Volume 301, number 1, 53–59 FEBS 10912 April 1992

Calcium signaling in lymphocytes and ELF fields

Evidence for an electric field metric and a site of interaction involving the calcium ion channel

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Received 30 January 1992; revised version received 24 February 1992

Calcium influx increased during mitogen-activated signal transduction in thymic lymphocytes exposed to a 22 mT, 60 Hz magnetic field ($E_{induced}$= 1.7 mV/cm, 37°C, 60 min). To distinguish between an electric or a magnetic field dependence a special multi-ring annular cell culture plate based on Faraday's Law of Induction was employed. Studies show a dependence on the strength of the induced electric field at constant magnetic flux density. Moreover, exposure to a pure 60 Hz electric field or to a magnetically-induced electric field of identical strength resulted in similar changes in calcium transport. The first real-time monitoring of $[Ca^{2+}]_i$ during application of a 60 Hz electric field revealed an increase in $[Ca^{2+}]_i$ observed 100 s after mitogen stimulation; this suggests that the *plateau phase* rather than the *early phase* of calcium signaling was influenced. The hypothesis was tested by separating, in time, the early release of calcium from intracellular stores from the influx of extracellular calcium. In calcium-free buffer, 60 Hz fields exerted little influence on the early release of calcium from intracellular stores. In contrast, addition of extracellular calcium during exposure enhanced calcium influx through the plasma membrane. Alteration of the *plateau phase* of calcium signaling implicates the calcium channel as a site of field interaction. In addition, an electric field exposure metric is mechanistically consistent with a cell-surface interaction site.

Intracellular calcium; Calcium transport; Signal transduction; Lymphocyte; Extremely-low-frequency (ELF) electric and magnetic field

1. INTRODUCTION

There is growing scientific interest in understanding how extremely-low-frequency (ELF) electromagnetic fields in our environment may influence biological systems. Two important questions currently addressed in our laboratory are (1) 'What is an appropriate exposure metric for biological responses - the electric or the magnetic field?', and (2) 'What is the mechanism or site of interaction?' The first question is important because human exposure guidelines must be based on a biologically relevant metric. Both questions are linked, however, since knowledge of the exposure field metric relates directly to a mechanism and site of interaction. Biological responses to ELF fields necessarily encompass both the potential health risks associated with ELF fields and the beneficial uses of ELF fields such as accelerating bone and soft tissue wound repair [1,2].

Recently we reported that 60 Hz magnetic fields (22 mT, $E_{induced}$ = 1 mV/cm, 37°C, 60 min) enhance mitogen-activated calcium influx in rat thymic lymphocytes; resting cells were unresponsive to the fields [3]. This synergistic interaction with mitogen indicates the fields as a *co-mitogen* to influence calcium signaling [4,5]. Moreover, lymphocytes from older animal donors, which displayed reduced or suboptimal mitogen-activation, were most responsive to the field [4,5]. This underscores the important role the *biological state* of the system plays in the field interaction. We have now used this cell model to address the question of an exposure metric using a special multi-ring annular cell culture plate. This device enabled all cells on the plate to be exposed to a uniform magnetic field, while cells in each annular well experienced an induced electric field which varied with the annular-well radii according to Faraday's Law of Induction. Calcium transport was found to scale with the induced electric field, and not with the applied magnetic field. As a further test, magnetically-induced and pure electric field exposures were compared and similar increases in calcium transport were observed, providing additional support for the electric field as an exposure metric.

The question of an interaction mechanism and site of interaction was addressed using a special cuvette exposure system, and we report the first measurements of $[Ca^{2+}]_i$ during signal transduction in lymphocytes exposed to 60 Hz fields using the fluorescent probe FURA-2. This approach allowed differentiation between a field effect of the influx of extracellular calcium

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across the outer cell membrane from that on calcium release from internal stores. $[Ca^{2+}]_i$ rises in response to ligand binding at the T-cell receptor complex in two temporally distinct phases. A rapid rise in $[Ca^{2+}]_i$ occurs immediately following ligand-binding as a result of calcium release from intracellular stores, mostly from the endoplasmic reticulum [6,7]. Following this *early phase*, a specialized, ligand-gated calcium channel is opened and extracellular calcium enters the cell to sustain a *plateau phase*, thought to be associated with continuous receptor occupancy and with T-cell activation [8–10]. We report here that when thymocytes are placed into calcium-free media, the initial rise in $[Ca^{2+}]_i$ in response to mitogen is minimally influenced by the presence of a 60 Hz electric field. After adding extracellular calcium to these cells an increase is observed in the influx of calcium across the cell membrane. These findings provide evidence that the cell plasma membrane and the calcium channel therein, in contrast to internal cellular structures, is involved in this field interaction. A cell-surface interaction site is mechanistically consistent with an electric field metric since the induced electric field in the media does not significantly penetrate the plasma cell membrane to act on internal cell structures.

Preliminary results of this work have been presented in abstract form [11].

2. EXPERIMENTAL

2.1. *Rat thymocytes*

Thymic lymphocytes from male Sprague–Dawley rats (250–350 g) were employed, as described [3–5]. Assay buffer was 145 mM NaCl, 1 mM $CaCl_2$, 5 mM Glucose, 10 mM Na-HEPES at pH 7.4, 285 mOsm, 1.685 S/m (37±0.05°C). Cell viability before and after field exposures determined by nigrosine dye exclusion was typically ≥ 97%.

2.2. *Calcium transport*

Calcium movement was followed using a rapid, one-step $^{45}Ca^{2+}$ centrifugation through a cushion of non-aqueous dibutyl phthalate (DBP) [5,12]; DBP effectively removes aqueous-phase calcium not compartmentalized inside the cell. Assay conditions: thymocytes at 1×10^7/ml, 3 μCi/ml $^{45}Ca^{2+}$, 1 μg/ml of *Concanavalia ensiformis* (Concanavalin A, Con A) (when required), 60 min at 37±0.05°C.

2.3. *Intracellular calcium measurements*

$[Ca^{2+}]_i$ was determined by ratio fluorescence spectroscopy using Fura-2-loaded thymocytes and a computer-controlled Perkin-Elmer LS-5B spectrofluorimeter [5]. Cells were loaded by incubation at 1×10^7/ml in assay buffer using 2 μM Fura-2/AM with gentle shaking in the dark for 45 min at 35°C. Viability as determined by nigrosine dye exclusion was typically >96% postloading. Fura-2 thymocytes were washed with assay buffer and an aliquot was checked to determine if biological activity to Con A was retained; Con A at a mitogenic dose of 1 μg/ml typically results in at least a doubling of $[Ca^{2+}]_i$ over 15 min at 37°C. In addition, Fura-2-loaded thymocytes were treated with 5 μM ionomycin and then with 2 mM $MnCl_2$ or EGTA to establish the dynamic range for the probe's response to changes in $[Ca^{2+}]_i$ [6,13,14]. This latter procedure determines the maximum (ionomycin) and the minimum ($MnCl_2$/EGTA) fluorescence intensity ratios of 340 nm/380 nm (emission wavelength, 509 nm), corresponding to R_{max} and R_{min}, respectively. Intracellular calcium was computed as $[Ca^{2+}]_i = K_d \times [(R - R_{min})/(R_{max} - R)][\beta]$, where R is the fluorescence intensity ratio of the sample at 340 nm/380 nm (emission wavelength, 509 nm), β is the ratio of fluorescence of Fura-2 at 380 nm in zero and in saturating Ca^{2+}, and K_d was taken to be 224 nM at 37°C [13,14]. Thymocytes in Ca^{2+}-free buffer were obtained immediately before use by pelleting cells in 5 s at 12,000 rpm (7,000 × g), aspirating assay buffer, and resuspending cells in Ca^{2+}-free buffer. Ca^{2+}-free buffer contained trace calcium at 3–4 μM as determined by pH titration to Ca-EGTA equimolarity [15].

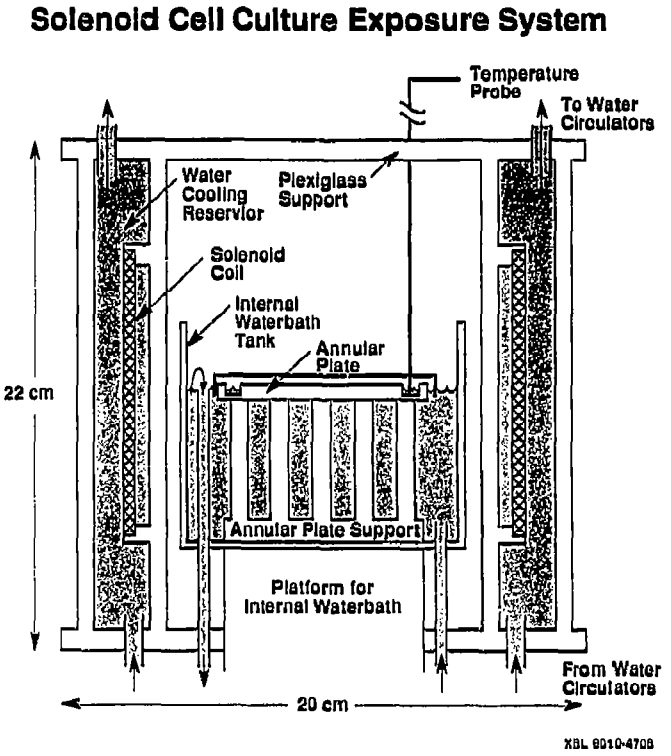

Fig. 1. Solenoidal cell culture exposure system. A uniform magnetic field is established in the center volume of a water-cooled solenoid (n = 400, 2.3 Ω, 20.1 mH) where a specialized multi-ring annular cell-culture plate is positioned. The cell-culture plate is isolated from the solenoid, vibration-free, and temperature-regulated to 37 ± 0.05°C.

2.4. *Solenoidal magnetic field exposure system*

A solenoidal coil encased in a water-jacketed, cooling housing (6 l/min) was fabricated at the Magnet Winding Facility at LBL (n = 400, 2.3 Ω, 20.1 mH) (Fig. 1). Magnetic flux density was mapped using an axial Hall effect probe (Bell, Model 620) calibrated at the Magnetic Field Measurements Group at LBL. A waterbath assembly was located in the center of the solenoid with the plane of the cell-culture plate perpendicular to the magnetic field. Temperature of media in the annular wells was 37±0.05°C; temperature rise due to Joule heating from a 22 mT 60 Hz magnetic field in a non-temperature-regulated plate over 60 min is less than 10^{-6} °C. Control treatment of cells took place simultaneously in time in a remote water bath tank using identical cell-culture plates having an acrylic plug. The ambient DC geomagnetic field and the ambient 60 Hz field perpendicular to exposed and control plates was 20.5 μT and 0.5 μT, respectively.

2.5. *Multi-ring annular cell culture plates*

Cells were placed in multi-ring annular wells, shown in Fig. 2A, and exposed to a uniform magnetic field flux density. According to Faraday's Law of Induction, $E_{induced}$ $(V/m) = (r)(f)(\pi)(B_o)$, where r is the annular radius in meters, f is the frequency in Hz, and B_o is the magnetic flux density in Tesla [16]. The electric field induced in the media is also associated with a current density given by $J = \sigma \times E_{induced}$,

where σ_{media} was measured to be 1.685 S/m (Yellow Springs Conductance Meter, Antioch, OH, USA). Annular plates were made from optical-grade acrylic with scaling factors for the radii of the wells of 1:3:5; inner annular ring diameter is 0.005 m. Plugged wells shown in Fig. 2A were used for control thymocytes placed in a remote waterbath tank. The electric field induced by the ambient 60 Hz background field of 0.5 μT (3.86×10^{-5} mV/cm; outer well) is 4.4×10^{4} less than that due to an applied 22 mT magnetic field (1.7 mV/cm; outer well). However, since acrylic plugs are present in the control treatment wells this 3.86×10^{-5} mV/cm electric field induced in the outer well by the ambient 0.5 μT 60 Hz magnetic field is further reduced to negligible values.

To compare a magnetically-induced and a pure electric field exposure, a multi-ring annular plate was modified to permit direct application of a 60 Hz electric field. Fig. 2B shows an annular-ring plate with acrylic plugs and two agarose salt bridges mounted to apply a 60 Hz electric field to the outer ring of the plate from a calibrated current source. Ultrapure DNA agarose (Cat. no. 162-0125, Bio-Rad, Richmond, CA) was employed at 3% (w/v) in assay buffer (σ = 1.685 S/m).

2.6. *Fluorescence cuvette for real-time measurements of $[Ca^{2+}]_i$ during exposure to 60 Hz electric fields*

The first real-time measurement of $[Ca^{2+}]_i$ during exposure to a spatially uniform 60 Hz electric field was accomplished with a cuvette fitted with two platinum electrodes embedded in agarose walls and positioned at opposing end of a standard 1 cm pathlength cuvette (Fig. 3); one platinum electrode had an optical slit to enable right-angle fluorescence measurements. In contrast, a solenoid coil placed around the cuvette will generate a uniform magnetic field in the cuvette but not a uniform induced electric field. $E_{induced}$ will vary from a maximal value at the periphery of the cuvette to zero at the center. As a result excitation light and emission light would each traverse this electric field gradient fluorescence measurements.

3. RESULTS

3.1. *60 Hz magnetic field exposures and calcium influx in mitogen-activated thymocytes during exposures: induced electric field dependence*

Thymocytes were exposed at 22 mT in the middle and in the outer rings with control-treated thymocytes simultaneously positioned in plugged-well plates located in a remote waterbath tank. Cells in the outer and in the

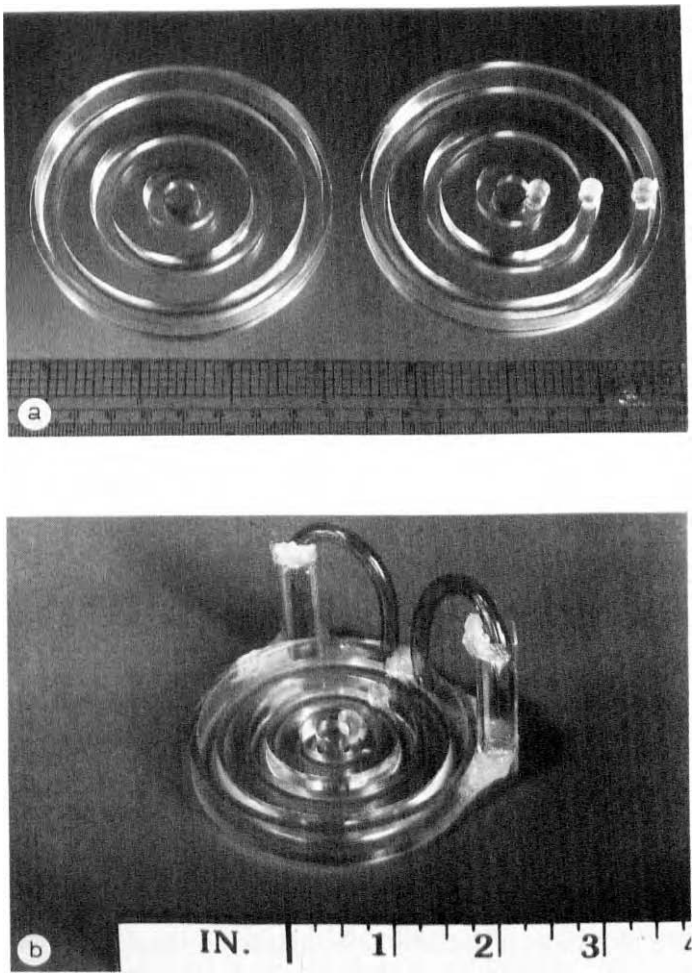

Fig. 2. Annular well cell-culture plates. A: acrylic cell-culture plates have three wells with radii scaled as 1:3:5 (inner diameter, 0.005 m). Specialized wells are permanently plugged with an acrylic dam to reduce $E_{induced}$ during magnetic field exposures. B: modified cell-culture plate to enable application of a 60 Hz electric field to a plugged outer well using agarose bridges to carry current.

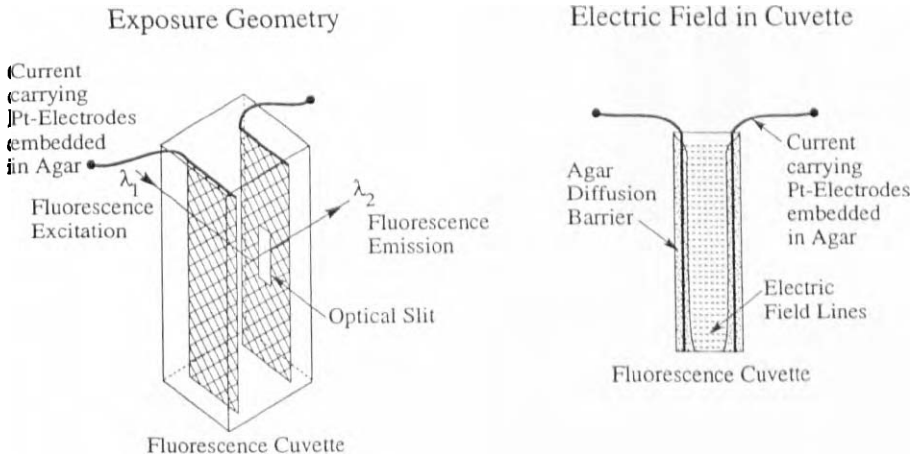

Fig. 3. Fluorescence cuvette exposure device. A spatially uniform electric field is established across the cuvette volume by opposing platinum electrodes embedded in agarose. Real-time monitoring of fluorescence is possible during application of an electric field.

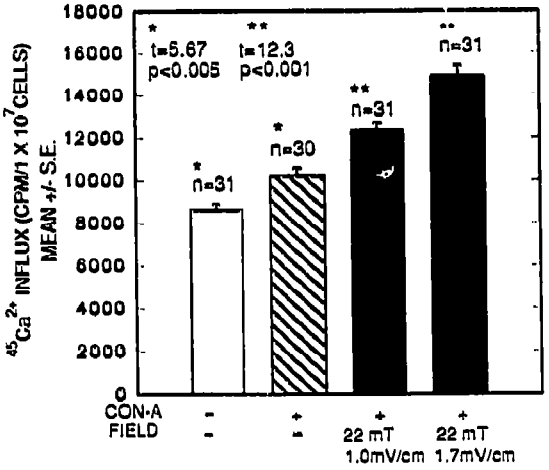

Fig. 4. Induced electric field dependence of calcium influx in activated thymocytes. Cells were exposed in a solenoid to a uniform, 60 Hz, 22 mT magnetic field and simultaneously to $E_{induced}$ of either 1.0 mV/cm (middle well) or 1.7 mV/cm (outer well): 1 μg/ml Con A, 37 ± 0.05°C, 60 min. Data subjected to a one-tailed *t*-test: * $t = 5.67$, $P < 0.005$, df = 59 for a comparison of resting to Con A-activated thymocytes (no field); ** $t = 12.3$, $P < 0.001$, df = 60 for a comparison of 1.0 to 1.7 mV/cm electric field treatment of Con A-activated thymocytes.

inner ring experienced an $E_{induced}$ of 1.7 mV/cm and 1.0 mV/cm, respectively. Fig. 4 shows thymocytes exhibited enhanced calcium influx during Con A activation in the presence of the 60 Hz magnetic field compared to cells activated in the absence of the magnetic field. We observed a 31%, 52%, and 82% increase in uptake above background levels (no Con A) for Con A treatment, Con A plus 1.0 mV/cm, and Con A plus 1.7 mV/cm, respectively. This corresponds to a 1.67-fold and a 2.65-

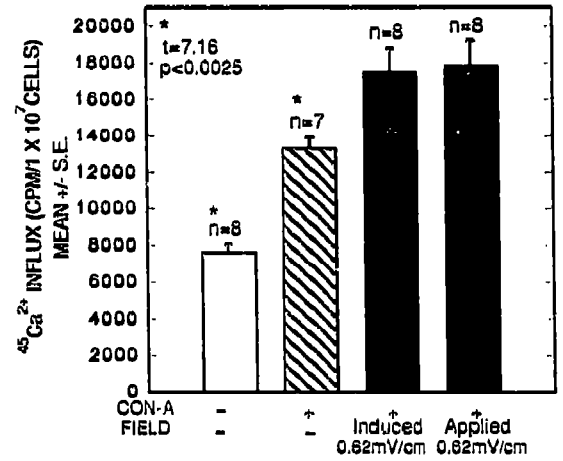

Fig. 5. Comparison of induced and applied electric fields on calcium influx in activated thymocytes. Cells were exposed to electric fields of 0.62 mV/cm using a magnetic field with an annular ring plate, as in Fig. 2A, or using a cell culture plate modified to apply a 60 Hz electric field via agarose bridges, as in Fig. 2B. Data subjected to a one-tailed *t*-test: * $t = 7.16$, $P < 0.0025$, df = 13 for a comparison of resting to Con A-activating thymocytes (no field). Induced and applied electric field treatments of Con A-activated thymocytes were not significantly different.

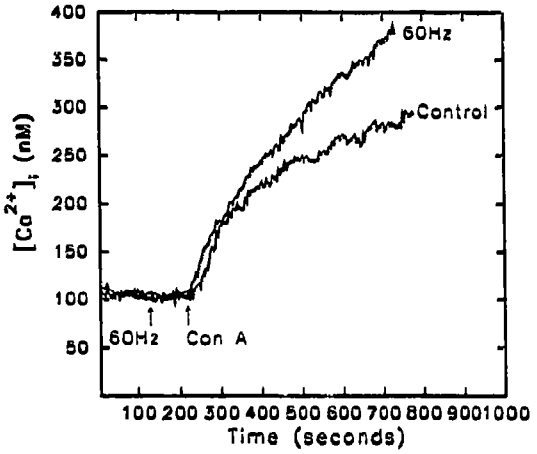

Fig. 6. Real-time measurements of $[Ca^{2+}]_i$ in thymocytes during exposure to 60 Hz electric fields: *plateau phase* involvement. Thymocytes activated with Con A at 1 μg/ml in the presence or absence of a 1.7 mV/cm electric field.

fold increase for $E_{induced}$ = 1.0 and 1.7 mV/cm, respectively. Thus, a synergistic effect was observed that scaled with the induced electric field at constant magnetic field strength.

3.2. *Both induced and applied 60 Hz electric fields enhance mitogen-activated calcium influx in thymocytes*

If the electric field is the metric of biological interest, a magnetically induced or a pure electric field, at comparable field strengths, should yield similar effects on calcium signaling. This hypothesis was tested by exposing thymocytes to a 13.7 mT, 60 Hz magnetic field ($E_{induced}$ = 0.62 mV/cm, middle well) or to an applied 60 Hz electric field of 0.62 mV/cm. Fig. 5 shows that comparable responses were observed for an applied or an induced 60 Hz electric field of 0.62 mV/cm.

3.3. *Real-time measurements of $[Ca^{2+}]_i$ in thymocytes during exposure to 60 Hz electric fields: early and plateau phase of mitogen-induced calcium signaling*

To evaluate early calcium responses to 60 Hz electric fields the first real-time measurements of $[Ca^{2+}]_i$ during field exposure were performed. Fig. 6 illustrates a typical time course for $[Ca^{2+}]_i$ during Con A activation in the absence or presence of a 1.7 mV/cm 60 Hz electric field. Before Con A was added to the thymocytes $[Ca^{2+}]_i$ was stable at about 105 nM. When Con A was added at approximately 200 s, $[Ca^{2+}]_i$ increased during the *early phase* of calcium signaling. $[Ca^{2+}]_i$ subsequently reached a value of approximately 300 nM at 800 s. Fig. 6 also depicts the time course of $[Ca^{2+}]_i$ for cells continuously exposed to a 60 Hz electric field (1.7 mV/cm). In the absence of Con A no change was detected in $[Ca^{2+}]_i$ when the electric field was applied at 130 s. At approximately 200 s, Con A was added and an increase

in $[Ca^{2+}]_i$ was observed that was identical to that shown for unexposed cells over the first 100 s. After this time point, however, $[Ca^{2+}]_i$ increased at a greater rate than for cells in the absence of the field. By 800 s the *plateau phase* of $[Ca^{2+}]_i$ reached approximately 380 nM. The above findings support the hypothesis that the electric field influences the *plateau phase* of calcium signaling. This implicates the calcium ion channel of the outer plasma membrane as a possible site of field interaction, in contrast to structures associated with calcium release from intracellular stores.

3.4. *Real-time measurements of $[Ca^{2+}]_i$ in thymocytes during exposure to 60 Hz electric fields: separation of the early vs. plateau phase of mitogen-induced calcium signaling*

To test the above hypothesis studies were performed in which the *early* and *plateau phase* of calcium signaling were separated in time. A temporal dissection is possible by placing cells in calcium-free media, and then adding calcium to the buffer.

Fig. 7 depicts this sequence of events for thymocytes in calcium-free buffer treated first with Con A and then calcium. At approximately 200 s mitogen was added and $[Ca^{2+}]_i$ increased to approximately 200 nM at 600 s corresponding to the *early phase* and release of calcium from intracellular stores. When extracellular calcium was added to the cells at approximately 670 s the *plateau phase* for $[Ca^{2+}]_i$ was observed to reach 390 nM at 800 s. Fig. 7 also depicts this sequence of events for thymocytes exposed to a 60 Hz electric field of 1.7 mV/cm. In the absence of Con A when the 60 Hz electric field was applied no change in $[Ca^{2+}]_i$ was detected; this is consistent with observations presented in Fig. 6. In Fig. 7, when Con A was added to non-field exposed cells in calcium-free buffer there followed an increase in $[Ca^{2+}]_i$, and an essentially identical increase was observed for Con A-treated cells exposed to the 60 Hz electric field. Thus, the electric field does not appear to significantly influence the *early phase* of calcium signaling. When extracellular calcium was added to thymocytes in calcium-free buffer during field exposure an increase in $[Ca^{2+}]_i$ was observed to levels of approximately 490 nM at 800 s, which was greater than that for control-treated cells. These observations plus those in Fig. 6 indicate that the electric field influenced the *plateau phase* of calcium signaling, and, thus, calcium movement across the outer cell membrane.

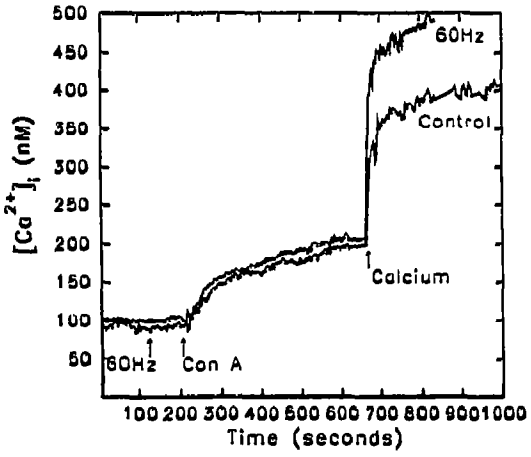

Fig. 7. Real-time measurements of $[Ca^{2+}]_i$ in thymocytes during exposure to 60 Hz electric fields: temporal dissection of *early* and *plateau phase*. Cells in calcium-free assay buffer were treated as in Fig. 6. This corresponds to the *early phase* response of calcium signaling. Extracellular calcium was subsequently added to initiate calcium influx and the *plateau phase* of calcium signaling.

4. DISCUSSION

In this study special exposure devices were designed to aid in hypothesis testing, and experimental findings indicate the electric field is a biologically relevant exposure metric, and that the calcium ion channel in the thymocyte cell membrane is a likely site for field interaction.

These two main findings are mechanistically complementary. At the cellular level an electric field, whether magnetically-induced or directly applied using electrodes, will exert its influence at the cell surface and not the cell interior [17]. At ELF frequencies the electric field does not penetrate the outer cell membrane effectively since the cell bilayer acts as an electrical insulator. The dominant physical effects involve generation of cell surface current, the movement or displacement of charged moieties on the outer leaflet of the bilayer membrane, and structural changes (e.g. protein shedding, or lipid/protein conformational alterations) associated with these effects. For these reasons, an applied electric field is not expected to directly influence internal cell structures or components such as DNA, RNA, and nuclear protein. In contrast, a 60 Hz magnetic field will uniformly penetrate the cell, and, if there are no ferromagnetic structures, the main interaction is induction of an electric field according to Faraday's Law, as discussed above. It is important to emphasize, however, that the induced electric field inside a lymphocyte is minute since the cell radius is of the order of microns. For example, a 22 mT magnetic field will induce an electric field inside a thymocyte of approximately 10^{-6} mV/cm. In contrast, the electric field induced in the outer well of the annular plate is 1.7 mV/cm which is experienced at the outer surface of the thymocyte cell membrane.

The cell surface has been suggested as a likely site of interaction for electromagnetic fields [18–21]. Both the erythrocyte and the lymphocyte, for example, experience alterations in calcium membrane transport and the release of proteins from the cell surface in response to 60 Hz and to microwave fields [19,22]. Altered calcium ion transport has been reported independently for

mouse spleen lymphocytes exposed to sinusoidal 60 Hz electric field [23]; this may relate to a suppression of T-lymphocyte cytotoxicity [24]. In synthetic phospholipid vesicle bilayers free of protein electromagnetic fields are reported to alter lipid bilayer permeability [25,26]. Antibody binding to signal transduction elements of the parathyroid hormone receptor in the cell membrane is altered during magnetic field treatment [27]. This suggests a way, in addition to protein shedding, in which fields may influence signal transduction events in intact cells. Interestingly, applied and induced electric fields of ≤ 4 mV/cm in the frequency range of 30–300 Hz have been shown to influence the activity of membrane associated Na-K/ATPase activity [28]; this field intensity is of the order employed here.

Although the findings presented here demonstrate that an electric field is associated with changes in calcium signaling in activated thymocytes, this does not rule out the possibility of magnetic field interactions. For example, magnetic field interactions have been reported for AC/DC magnetic field combinations with the magnetic fields oriented perpendicular [29] or parallel [30,31] to each other. Recently, we reported that a relatively low intensity 16 Hz magnetic field of 42.1 μT in combination with a parallel static (DC) magnetic field of 23.4 μT results in an inhibition of calcium influx in activated thymocytes [12]. With regard to the present study it is important to note that the time-varying magnetic field alone did not influence calcium influx (16 Hz; 42.1 μT; $E_{induced}(max)$ = 0.89 μV/cm). This suggests an operational lower limit for an induced electric field effect. Studies are underway to define a dose–response relationship for the electric field; for example, at constant applied magnetic field and annular radius, the induced electric field can be systematically varied by changing frequency according to Faraday's Law. In addition, the induced current associated with a constant electric field can be varied by changing the conductivity of the media. Based on the above reports and our findings presented here it seems likely that different interaction mechanisms may operate over a range of magnetic field flux densities. Further research is required to establish these relationships.

What biological significance do these findings hold? The phenomenon of signal transduction is central to a wide range of cellular activities triggered by ligand-gated binding of hormones, antigen molecules, autocrine and paracrine substances, and other cell-surface agonists. Calcium signaling is an early step in T-lymphocyte activation, and up or down regulation of calcium signaling by ELF fields is expected to influence subsequent events in mitogenesis [4,5,12]. Alteration of 'downstream' events such as RNA, DNA, and protein synthesis has been reported in cells exposed to time-varying fields [32–34]. Observations presented here suggest a paradigm for understanding ELF field alterations of 'downstream' events distal to calcium signaling: an early field interaction at the cell surface involving calcium movement through membrane channels could represent an important interaction site triggering subsequent changes in RNA, DNA, and protein synthesis.

How could the cell surface mediate up or down regulation of the *plateau phase* of calcium signaling? One way is to modulate Con A binding and we are currently testing this hypothesis: Con A is believed to the TCR-CD3 complex, and can be blocked by α-methyl-D-mannoside [9,35]. In addition, the human Jurkat leukemic T-cell is a good candidate for such studies since somatic mutants are available with alteration in the α and β chains of the TCR [12,36]. Alternatively, since calcium influx is controlled through opening and closing of the ligand-gated calcium channel, it is possible that the ELF field may directly interact with this structure, or with inositol-1,4,5-triphosphate and its phosphorylation product IP_4, which are involved in regulating the opening of this channel. These inositol phosphates regulate the *plateau phase* of calcium signaling, which, in bulk suspensions, as employed here, is observed as a sustained elevation of $[Ca^{2+}]_i$. Studies are planned at the single-cell level which are capable of resolving this average signal into repetitive oscillations characterized by cycling of $[Ca^{2+}]_i$ between resting level troughs and transient peaks [10,37]. Several non-linear oscillator models have been proposed to explain calcium oscillations, and it will be of interest to determine if frequency-encoded signaling at the single-cell level is influenced by ELF fields.

Acknowledgements: Support provided in part by the Office of Energy Management, Utilities Systems Division, the Office of Health and Environmental Research, U.S. Department of Energy under Contract DE-AC-3- 76SF00098, and the NIH under Grant CA53711 from the NCI. Technical assistance was provided by D.J. de Manincor. The helpful comments of D. Callahan, M. Wiessendanger, and M. Roos in preparing this paper are gratefully acknowledged.

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