2+})_i$ increases (6-fold) in a *frequency-dependent* manner. We hypothesize that the latter increases in $(Ca^{2+})_i$ are mediated by selective activation of verapamil-sensitive Ca^{2+}channels, and that T-type VGCCs could represent a potential candidate.

3. MATERIALS AND METHODS

3.1. Cell culture

Human foreskin fibroblasts (HS27, Naval Bioscience Lab, Oakland, CA) were grown in Alpha Minimal Essential Medium supplemented with 10% fetal calf serum (Sigma, St. Louis, MO), 100 U/ml penicillin-100 μg/ml streptomycin and 100 mM L-glutamine (Sigma) at 37 ^{o}C in a 5% CO_2 humidified incubator. Cells at passages 16 - 20 were subcultured at 60-70% confluence onto 35 x 50 mm No. 2 coverslips 48 to 60 hr before each experiment to ensure log phase of growth. These cells can be propagated beyond passage 42.

3.2. Electrical stimulation

The chamber used to expose cells to ES, described elsewhere (32), is shown in Figure 1. Briefly, sinusoidal signals were generated by a function generator (Model 19, Wavetek, San Diego, CA), fed into a 100 W amplifier (BOP100, Kepco, Flushing, NY), and monitored by an oscilloscope (Model 2205, Tektronix, Beaverton, OR). The computation of ES strength followed Ohm's law, $J = \sigma E$, where J is the electric current density and σ is the conductivity of the medium. Unless otherwise stated, the oscillatory ES strength represents the peak to peak value. The calculated ES strength is accurate to within 20%. All experiments were performed at room temperature. The

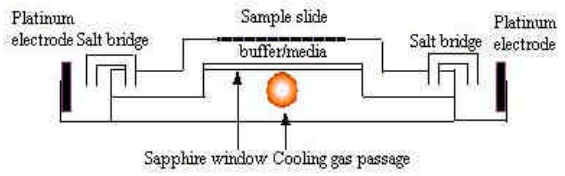

Figure 1. Schematic for the ES chamber. This chamber was designed to allow the direct visualization of cellular responses using imaging techniques. Changes in $(Ca^{2+})_i$ were recorded in real time, stored, and analyzed. The design of this chamber resists changes in pH and temperature. The cross section of the chamber is machined and measured with μm-precision. Cell viability studies using this chamber have been described elsewhere (33).

strongest ES used in the experiments (10 V/cm) induced a maximum temperature rise of < 2.2 ^oC in our experimental apparatus, resulting in a maximum sample temperature of ~24 ^oC. Application of the weaker ES (2 V/cm) resulted in < 0.5 ^oC, and this degree of heating was independent of ES frequency in the 1-100 Hz frequency range.

3.3. Calcium dye loading and fluorescence imaging

The Ca^{2+}-sensitive fluorescent dye Fluo-3 AM ester (Fluo-3, Molecular Probes, Eugene, OR) was dissolved in DMSO to make a 1 mM stock solution, then dissolved at 10 μM final concentration in Hank's balanced salt solution (HBSS). About 1 ml of 10 μM Fluo-3 solution in HBSS was carefully layered on top of the coverslip bearing the cells, then incubated for 60 min in the dark at room temperature. Coverslips were then washed twice with HBSS and used immediately in quantitative fluorescence microscopy experiments. In some experiments, Ca^{2+}-free conditions were ensured by washing and incubating cells in modified HBSS containing 0 mM $CaCl_2$, 2 mM $MgCl_2$, and 1 mM EGTA.

3.4. Fluorescence microscopy

Epifluorescence video microscopy was used to obtain digitized fluorescence images of Fluo-3 loaded cells. Cells were observed using a Zeiss Axioskop microscope (Carl Zeiss Inc., Thornwood, NY). The illumination source was a 100 W mercury arc lamp. Illuminating light was passed through a dichroic filter and focused on the sample through a 25x/0.8 NA oil immersion objective. Fluorescence emission was imaged using a cooled CCD camera (Roper Scientific, Tucson, AZ), and processed by an image processor (Metamorph, Universal Imaging, West Chester, PA). Background intensity was subtracted from each image. All operations were controlled by a computer.

Fluorescence images of fibroblasts loaded with Fluo-3 were recorded in real time before and after ES application. Typically, 3 to 8 cells were identified in a field of view, and changes in fluorescence intensity in each of the cells were monitored. Cell boundaries were drawn using the image processor, and fluorescence intensity was integrated over all pixels within the boundary of each individual cell, as described previously (19). To account for the variability in size and shape of human fibroblasts, and to eliminate effects due to variation in Fluo-3 dye loading, the fluorescence intensities from each image were normalized by those from the reference image recorded before application of an ES. The small temperature rise induced by ES application did not cause changes in the Fluo-3 fluorescence intensity.

3.5. $(Ca^{2+})_i$ calibration

$(Ca^{2+})_i$ was estimated from the fluorescence intensity of Fluo-3 by using the equation, $(Ca^{2+})_i = K_d (F - F_{min})/(F_{max} - F)$, where K_d is 400 nM, and F_{max} and F_{min} are the maximum and minimum fluorescence intensities determined according to a previously described method (19, 35). In resting human fibroblasts loaded with Fluo-3, $(Ca^{2+})_i$ was determined to be 25 ± 10 nM. This calculation is consistent with the previously reported findings that, in resting fibroblasts, the baseline $(Ca^{2+})_i$ ranges from 20 to 100 nM (36, 37).

3.6. Cell treatment with verapamil

Fibroblasts were incubated with verapamil (a selective VGCC inhibitor, 25 μM final concentration, Sigma) for 30 min at room temperature, washed twice in HBSS, loaded with Fluo-3 as described, washed twice in HBSS, and mounted on the chamber for quantitative fluorescence microscopy experiments. To ensure that verapamil treatment did not interfere with Fluo-3 loading, cells were loaded with Fluo-3 first and then treated with verapamil. Reversing the order of the Fluo-3 loading and verapamil treatment protocols did not affect the experimental results. For those experiments in which verapamil was used to inhibit Ca^{2+}channels, the HBSS medium was supplemented with 25 μM verapamil.

4. RESULTS

4.1. $(Ca^{2+})_i$ increase induced by strong electrical stimulus

We first quantified the changes in $(Ca^{2+})_i$ induced by a strong dc ES that was sufficient to activate VGCCs directly. Previous reports have demonstrated that application of a 10 V/cm dc ES induces a > 20-fold $(Ca^{2+})_i$ increase in fibroblasts, and that this increase is prevented by treatment of cells with VGCC inhibitors including cobalt and D-600 (17). To calibrate our experimental conditions against the previous results, human fibroblasts were exposed to dc 10 V/cm ES in the presence or absence of verapamil (25 μM, a VGCC inhibitor). Figure 2 shows that application of a dc 10 V/cm ES induced a 25-fold increase in $(Ca^{2+})_i$; the maximum $(Ca^{2+})_i$ increase was obtained 38 ± 19 s after the initiation of ES exposure, followed by diminished fluorescence intensity due to possible damage to Ca^{2+}channels or cell membrane. Treatment of cells with verapamil prevented the ES-induced $(Ca^{2+})_i$ increase (Fig. 2), indicating that the induced $(Ca^{2+})_i$ increase required Ca^{2+}influx through Ca^{2+}channels. Interestingly, application of a 1 Hz, 10 V/cm ES also caused a 20-fold increase in $(Ca^{2+})_i$. Treatment of cells with 25 μM verapamil prior to and during exposure to a 1 Hz, 10 V/cm ES also inhibited the ES-induced $(Ca^{2+})_i$ increase. Thus,

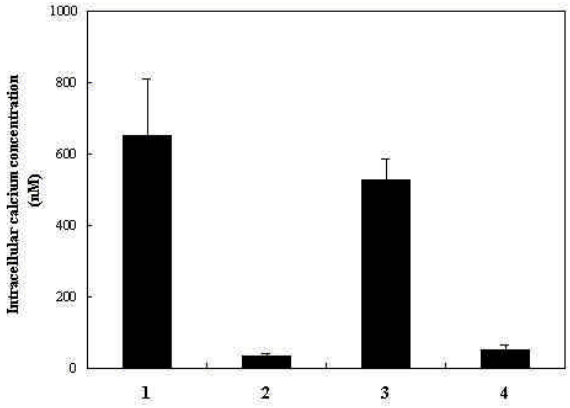

Figure 2. Strong ES-induced $(Ca^{2+})_i$ increase is mediated by Ca^{2+}influx through the plasma membrane. Cells were loaded with Fluo-3 and exposed to a dc or 1 Hz ES at room temperature. The integrated fluorescence intensity of each cell was monitored and recorded at 10 s intervals and normalized against the fluorescence intensity of that cell at t = 0. The maximum fluorescence intensity was calculated and converted to $(Ca^{2+})_i$, as described in the text. Application of a dc 10 V/cm ES increased the $(Ca^{2+})_i$ by 25-fold over the baseline level of 25 nM (1); this increase was inhibited by treatment of cells with 25 μM verapamil (2). Similarly, application of a 1 Hz, 10 V/cm ES increased the $(Ca^{2+})_i$ by 20-fold (3); this increase was also inhibited by treatment of cells with verapamil (4). Each data point represents the mean ± SD from 6-8 individual cells.

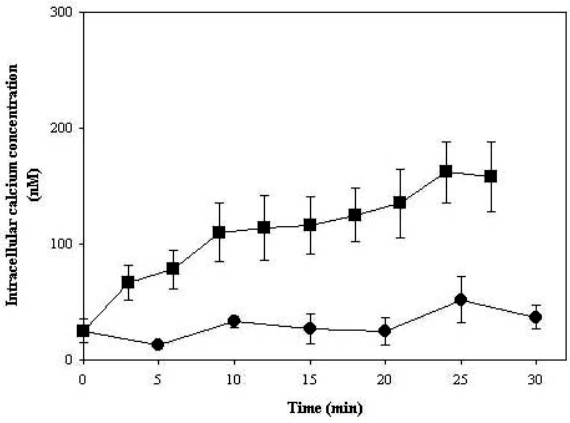

Figure 3. Time-dependent $(Ca^{2+})_i$ increase is induced by weak oscillatory ES. Cells were loaded with Fluo-3 and exposed to a 1 Hz, 2 V/cm ES for 30 min at room temperature (squares). Control cells (circles) were treated identically, except that these cells were not exposed to ES. The integrated fluorescence intensity of each cell was monitored and recorded at 3 or 5 min intervals, and normalized against the fluorescence intensity of that cell at t = 0. Each data point represents the mean ± SEM of 3-4 independent experiments from 10-17 individual cells.

changes in MEP induced by strong dc or oscillatory ES are likely to activate Ca^{2+}channels and induce Ca^{2+}influx, leading to a 20 to 25-fold increase in $(Ca^{2+})_i$.

4.2. Time dependence of $(Ca^{2+})_i$ increase induced by oscillatory electrical simulation

Application of oscillatory ES of a smaller but physiologically relevant strength (i.e., 2 V/cm) was also found to induce increases in $(Ca^{2+})_i$. Figure 3 represents the time course of the $(Ca^{2+})_i$ increase observed in response to this oscillatory ES. Control fibroblasts (i.e., cells not exposed to an ES) showed no significant changes in $(Ca^{2+})_i$ over a 30 min period of observation. Unlike the rapid and large $(Ca^{2+})_i$ increases induced by a 1 Hz, 10 V/cm ES, cells exposed to a 1 Hz, 2 V/cm ES showed a maximum 6-fold increase in $(Ca^{2+})_i$ that was gradual in onset and appeared to saturate after 30 min of ES exposure. Monitoring of the $(Ca^{2+})_i$ level for 45 to 60 min after the onset of ES exposure did not show a further increase in $(Ca^{2+})_i$. To investigate the possibility that a 1 Hz, 2 V/cm ES could induce rapid $(Ca^{2+})_i$ transients in the early phase of the ES application (i.e., within the first 3 min of ES exposure), fluorescence images of Fluo-3 loaded fibroblasts were recorded at 10 s intervals after the onset of ES exposure. No significant $(Ca^{2+})_i$ transient was observed during the first 3 min of ES application (data not shown).

4.3. Frequency dependence of $(Ca^{2+})_i$ increase

Based on our previous findings that cell surface receptor (CSR) redistribution (32) and cytoskeletal reorganization (33) depend critically on the frequency of an applied ES, we next tested the hypothesis that the ES frequency regulates $(Ca^{2+})_i$ increases in human fibroblasts. Fibroblasts were exposed to 1 Hz, 10 Hz, and 100 Hz ES of constant strength (2 V/cm), and the $(Ca^{2+})_i$ level was monitored over time. Like cells exposed to a 1 Hz ES, cells exposed to a 10 Hz ES showed a maximum 6-fold $(Ca^{2+})_i$ increase ($p = 0.54$, Figure 4). The time course of the $(Ca^{2+})_i$ increase in response to a 10 Hz ES was also similar to that observed for a 1 Hz ES application. In contrast, application of a 100 Hz ES caused no statistically significant $(Ca^{2+})_i$ increase compared to the $(Ca^{2+})_i$ in control cells ($p = 0.17$). It therefore appears that the ES-induced $(Ca^{2+})_i$ increase depends critically on the *frequency* of the applied ES, and that there exists a "frequency window" (< 100 Hz) beyond which an increase in $(Ca^{2+})_i$ is not induced by weak oscillatory ES. It is interesting to note that the band width of the frequency window is consistent with that found for oscillatory ES-induced cell surface receptor (CSR) redistribution and cytoskeletal reorganization, suggesting that the three ES-induced cellular responses could be mechanistically coupled.

4.4. Role of extracellular $Ca^{2+}$and involvement of VGCCs

Ca^{2+}influx across the plasma membrane is one potential mechanism for the $(Ca^{2+})_i$ increase induced by weak ES (i.e., 2 V/cm). To test this hypothesis, cells were exposed to a 1 Hz, 2 V/cm ES in the absence of extracellular Ca^{2+}, and the $(Ca^{2+})_i$ was examined over

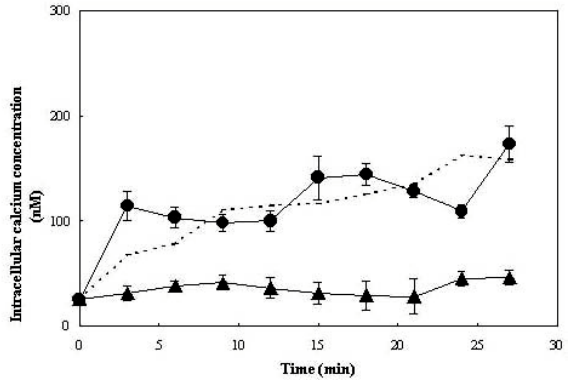

Figure 4. Weak oscillatory ES-induced $(Ca^{2+})_i$ increase is frequency-dependent. Cells were loaded with Fluo-3 and exposed to ES of varying frequencies. The ES strength was maintained at 2 V/cm. While 1 Hz (dashed line) or 10 Hz (circles) ES applications showed similar $(Ca^{2+})_i$ increases, a 100 Hz ES application did not cause a significant $(Ca^{2+})_i$ increase (triangles). Each data point represents the mean ± SEM of 2-3 independent experiments from 8-14 individual cells. Data from cells exposed to 1 Hz ES are reproduced from Figure 3.

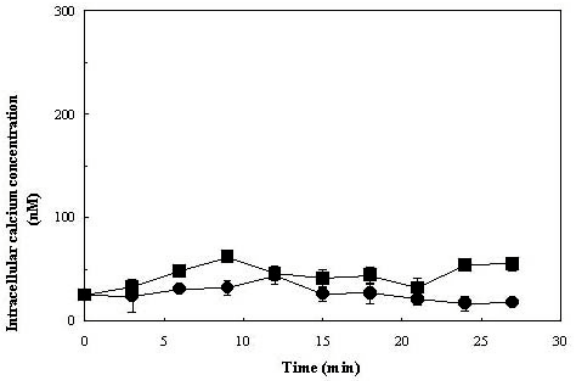

Figure 5. Weak oscillatory ES-induced $(Ca^{2+})_i$ increase requires influx of extracellular Ca^{2+}. Cells were loaded with Fluo-3 and exposed to a 1 Hz, 2 V/cm ES. Either removal of $Ca^{2+}$from the extracellular medium (squares) or treatment of cells with verapamil (circles) inhibited the ES-induced $(Ca^{2+})_i$ increase. Data points represent the mean ± SEM of 2-4 independent experiments from 8-20 individual cells.

time. As shown in Figure 5, application of a 1 Hz, 2 V/cm ES did not cause an $(Ca^{2+})_i$ increase when extracellular $Ca^{2+}$was depleted, indicating that Ca^{2+}influx across the plasma membrane was required for the oscillatory ES-induced $(Ca^{2+})_i$ increase. This finding suggests further that VGCC activation is the likely electrocoupling mechanism between weak oscillatory ES and the observed $(Ca^{2+})_i$ increase. To test this hypothesis, cells were incubated with verapamil prior to and during weak ES exposure. Treatment of cells with verapamil inhibited the ES-induced $(Ca^{2+})_i$ increase (Fig. 5). These results indicate that the weak ES-induced $(Ca^{2+})_i$ increase in human fibroblasts is mediated by Ca^{2+}influx across the plasma membrane, and that activation of internal Ca^{2+}stores is not likely involved.

5. DISCUSSION

Results from the present study show that oscillatory ES is capable of inducing $(Ca^{2+})_i$ increases in human fibroblasts. Application of a strong ES (i.e., 10 V/cm) causes a > 20-fold $(Ca^{2+})_i$ increase, independent of the mode of ES exposure; these $(Ca^{2+})_i$ increases are mediated by Ca^{2+} influx through verapamil-sensitive Ca^{2+}channels. Application of a 1 Hz, 2 V/cm ES induces a maximum 6-fold increase in $(Ca^{2+})_i$ over a 30 min period of ES exposure. Either depletion of extracellular Ca^{2+}or treatment of cells with verapamil inhibits the ES-induced $(Ca^{2+})_i$ increase, indicating that weak oscillatory ES also activates Ca^{2+}channels. Application of a 10 Hz, 2 V/cm ES induces an $(Ca^{2+})_i$ increase similar to that induced by a 1 Hz, 2 V/cm ES exposure. In contrast, application of a 100 Hz, 2 V/cm ES causes no significant increase in $(Ca^{2+})_i$, however. Taken together, these findings suggest that Ca^{2+}influx across the plasma membrane is responsible for oscillatory ES-induced $(Ca^{2+})_i$ increases, and that such increases are mediated by activation of voltage-dependent Ca^{2+}channels in a frequency-dependent manner.

VGCCs have been identified in the plasma membrane of non-excitable as well as excitable cell types, including osteoclasts, astrocytes, and fibroblasts (38). Direct activation of VGCCs by *oscillatory* ES could therefore represent a potential electrocoupling mechanism mediating ES-induced $(Ca^{2+})_i$ increases in human fibroblasts. This mechanism requires MEP depolarization. Changes in MEP (δV) depend on many factors, including the ES strength, cell shape and size, and the orientation of the cell with respect to the electric field vector. For a spherical cell, $\delta V = 3/2\ E\ r \cos\theta$ (22), where r is the radius of cell and θ is the angle formed by the electric field axis and a point on the cell surface. Application of a 10 V/cm ES is predicted to depolarize the cathode-facing surface of a 50 μm diameter cell by a maximum of 40 mV. A δV in the range of 40 to 50 mV is likely to be sufficient for direct VGCC activation. It is difficult to formulate a simple model to predict δV for irregularly shaped cells such as fibroblasts, however. Instead, the magnitude of the maximum MEP depolarization can be approximated by treating fibroblasts as ellipsoidal non-conducting objects. By solving the Laplace equation, solutions for the potential difference across the membranes of ellipsoidal cells in response to externally applied ES have been determined. Assuming 10 and 25 μm for the semimajor axes of an ellipsodial cell (these are representative values for human fibroblasts measured from video images), the ES-induced δV is expected to differ by no more than 10% from that for a spherical cell (39). Thus, the δV induced by a strong ES is likely to be sufficient for activation of VGCCs in fibroblasts.

We find here that both 1 Hz and dc 10 V/cm ES are capable of causing large increases in $(Ca^{2+})_i$, presumably due to VGCC activation caused by MEP depolarization. Thus, the mode of ES is not important if the ES strength is sufficiently large. Two alternate explanations could be offered. First, this result is consistent with the theoretical prediction that ES-induced changes in MEP are independent of frequencies < 1 MHz (40). Second, the duration for a one-half cycle of 1 Hz ES is 0.5 s, sufficiently long compared to the time course of Ca^{2+} channel opening. We further note that, in response to a 10 V/cm dc ES, the time required to reach the maximal $(Ca^{2+})_i$ increase is about 40 s. One explanation of this "delay" could be localized VGCC activation. ES-induced Ca^{2+} influx is likely to be initiated at the cathode-facing side of the cell, and intracellular Ca^{2+} is likely then to diffuse down its concentration gradient toward the anode-facing side of the cell. By approximating the characteristic area of cell as that of an ellipsoid, we estimate from the 40 s time course of the $(Ca^{2+})_i$ increase that the diffusion coefficient of Ca^{2+} inside the human fibroblast is approximately 2×10^{-7} cm^2/s. This rough estimate is within about an order of magnitude of the previously reported diffusion coefficient for intracellular Ca^{2+} (3×10^{-6} cm^2/s) (23).

Increases in $(Ca^{2+})_i$ can be mediated by release of Ca^{2+} from internal stores and/or by Ca^{2+} influx across the plasma membrane. The observation that Ca^{2+} depletion from the extracellular medium inhibits the oscillatory ES-induced $(Ca^{2+})_i$ increase provides evidence that Ca^{2+} influx across the plasma membrane is the mechanism likely to be responsible for the $(Ca^{2+})_i$ increase. Based on theoretical considerations and experimental findings, oscillatory ES of frequencies 1 to 100 Hz does not penetrate inside the cell (20, 41). Therefore, direct activation of internal Ca^{2+} stores by an oscillatory ES in this frequency range is unlikely. Release of Ca^{2+} from internal Ca^{2+} stores could be mediated by receptor activation at the cell surface, however. We have previously shown that inhibition of phospholipase C (PLC) does not prevent the ES-induced increase in $(Ca^{2+})_i$ in hepatocytes, indicating that PLC-mediated activation of internal Ca^{2+} stores is unlikely (19). Ca^{2+} influx pathways other than VGCCs could also be responsible for the $(Ca^{2+})_i$ increase in response to oscillatory ES. For example, fibroblasts are known to express stretch-activated cation channels (SACCs), which mediate influx of cations including Ca^{2+} across the plasma membrane (42). Because we find here that the verapamil treatment of cells completely prevents the ES-induced $(Ca^{2+})_i$ increase, the role of SACC activation in mediating the $(Ca^{2+})_i$ increase appears to be negligible. Although verapamil can inhibit SACCs at high concentration, the verapamil concentration used in the present study (25 μM, final concentration) does not inhibit SACCs (43).

In response to weak oscillatory ES, increases in $(Ca^{2+})_i$ are gradual and appear to saturate after 30 min of ES exposure. Presumably, after 30 min the fibroblasts have reached a new equilibrium in which the increased Ca^{2+} influx is again balanced by increased Ca^{2+} efflux and sequestration, at a new steady-state $(Ca^{2+})_i$ level. The simplest explanation for our observation that weak oscillatory ES induces $(Ca^{2+})_i$ increases is the activation of Ca^{2+} channels in the plasma membrane. Several electrically and pharmacologically distinguishable Ca^{2+} channels have been identified. Of these different types of Ca^{2+}channels, fibroblasts are known to possess both the high voltage-activated (L-type) and low voltage-activated (T-type) Ca^{2+} channels (36, 44-47). Activation of the L-type Ca^{2+}channels requires changes in MEP that are about 3-fold greater than those required to activate the T-type Ca^{2+} channels (21, 48). Results from the present study are consistent with such findings. For example, application of a 10 V/cm ES is likely to induce a change of about 40 mV in MEP, which is probably sufficient to activate both the L- and T-type Ca^{2+} channels. In contrast, application of a 2 V/cm ES is predicted to induce a change of less than 10 mV in MEP, which is unlikely to be sufficient to cause L-type Ca^{2+} channels activation. In the latter case, a partial activation of Ca^{2+} channels (e.g., T-type) is postulated. Based on these observations, we propose that Ca^{2+} homeostasis could be regulated by using optimal ES parameters to activate Ca^{2+} channels selectively.

At least two molecular mechanisms could be responsible for selective Ca^{2+} channel activation by weak oscillatory ES. First, because the oscillatory ES-induced $(Ca^{2+})_i$ increase requires 30 min of continuous exposure to reach a maximum, exogenous weak electrical signals may be temporally integrated and rectified to cause Ca^{2+} channel activation. Such mechanisms have indeed been proposed and shown to be theoretically plausible (49, 50). Second, redistribution of charged CSRs could alter the MEP and, thereby, cause partial activation of Ca^{2+} channels. This mechanism is supported by our previous observations that at least three different types of CSR do redistribute on the surface of fibroblasts in response to oscillatory ES of frequencies similar to those used in the present study. The time scale for CSR redistribution is consistent with the time course of the ES-induced $(Ca^{2+})_i$ increase. For example, a 1 Hz or 10 Hz ES induces CSR redistribution on a time scale of ~15 min (32). Further, theoretical treatment shows that redistribution of laterally mobile and electrically charged CSRs (e.g., glycoproteins) does alter MEP (51). We cannot rule out the possibility that Ca^{2+} channels (i.e., VGCCs) themselves are laterally mobile, and are thereby redistributed on the cell surface in response to ES. Although VGCC expression in the cell membrane of fibroblasts could be observed using fluorescently conjugated anti-VGCC antibodies, direct experimental observation of VGCC redistribution is difficult, due to poor signal-to-noise ratio. More sophisticated experiments using single particle tracking

techniques are underway to quantify, with nm-precision, the motion of individual cell surface receptors, including ion channels, in response to strong and weak oscillatory ES.

Finally, the $(Ca^{2+})_i$ has been shown to be an important regulator of cell adhesion (52). Ca^{2+} influx can induce cell adhesion and migration in platelets (53), neutrophils (54), endothelial cells (55), and fibroblasts (12). Stretch-induced calcium channel activation in keratinocytes leads to cell detachment and subsequent cell release (56), suggesting that Ca^{2+} influx controls cellular mechanics. Because Ca^{2+}-dependent cell adhesion and motility are two of the critical factors that determine tissue integrity and function, methods of control of the $(Ca^{2+})_i$ level could play an important role in cell and tissue engineering. By applying optimal ES non-invasively, we have demonstrated that the $(Ca^{2+})_i$ level can be regulated without causing cellular damages.

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Abbreviations: $(Ca^{2+})_i$, intracellular calcium ion concentration; CSRs, cell surface receptors; ES, electrical stimulation; HBSS, Hank's balanced salt solution; MEP, membrane electrical potential; SACCs, stretch-activated cation channels; VGCCs, voltage-gated calcium, channels

Key Words: Electrical Stimulation, Calcium Channel Activation, Intracellular Calcium Ion Concentration

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