Quantitative study of calcium uptake by tumorigenic bone (TE-85) and neuroblastoma×glioma (NG108-15) cells exposed to extremely-low-frequency (ELF) electric fields

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Abstract To verify the effect of cell culture state on frequency dependent increase in proliferation as well as Ca^{2+} flux across the plasma membrane, tumorigenic bone (TE-85) and neuroblastoma×glioma (NG108-15) cells cultured in the presence of fetal bovine serum (FBS) were exposed to capacitively coupled electric (CCEF) fields in the extremely low frequency (ELF) range of 10 to 18 Hz. [^{3}H]Thymidine incorporation and $^{45}Ca^{2+}$ uptake were used as endpoints. TE-85 cells cultured in the presence of 10% FBS did not exhibit a frequency dependent increase in proliferation in contrast to previous studies under growth arrested culture conditions, in which the cells were deprived of FBS. However, both TE-85 and NG108-15 cells had an increase in $^{45}Ca^{2+}$ uptake in response to a 16 Hz 18.3 mV/cm CCEF. Fura-2 digital imaging microscopy was used to verify addition of 0.5 mM La^{3+} and 0.5 mM ionomycin as negative and positive controls, respectively. Imaging microscopy data was combined with $^{45}Ca^{2+}$ incorporation results to quantify free intracellular calcium ($[Ca^{2+}]_i$) increase in response to CCEF exposure. TE-85 $[Ca^{2+}]_i$ increased from 140 to 189–210 nM where as NG108-15 $[Ca^{2+}]_i$ increased from 67 to 189–210 nM. These results suggested that serum deprivation may be a requirement for a frequency dependent increase in proliferation in TE-85 cells but is not necessary for the electric field induced increase in $^{45}Ca^{2+}$ uptake in both TE-85 and NG108 cells. The present study also represents the first demonstration of increased $^{45}Ca^{2+}$ uptake by neuroblastoma and/or glioma cells in response to an electric field exposure.

Key words: Extremely-low-frequency electric field; Calcium; Osteoblast; TE-85; NG108-15

1. Introduction

The molecular mechanism to explain how cells may respond to external electromagnetic fields (EMF) in the extremely low frequency range of 0–300 Hz (ELF) to induce disease processes like cancer [1,2] or promote beneficial effects like increased fracture healing rates [3–5] is not clearly understood. Based on the observation that bone naturally produces electric fields in response to mechanical loading, called stress-generated potentials [6], numerous laboratories have investigated the hypothesis that bone cells detect and respond to electric fields [7–9]. The results of such investigations have been instrumental in the development of FDA approved and physician administered clinical electromagnetic treatment for osteogenesis [5,10].

Fitzsimmons and coworkers [11,12] were some of the first investigators to demonstrate a frequency-dependent increase in cell proliferation and mitogen activity in skeletal tissue exposed to extremely-low-frequency (ELF) capacitively coupled electric fields (CCEF). Given that increases in free intracellular calcium ion concentration ($[Ca^{2+}]_i$) have been associated with EMF exposure in certain cells [13–16], Fitzsimmons et al. [15] further investigated ELF field exposure in partially growth arrested TE-85 osteosarcoma cells with $^{45}Ca^{2+}$ uptake as the endpoint. The cells were partially growth arrested in the G_0/G_1 stage by culturing in serum-free medium, 24 h before ELF-CCEF exposure. Interestingly, the net $^{45}Ca^{2+}$ uptake was found to be frequency-dependent, with the peak occurring in a range similar to one found in their earlier cell proliferation/mitogen release work [11,12].

TE-85 cells exhibit several markers characteristic of the osteoblastic phenotype, including type I collagen production [12] and alkaline phosphatase rich membranes [17]. Also, in response to 1,25-dihydroxyvitamin D_3, under serum free growth conditions, TE-85 cells exhibit increased alkaline phosphatase activity and produce osteocalcin [12,18]. Further, TE-85 cells exhibit increased proliferation when stimulated by parathyroid hormone under reduced serum conditions [19]. In view of these observations, TE-85 cells are commonly used as a cellular osteoblast model. However, in the presence of 10% fetal bovine serum (FBS) TE-85 cells exhibit no increase in alkaline phosphatase levels and do not produce osteocalcin in response to 1,25-dihydroxyvitamin D_3 stimulation [20,17], suggesting that FBS compromises the ability of TE-85 cells to model osteoblast responses.

Assuming that culture conditions in the presence of serum are more likely to mimic in vivo conditions when compared to the serum-depleted environment, we set out to determine whether TE-85 cells cultured in serum-containing medium before ELF-CCEF exposure also exhibit a frequency-dependent increase in proliferation, and if so, to quantitatively verify Ca^{2+} uptake in response to ELF-CCEF exposure in TE-85 and excitable cells.

2. Materials and methods

2.1. Cell lines and culture conditions

The human osteosarcoma TE-85 of passage 28 was obtained from the American Type Culture Collection (Rockville, MD) and the neuroblastoma×glioma hybrid NG108-15 of passage 12 was obtained from Dr. Nirenberg, National Institute of Health (Bethesda, MD). The cells were maintained in a Forma Scientific incubator model 3860 at 37°C, 10% CO_2 and 90% relative humidity in Dulbecco's modified Eagle's medium supplemented with 0.37% $NaHCO_3$ (w/v), 2 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin.

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and 10% FBS (DMEM/10% FBS). TE-85 cells were maintained in T-flasks and passaged every 4 to 5 days. For use in experiments, TE-85 cells were trypsinized with 0.25% trypsin (w/v) and 2% EDTA (w/v) in phosphate buffered saline (PBS) [21] for 1.5 min. The PBS was removed and the flasks were placed in the CO_2 incubator for 6 min followed by cell resuspension in DMEM/10% FBS. Viable cells (able to exclude trypan blue) were plated at 50 000 cells/cm^2 in six-well plates (Costar, Cambridge, MA) in DMEM/10% FBS and the medium was refreshed after 24 h. The cells were exposed to ELF-CCEF 24 h after medium change for either [^{3}H]thymidine incorporation or $^{45}Ca^{2+}$ uptake studies.

NG108-15 cells were maintained in 75-cm^2 T-flasks (Costar, Cambridge, MA) and passaged following previously published procedures [22]. The growth medium for NG108-15 cells was DMEM/10% FBS, supplemented with 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 160 μM thymidine [DMEM/10% FBS/HAT]. For use in experiments, confluent cultures were aspirated from the base of the flask with a Pasteur pipet. The suspension was centrifuged (500×g; 10 min) and viable cells were plated at 30 000 cells/cm^2 in six well plates. As with TE-85, the medium was changed after 24 h and the cells were exposed to ELF-CCEF 24 h after medium change.

2.2. Capacitively coupled electric field exposure system

The exposure system was based on a design previously reported by Brighton and Townsend [23]. As shown in Fig. 1, the system was composed of two aluminum electrodes, designed and fabricated to fit the Costar polystyrene six-well-plates, a Leader LFG-1300S function generator, a custom built high voltage amplifier, and a Tektronix 2232 digital storage oscilloscope. The applied field strength (E, V/cm) was computed as $E=\rho I/A$, where ρ is the DMEM/10% FBS resistivity (equal to 95.2 Ω cm), I is the current in amperes and A is the exposure well cross-section area in cm^2. The stray magnetic field intensity in the Forma Scientific incubator (model #3860) was measured with W.H. Bell Gauss/Teslameter (model #9550) and found to range between 0 and 14 μT. The vertical geomagnetic field was found to be 48 μT.

2.3. [^{3}H]Thymidine incorporation

The ability of the externally applied electric field to stimulate DNA synthesis versus similarly treated controls was assessed by monitoring the incorporation of [^{3}H]thymidine into DNA. The procedure previously described by Gospodarowicz et al. [24] and validated by Puzas et al. [25] was followed. Just prior to CCEF exposure, the medium was replaced with 5 ml fresh DMEM/10% FBS, the top electrode was inserted into the well, and the cover was placed over the electrode. The exposure was started by applying an 800 V (peak-to-peak) signal at the desired frequency (10–18 Hz). The applied frequency and amplitude of the voltage together with the resultant current were continuously monitored by the digital oscilloscope throughout the 30 min CCEF exposure period and was stopped by disconnecting and removing the electrodes. Control plates were similarly treated, but were not subjected to an electrical signal.

After CCEF exposure, the cells were incubated for a total of 18 h. At 16 h of incubation, [^{3}H]thymidine (final activity of 1.0 μCi/ml), deoxycytidine (final concentration of 0.1 mM) and cold thymidine (final concentration of 0.1 mM) were added to wells 1 through 3 that were labeled for [^{3}H]thymidine incorporation [26]. Deoxycytidine (final concentration of 0.1 mM) and cold thymidine (final concentration of 0.1 mM) were added to wells 4 through 6 that were labeled for DNA analysis. [^{3}H]thymidine incorporation was stopped after 2 h by removing the medium and washing the cell layer twice with PBS. A cotton swab moistened with 12.5% trichloroacetic acid (TCA) was used to collect the cell layer from each of the wells labeled for [^{3}H]thymidine incorporation. The swabs were washed twice in 12.5% TCA and once in 95% ethanol for 10 min each. The dry cotton tips were cut from the swab and placed in 6 ml of a scintillation cocktail (Scintiverse BD, Fisher Scientific) for counting on a Beckman LS 6000 Series Liquid Scintillation Counter. The radioactivity was corrected for background and recorded as counts per minute (CPM).

DNA analyses were performed on cells from wells 4 through 6 according to the methods by Labarca and Paigen [27]. This assay takes advantage of the fluorochrome Hoechst (2-[2-(4-hydroxypheny)-6-bnzimidazolyl]-6-(1-methyl-4-piperazyl)-benzimidazol 3HCl) a membrane permeable dye which specifically binds AT rich regions of DNA [28]. Hoechst binds DNA such that its fluorescence intensity is linearly proportional to the DNA concentration. Four ml of PBS-2 mM EDTA-Hoechst solution (1.5 nM final concentration of Hoechst 33342, Molecular Probes, Eugene, OR) was added and the wells were allowed to sit overnight at room temperature. The fluorescence of the aspirated suspension was determined with a Perkin Elmer Luminescence Spectrometer LS50 at excitation and emission wavelengths of 356 and 458 nm, respectively, under Perkin Elmer Fluorescence Data Manager Software control. The DNA calibration curve was generated with the sodium salt form of calf thymus DNA (Sigma). The degree of proliferation was expressed as CPM/μg DNA. Statistical analysis was performed using the Student's t-test because of the paired nature (exposed versus unexposed) of the experiments.

2.4. ^{45}Calcium uptake determination

$^{45}Ca^{2+}$ uptake by cells was performed following a procedure described by Farley et al. [29]. Briefly, the cells were removed from the CO_2 incubator and washed twice with 4 ml/well of Tris-BSS pH 7.4 (10 mM Tris-HCl, 140 mM NaCl, 0.8 mM $MgCl_2$, 5.3 mM KCl, 1 mM $CaCl_2$, pH 7.4), at room temperature. $^{45}Ca^{2+}$ was added to wells 1 through 3 labeled for $^{45}Ca^{2+}$ uptake experiments to a final radioactivity of 3.0 mCi/ml. Wells 4 through 6 were reserved for DNA analysis as described above. All wells were exposed in the CO_2 incubator by applying a 16 Hz sinusoidal 800 V (peak-to-peak) signal for 30 min. The signal induced a calculated CCEF of 18.3 μV/cm. At the end of the exposure, the plates were removed from the CO_2 incubator and the wells were washed three times with ice-cold Tris-BSS supplemented with 0.5 mM $LaCl_3$. To the wells labeled for $^{45}Ca^{2+}$ uptake experiments, 1.0 ml of 0.03% triton X-100 (v/v) was added. The wells were allowed to sit overnight at room temperature. Contents were mixed well and 800 μl of the homogenate was added to 6 ml of a scintillation cocktail (Scintiverse BD, Fisher Scientific) for counting on a Beckman LS 6000 series liquid scintillation counter. The radioactivity was corrected for background and recorded as CPM. DNA was analyzed as described before under the [^{3}H]thymidine incorporation section. The degree of $^{45}Ca^{2+}$ incorporation was expressed as CPM/μg DNA. Control plates were similarly treated, but were not subjected to an electrical signal. Additional negative and positive controls were included in each run by adding $LaCl_3$ (final concentration of 0.5 mM) and ionomycin (final concentration of 0.5 μM), respectively, to wells before CCEF exposure. The additional positive and negative control wells were not exposed to the electric field. For digital imaging microscopy experiments run at neutral to physiological pH, the calcium ionophore 4-Bromo-A231827 was used, because ionomycin exhibits limited activity in this range.

2.5. Free intracellular calcium ($[Ca^{2+}]_i$) measurements

$[Ca^{2+}]_i$ was determined by ratio fluorescence spectroscopy using Fura-2 loaded cells and computer controlled Photon Technology International (PTI) Delta Scan System. Both TE-85 and NG108-15 cells were plated on sterile #1, 25-mm cover slips (Fisher Scientific) at 10 000 cells/cm^2 in six-well plates with respective growth medium. The cultures were incubated overnight allowing cells to attach to the cover slips. Cells on cover slips were washed twice with Tris-BSS and were loaded with Fura-2 by incubating in Tris-BSS containing 2 mM Fura-2AM (Molecular Probes, Eugene, OR) for 45 min at 37°C. The unincorporated Fura-2AM was removed by washing the cover slips twice with Tris-BSS. Cells were then incubated at 37°C for 30 min to ensure thorough hydrolysis of the Fura-2AM. The coves slips were then mounted in a stainless steel flow-cell (Atto Instruments, Rockville, MD) for Fura-2 digital ratio imaging.

The PTI Delta Scan System was coupled to an Olympus I×70 Inverted Microscope fitted with an UApo/340 40×Objective, a Chroma Technology Fura-2 filter set and PTI IC100 digital camera. The PTI Delta Scan System was run under Imagemaster (Release II) software control. Fluorescence intensity ratios 340 nm/380 nm (emission wavelength, 510 nm) were collected every 5 s and $[Ca^{2+}]_i$ was computed as:

$$[Ca^{2+}]_i = K_d \frac{S_{f2}}{S_{b2}} \frac{(R-R_{min})}{(R_{max}-R)}$$

where R is the fluorescence intensity ratio from a region of interest of a single cell, S_{f2} and S_{b2} are the fluorescence intensities of Fura-2 at 380 nm in zero and saturated Ca^{2+} condition, respectively. K_d, the Fura-2-Ca^{2+} equilibrium dissociation constant, was taken to be 170 nM [28]. Fura-2 loaded cells were treated with 0.5 μM ionomycin and 5 mM EGTA in Tris-BSS, pH 8.4, to establish the dynamic range of

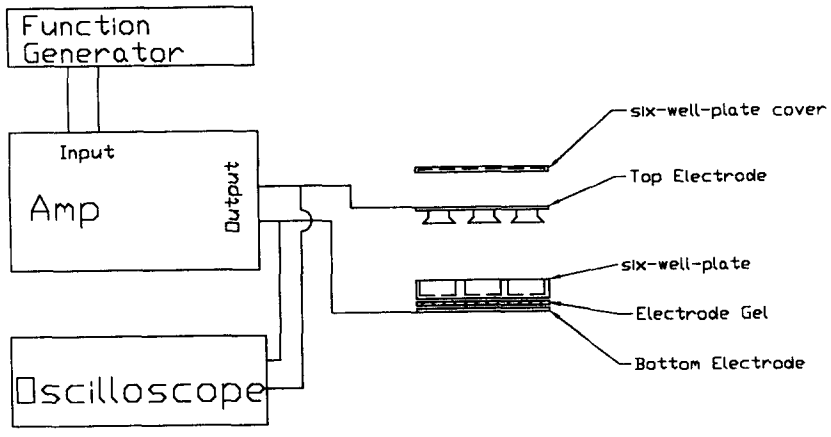

Fig. 1. A schematic of the capacitively coupled electric field (CCEF) exposure apparatus depicting the culture dish and the electrical components.

Fura-2 response to changes in $[Ca^{2+}]_i$ [30,31]. This procedure established the maximum (ionomycin) and minimum (EGTA) fluorescence intensity ratios of 340 nm/380 nm (emission wavelength, 510 nm), corresponding to R_{max} and R_{min}, respectively.

3. Results

Based on data which showed that a 30-min exposure to ELF-EMF increased cell proliferation in the partially growth arrested osteosarcoma cell line TE-85 [11], frequency-dependence of [^{3}H]thymidine incorporation in non-growth-arrested (with serum) TE-85 was investigated. [^{3}H]thymidine incorporation was not significantly different between control and exposed cells (Fig. 2). As shown, [^{3}H]thymidine incorporation in CCEF exposed cells was approximately equal to that found in unexposed cells for all frequencies tested. Since these results did not provide a peak frequency, we decided to investigate $^{45}Ca^{2+}$ uptake at 16 Hz, previously identified by Fitzsimmons et al. [12] as the peak frequency in partially growth arrested TE-85 cells.

The positive control (ionomycin addition) was verified by digital imaging microscopy. Addition of 0.5 μM ionomycin raised $[Ca^{2+}]_i$ from a resting level of approximately 140 nM to the maximum level of approximately 1500 nM in a little over 100 seconds. The trivalent cation lanthanum acts like a nonspecific Ca^{2+} channel inhibitor by replacing calcium at calcium binding sites. Additionally, La^{3+} can mimic the properties of calcium or inhibit the effects of calcium by binding at calcium binding sites. Based on these properties, $LaCl_3$ was used as a negative control. Efforts to verify the negative control ($LaCl_3$ addition) by spectrofluorometry and digital imaging microscopy revealed that Fura-2 bound La^{3+} emitted fluorescence in the same wavelength neighborhood as the Ca^{2+}-bound form (Fig. 3A). As shown in Fig. 3A, the fluorescence spectra for Fura-2-La^{3+} and Fura-2-La^{3+}/Ca^{2+} solutions were identical, suggesting that Fura-2 exhibited a greater affinity for La^{3+} in comparison to Ca^{+2}. Addition of La^{3+} to cells with depleted intracellular Ca^{2+} stores in Ca^{2+}-free extracellular environment revealed that La^{3+} enters TE-85 cells as depicted by the gradual increase in the fluorescence ratios in Fig. 3B. Further, addition of Ca^{2+} and later 4-Bromo-A23187 in the presence of La^{3+} revealed that La^{3+} inhibits but does not eliminate Ca^{2+} entry in TE-85 cells (Fig. 3C). Taken together, these results fully accounted for the relative difference in $^{45}Ca^{2+}$ uptake when La^{3+} was added to TE-85 cells (Fig. 4) both in the presence and absence of ionophores and are consistent with results previously reported by Pillai and Bikle [32] on keratinocyte $[Ca^{2+}]_I$ levels in the presence of La^{3+}. The purpose of inomycin/La^{3+} experiment was to verify the effect of La^{3+} as a negative control in the presence of ionomycin, a powerful Ca^{2+} ionophore. It should be pointed out that 4-Bromo-A23187 was used in the experiments reported in Fig. 4 instead of ionomycin because ionomycin exhibits limited activity at pH 7.0. A pH of 7.0 was preferred for these experiments as opposed to 8.4 to enhance La^{3+} solubility.

The net $^{45}Ca^{2+}$ uptake into both TE-85 and NG108-15 cells were significantly increased by CCEF exposure (18.3 μV/cm) at 16 Hz (Figs. 4 and 5). The La^{3+} controls in NG108-15 experiments are not presented since they were considered unreliable as La^{3+} seemed to enhance NG108-15 cell attachment. DNA from wells treated with 0.5 mM La^{3+} was found to be

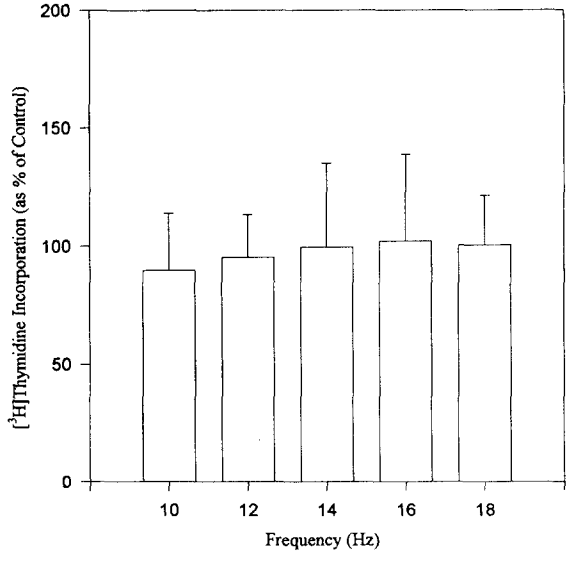

Fig. 2. Effect of capacitively coupled electric field (CCEF) frequency on osteosarcoma TE-85 proliferation, assessed by [^{3}H]thymidine incorporation. The exposure involved applying a sinusoidal 800 V peak-to-peak signal. Presented are means ± standard deviation from 18 to 26 replicates.

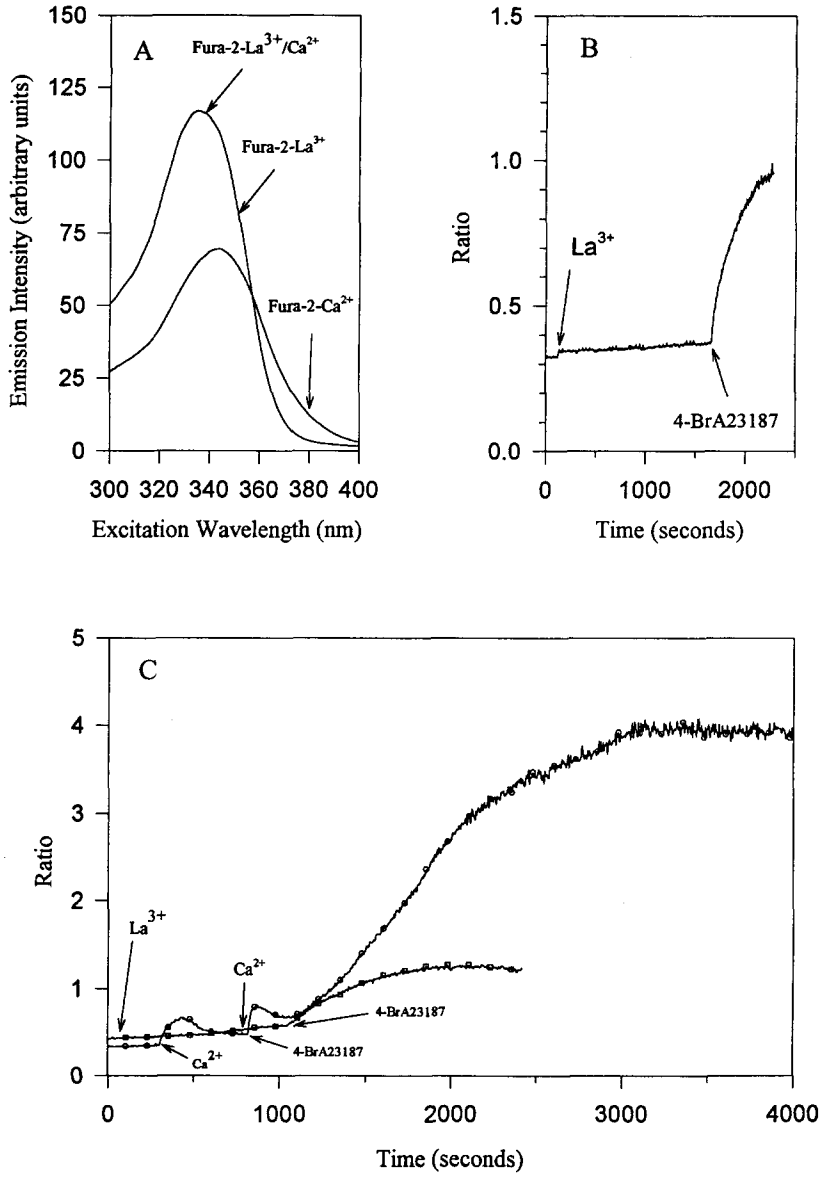

Fig. 3. [A] The 510 nm fluorescence of Ca^{2+}- and La^{3+}-bound Fura-2 overlap extensively at the critical 340/380 nm excitation wavelengths. Also, fluorescence spectra for Fura-2-La^{3+} and Fura-2-La^{3+}/Ca^{2+} solutions were identical, suggesting that Fura-2 exhibited a greater affinity for La^{3+} in comparison to Ca^{2+}. The spectra were obtained with a Perkin Elmer Luminescence Spectrometer LS50. Ca^{2+} and La^{3+} were separately added to 1.0 μM Fura-2 in Tris buffer (pH 7.4) to final concentrations of 0.1 mM, before measurement. [B] Effect of La^{3+} and 4-Bromo-A23187 (calcium ionophore) addition to TE-85 cells. Intracellular Ca^{2+} stores were depleted by incubating cells in Ca^{2+}-free Tris-BBS, pH 8.4, containing 5.0 mM EGTA for 45 min prior to experimentation. The gradual increase in fluorescence after La^{3+} (0.5 mM) addition was attributed to slow entry of La^{3+} into the cells. As expected, addition of 4-Bromo-A23187 (10 μM) facilitated La^{3+} entry in the cells. Data represents average of 16 cells. [C] Effect of La^{3+} on the rate of Ca^{2+} uptake by TE-85 cells. The fluorescence ratio changes on addition of Ca^{2+} (1.0 mM) in the presence (□) and absence (○) of La^{3+} (0.5 mM) suggested that La^{3+} inhibited but did not completely eliminate Ca^{2+} uptake by cells. Data represents averages of 16 cells.

approximately 25% higher that the nontreated control. The quantitative estimates of average changes in $[Ca^{2+}]_i$ were based on the assumption that $[Ca^{2+}]_i$ as determined with Fura-2 is linearly related to $^{45}Ca^{2+}$ uptake and the resting and maximum (in presence of ionomycin) $[Ca^{2+}]_i$ levels correspond to the control (unexposed) and maximum (in presence of ionomycin) cell $^{45}Ca^{2+}$ uptake, respectively. Using resting $[Ca^{2+}]_i$ levels of 67 ± 1 nM [33] for NG108-15 cells and 140 nM ($n = 6$) for TE-85 cells as well as R_{min} of 0.32 and R_{max} of 6.21, the following $[Ca^{2+}]_i$ levels were calculated: 210.3, 203.6 and 188.5 for TE-85; 254.9 and 291.3 for NG108-15 ($n = 3$) from separate experiments. Based on the above calculations, NG108-15 cells exhibited a higher $^{45}Ca^{2+}$ uptake in comparison to TE-85 cells.

4. Discussion

A frequency-dependent increase in TE-85 cell proliferation similar to that previously seen with embryonic chick calvarial cells was reported by Fitzsimmons et al. [12]. The absence of a similar response in the present study was attributed to use of serum in the growth medium, 24 h before ELF-CCEF exposure. Serum deprivation apparently results in growth arrested cell cultures at the G0/G1 transition point [35]. It is possible

that in the present study, the number of cells responding were too few to be detectable with the [^{3}H]thymidine incorporation assay. It is interesting to note that the maximum error in [^{3}H]thymidine incorporation in this study was observed at 16 Hz, previously found to be the peak for net $^{45}Ca^{2+}$ uptake [35].

The CCEF exposure apparatus was composed of a capacitively coupled system patterned after one previously used by Brighton and Townsend [23]. This system exposed cells to electric fields only. The choice of the electric field component was based on detailed previous studies that have demonstrated that the electric field is the critical metric involved in increasing the calcium influx in the lymphocyte model system [34]. The exposure system in these studies was calculated to induce a maximum potential gradient of 18.3 μV/cm in each tissue culture well. The observed increase in $^{45}Ca^{2+}$ uptake by TE-85 in response to ELF-CCEF exposure is consistent with findings by Fitzsimmons et al. [35]. To extend these findings to other cell types we chose NG108-15, an excitable nervous system-like cell. The choice of an excitable cell was based on several factors. First, epidemiological studies have suggested that ELF-EMF exposure may have adverse effects on the nervous system [36]. Second, the ability of ELF-EMF to directly affect cellular calcium homeostasis through unknown mechanism(s), coupled with the ability of specific voltage-dependent Ca^{2+} channel blockers to antagonize the effect [37–39], suggests the involvement of voltage-dependent Ca^{2+} channels. Yet, the majority of ELF-EMF-induced $[Ca^{2+}]_i$ changes have been demonstrated on immune system and cells/tissues of the neuroendocrine system [40,41] that are non-excitable. NG108-15 (glioma×neuroblastoma hybrid) cells have been shown to express a range of calcium currents similar to those observed in neurons and therefore serve as a convenient alternative model for the study of biological phenomena involving voltage-sensitive calcium channel conductances [21]. An increase in $^{45}Ca^{2+}$ uptake by NG108-15 in response to ELF-CCEF exposure was observed. Previous ELF-EMF exposure studies with neuroblastoma cells have mainly focussed on neurite damage or outgrowth [42,43] as endpoints. The present study therefore constitutes the first demonstration of $^{45}Ca^{2+}$ uptake by neuroblastoma and/or glioma cells in response to ELF-CCEF exposure.

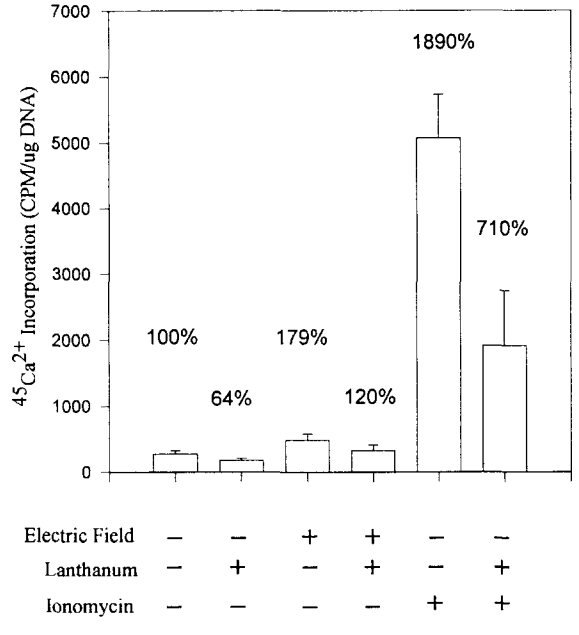

Fig. 4. CCEF exposure (16 Hz 800 V peak-to-peak, 30 min) significantly increased $^{45}Ca^{2+}$ uptake by TE-85 cells in comparison to the unexposed control ($P=0.000049$). Data represents three replicates and n = 3 in each run. + and − mean presence and absence, respectively. Numbers above each bar represent percentage change in $^{45}Ca^{2+}$ incorporation in comparison to the control.

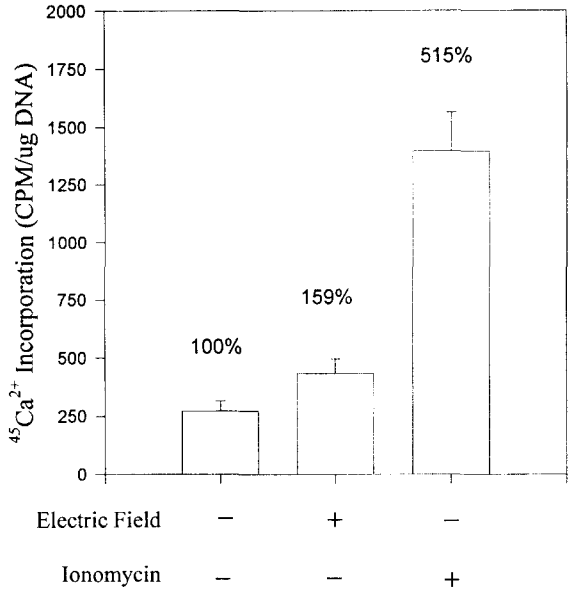

Fig. 5. CCEF exposure (800 V peak-to-peak, 16 Hz, 30 min) significantly increased $^{45}Ca^{2+}$ uptake by NG108-15 cells in comparison to the unexposed control ($P=0.00005$). Data represents three replicates and $n=3$ for each run. + and − mean presence and absence, respectively. Numbers above each bar represent percentage change in $^{45}Ca^{2+}$ incorporation in comparison to the control.

The quantitative estimates of average changes in $[Ca^{2+}]_i$ were based on the assumption that $[Ca^{2+}]_i$ as determined with Fura-2 is linearly related to $^{45}Ca^{2+}$ uptake and the resting $[Ca^{2+}]_i$ level corresponds to the control (unexposed) cells $^{45}Ca^{2+}$ uptake. The estimated $[Ca^{2+}]_i$ levels; 189-210 nM for TE-85 and 255–291 nM for NG108-15 are within the general range of $[Ca^{2+}]_i$ changes published by Lindström et al. [40], who exposed Jurkat cells to a 5–100 Hz 0.15 mT EMF and examined $[Ca^{2+}]_i$ in individual cells using Fura-2. Within a minute of 25 Hz exposure, $[Ca^{2+}]_i$ increased from baseline levels of 70–120 nM to 200–245 nM. It should be pointed out that the $[Ca^{2+}]_i$ changes reported in the present study are averages from the whole cell population. Since in most single cell studies not all cells respond [40], it is reasonable to suggest that the actual single cell $[Ca^{2+}]_i$ changes in response to ELF-CCEF exposure to cells in this study would probably be higher than the reported values.

The calculated $[Ca^{2+}]_i$ changes in response to ELF-CCEF exposure were 30–40% higher for NG108-15 in comparison to TE-85 cells. Although no definitive experimental evidence has been provided to support the hypothesis that the regulation of calcium conductivity at the cell plasma membrane is a potentially critical process through which ELF-EMF could significantly interact with cells [44], it is tempting to attribute the difference in $[Ca^{2+}]_i$ changes between TE-85 and NG108-15 cells to the differences in voltage-activated Ca^{2+} channels in their plasma membranes which may lead to different trans-

membrane calcium conductances. In excitable cells, transmembrane calcium conductances are dominated by high-voltage acting L- and N-type channels, while nonexcitable cells commonly demonstrate the low-voltage activating T-type channels [45,46].

The results from the present study have suggested that serum deprivation, which results in a large percentage of growth arrested cells (i.e., partially synchronized) may be a requirement for frequency-dependent increase in proliferation, but is not necessary for $^{45}Ca^{2+}$ uptake effects in response to ELF-CCEF exposure. The present study also constitutes the first demonstration of $^{45}Ca^{2+}$ uptake by neuroblastoma and/or glioma cells in response to an electric field exposure.

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