

Time-varying and static magnetic fields act in combination to alter calcium signal transduction in the lymphocyte

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We have tested the hypothesis that extremely low frequency (ELF) time-varying magnetic fields act in combination with static magnetic fields to alter calcium signalling in the lymphocyte. Results indicate that a 60-min exposure of thymic lymphocytes at $37 \pm 0.05^\circ\text{C}$ to a 16 Hz, 421 mG (42.1 μT) magnetic field simultaneously with a colinear static magnetic field of 234 mG (23.4 μT) (a.c./d.c. field intensity ratio = 1.8) inhibits calcium influx triggered by the mitogen Concanavalin A. Significantly, *resting* lymphocytes do not respond to the fields, thus, only *mitogen-activated* cells undergoing calcium signalling exhibit a field response. These results indicate that signal transduction involving calcium is an important *biological constraint* which operates to mediate this field interaction. Additional split field exposures show that the presence of the a.c. field or the d.c. field alone does not produce an effect. This is consistent with a proposed parametric resonance theory of interaction of low intensity magnetic fields with biological systems (L.L. Lednev (1991) *Bioelectromagnetics* 12, 71–75), which predicts the occurrence of biological effects at specific values for the frequency and field intensity of the ELF and static magnetic fields.

Signal transduction; Calcium signalling; Electromagnetic field; Mitogen activation; Lymphocyte

1. INTRODUCTION

A question that is central to interaction mechanisms of magnetic fields and biological systems is whether a time-varying (a.c.) magnetic field interacts in a fundamentally different manner at the cellular level when a static (d.c.) magnetic field is present [1–7]. This question is of interest since both ELF magnetic fields and geomagnetic static magnetic fields are present in the environment and workplace, and since it is critically important to identify field metrics that have biological relevance.

Recent work from our laboratory has shown that time-varying 60 Hz magnetic fields produce changes in calcium transport in normal thymic lymphocytes that scale according to Faraday's law of current induction; in addition, we show that changes in $[\text{Ca}^{2+}]_i$ triggered by direct application of a 60 Hz current correlate with calcium transport changes triggered by similar levels of 60 Hz current induced by magnetic fields [8,9]. In these studies we assessed calcium signalling in the thymic T-lymphocytes during mitogen activation and we used a special multi-ring annular plate Petri dish designed so that lymphocytes would experience a constant magnetic field, and, at the same time, an induced electric field that

varies with the radii of the annular ring into which the cells were placed ($2\text{--}27 \mu\text{A}/\text{cm}^2$, $16\text{--}220 \text{ Gauss}_{\text{rms}}$ (1.6–22 mT), 60 Hz) [8,9]. Since mitogen-activated calcium transport varied directly with the electric field while the magnetic field was held constant, and since applying a 60 Hz electric current to match the induced current from the magnetic field resulted in similar changes, these studies indicate that the electric field induced by the applied magnetic field is a biologically relevant metric.

In the above 60 Hz studies no attempts were made to alter the ambient linear static magnetic field in our laboratory, which was approximately 350 mG total for exposed and control sample environments. In fact nearly all previously published experimental research from other laboratories employing 50/60 Hz time-varying fields do not control or even measure the static magnetic field. Based on the findings presented here we feel it is important to carefully measure background magnetic fields in the laboratory where exposure and control samples are to be placed. This is particularly important when exposure systems are placed inside cell culture incubators which can exhibit relatively high and spatially non-uniform background static and time-varying magnetic fields. In the studies presented here we have taken the additional precaution of using μ -metal shielding around our exposure and control chambers to significantly reduce stray background magnetic fields.

A.c. magnetic field exposures to humans usually occur in combination with d.c. magnetic fields. In the home and environment these exposures involve magnet-

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ic field flux densities in the 2–400 mGauss range. The experimental evidence supporting the possibility that such low-intensity combined a.c./d.c. fields interact with biological systems comes from studies in which the a.c./d.c. fields are oriented perpendicular [2] or parallel [3] to each other. Perpendicular orientation can be thought of as a nuclear magnetic resonance-type interaction, while the parallel orientation can be thought of as a ‘ q/m ’ cyclotron interaction.

Recently a report has specified field parameters in sufficient detail for testing the hypothesis that a.c./d.c. field combinations interact with biological systems [1]. This parametric resonance theory states that protein-bound ions can behave as a spatial oscillator with a set of vibrational frequencies that depend on the bond energy, charge q , and mass m of the ligand bound ion. The bound ion continuously oscillates about an energy level as coordination bonds are broken and reformed due to random thermal motion. The presence of a static magnetic field splits the energy level of the bound ion into two sublevels, with amplitudes A_1 and A_2 corresponding to electromagnetic frequencies ω_1 and ω_2 in the infrared band. The difference between these two energy levels equals the cyclotron frequency, $\omega_c = (\omega_1 - \omega_2) = (q/m)B_{d.c.}$.

Applying a co-linear a.c. magnetic field modulates the two energy sublevels established by the static field. An a.c. field at the cyclotron frequency modifies the probability, P , of ion transitions between energy states that depends on a Bessel function argument defined by the intensity ratios of the a.c. and d.c. fields.

$$P = 2(A_1)(A_2) [J_1(B_{a.c.}/B_{d.c.})]$$

where J_1 is a Bessel function of order 1. Fig. 1 shows the Bessel function response curve which has a first maxima at $B_{a.c.}/B_{d.c.} = 1.8$. We interpret this to mean that at this field intensity ratio a change in biological response will be maximal; this could be enhancement or inhibition of a process since there is no direct interpretation for the change in ion probability, P . This analysis leads to the same predicted frequency for a given ion as previously proposed for ‘ q/m ’ interactions [3,4], however, it has the additional feature that the a.c./d.c. field intensity ratio is specified. Such specifications enable experimental testing as we report here. This approach also resolves apparent theoretical difficulties associated with ions following spiral cyclotron trajectories in viscous biological fluid since the a.c./d.c. field interaction influences ion binding processes and does not require spiralling cyclotron motion of the ion.

We report here that a combination of a.c./d.c. fields that satisfies the requirements a.c./d.c. = 1.8 = 421 mG/234 mG, 16 Hz, for $^{45}\text{Ca}^{2+}$, leads to decreased calcium influx during mitogen-activated signal transduction in normal rat thymocytes. When the a.c. or d.c. field alone was employed no effect was detected. These findings are

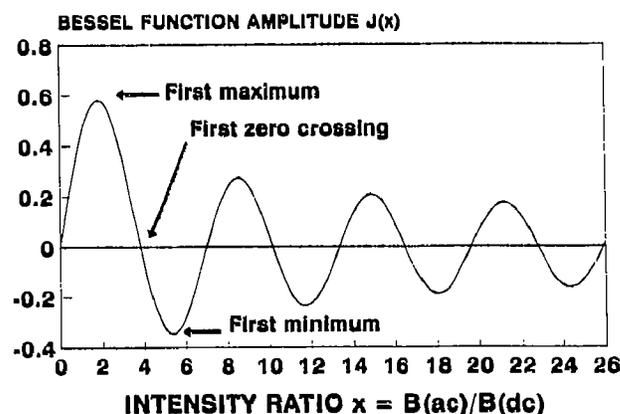


Fig. 1. First order Bessel function, order $n = 1$. The shape of the curve resembles a damped sine wave with a first maximum, a first zero crossing, and a first minimum. Y axis, amplitude of the Bessel function. X axis, argument of the Bessel function which defines the a.c./d.c. magnetic field intensity ratio.

consistent with the theoretical predictions, however, a complete test requires that a.c. frequency be altered to ‘detune’ field coupling, and that other a.c./d.c. field intensity ratios be tested based on the Bessel function response curve.

2. EXPERIMENTAL PROCEDURES

2.1. Biological methods

Male Sprague–Dawley rats between 70–130 g were employed in these studies as a source of thymic T-lymphocytes. Thymocytes were harvested and maintained in assay buffer which consisted of 145 mM NaCl, 1 mM CaCl_2 , 5 mM glucose, 10 mM Na-HEPES at pH 7.4 ($37.0 \pm 0.05^\circ\text{C}$), 1.685 S/m and 285 mOsm. Cell viabilities before and after field exposures were determined by nigrosine dye exclusion and were typically $\geq 95\%$.

Calcium transport across the lymphocyte membrane was monitored using a rapid, one-step $^{45}\text{Ca}^{2+}$ centrifugation procedure [8,9]. Thymic lymphocytes were suspended at 1×10^7 cells/ml in assay buffer at $37.0 \pm 0.05^\circ\text{C}$ and nuclide added to give 3 $\mu\text{Ci/ml}$ at time zero. Five minutes later this sample was split into two aliquots with one aliquot receiving the mitogen Concanavalin A (Con A) to give a final concentration of 1–3 $\mu\text{g/ml}$; cells were housed in a Coulter counter-type square plastic cube filled with 10 ml of sample volume. Samples to be exposed to fields as well as the control samples were then immediately placed into the shielded exposure device or the shielded control chamber for 60 min. At the end of the 60-min exposure period, cell suspensions were removed and 1 ml aliquots immediately and carefully pipetted on top of a 1.0 ml layer of non-aqueous dibutyl phthalate [DBP] residing in 2.5 ml microcentrifuge plastic tubes. These tubes were immediately spun at 12000 rpm for 10 min (25°C) to rapidly, in one-step without cell manipulation, isolate the lymphocytes from the aqueous phase assay buffer; this procedure removes loose cell-surface bound calcium and results in a cell pellet that represents calcium transported into the cell interior. The aqueous phase was then carefully aspirated, followed by the DBP phase, and the tube was swabbed with a cotton-tipped applicator from the bottom up, without disturbing the cell pellet. The cell pellet was carefully snipped into liquid scintillation media for spectroscopy analysis.

2.2. Exposure system and dosimetry

Fig. 2 depicts the coils employed in these experiments. One set of opposing coils was oriented with the horizontal axes co-linear with the geomagnetic N–S direction established in our laboratory (160 turns, 16 gauge copper magnet wire, coil diameter_{int} = 15 cm, opposing coil

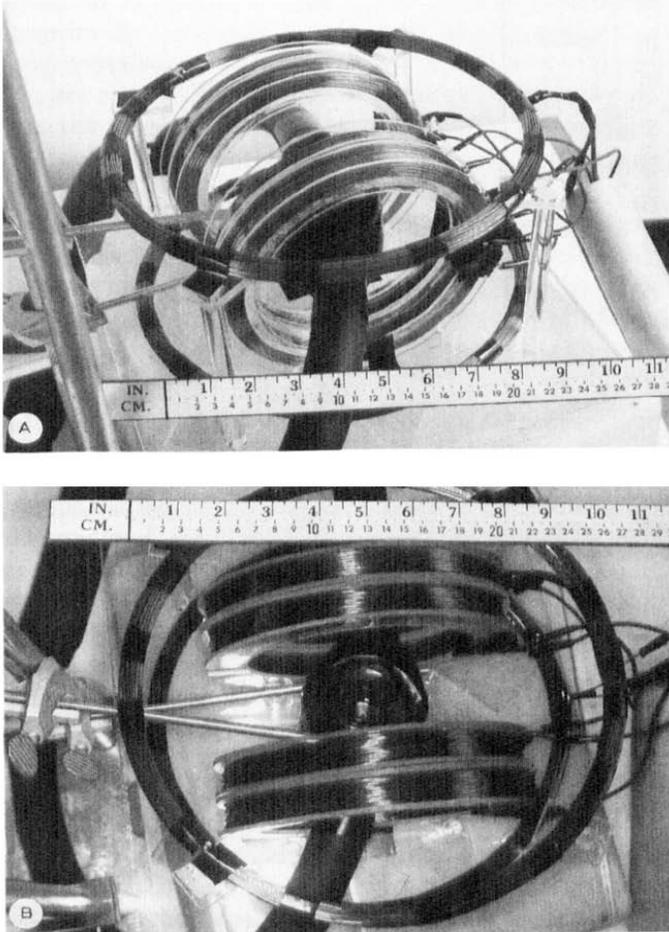


Fig. 2. a.c./d.c. exposure coil device. (A) Two sets of coils were oriented perpendicular to each other such that the inner set established co-linear a.c. and d.c. magnetic fields used to expose samples, and an outer set that nulled the ambient vertical geomagnetic field. The entire exposure device was placed inside a μ -metal shielded box which reduced ambient a.c. and d.c. magnetic fields at least 10-fold. (B) A glass water-jacketed sample holder was located at the center of the coil system so that a 10 ml volume of cells could be exposed at $37 \pm 0.05^\circ\text{C}$. A separate μ -metal shielded styrofoam water bath chamber remote from the exposure device was employed for control samples. (See text for details).

spacing_{ave} = 9 cm). This set of coils was used to generate co-linear a.c. and the d.c. magnetic fields for exposures. A second set of opposing coils was placed perpendicular to the first set such that their axis was vertical (coil diameter_{ave} = 26 cm, coil spacing_{ave} = 13 cm). This set of coils when energized with d.c. current nulled the vertical d.c. field component. This coil arrangement enabled the application of an a.c. and co-linear d.c. magnetic field along the axis of the first set of coils. A field uniformity to within $\pm 3\%$ was achieved for an active exposure volume of 5.0 cm^3 in the geometric center of this device. Field mapping was performed using three d.c. magnetic field probes: FGM-3D1 (Walker Scientific, Inc., Worcester, MA, USA); MAG-01 (Bartington, Instruments, Ltd, Oxford, UK); Model 428B fluxgate meter (Hewlett Packard, Cupertino, CA, USA). Measurements agreed to within $\pm 3\text{--}5\%$. The time-varying a.c. magnetic field was mapped using the Model 428B fluxgate meter from Hewlett Packard, frequency was checked using a calibrated oscilloscope and a rms digital multimeter (Fluke 87, Everett, WA, USA).

It is important to shield the exposure coils from any stray background electromagnetic fields present in the laboratory. We placed our

exposure coils in a plastic cube frame (35 cm^3) which was completely wrapped in μ -metal foil of 0.254 mm thickness (CO-Nectic AA foil, Magnetic Shielding Corp., Bensenville, IL, USA). This reduced ambient background a.c. and d.c. field intensities inside of the shielded chambers to values $\leq 5\text{ mGauss}$ (60 Hz a.c.) and 20 mGauss (d.c.).

For field exposures, a 10 ml suspension of thymocytes at $1 \times 10^7/\text{ml}$ in a plastic square Coulter counter-type tube was placed inside a glass water-jacketed vessel which enabled temperature of the cell suspension to be maintained at $37 \pm 0.05^\circ\text{C}$ (Fig. 2). One flat surface of the square test tube was oriented perpendicular to the incident a.c. and d.c. magnetic fields. This insured that the incident a.c. and d.c. field vectors were perpendicular to a spatially uniform surface element of the sample as the fields traversed the sample volume from front to back. Temperature was monitored using thermistor-type microprobes calibrated against a platinum resistance temperature standard traceable to the National Institute of Technology and Standards, USA.

A control water-bath chamber, built of styrofoam and shielded in μ -metal foil, was located remotely from the exposure samples and was maintained isothermal with the field exposure samples at $37 \pm 0.05^\circ\text{C}$. All experiments involved the simultaneous treatment in time of samples to the a.c./d.c. fields or to the non-field control environment inside the remote water-bath chamber.

The a.c. field frequency chosen in these experiments was 16 Hz since it represents an ELF frequency that is not a harmonic of ambient 50 or 60 Hz fields, and since this a.c. field frequency has been employed in previous a.c./d.c. studies [4]. Given this frequency and the q/m ratio for $^{45}\text{Ca}^{2+}$, we established a d.c. magnetic field of 234 mGauss according to $16\text{ Hz} = 1/2 \pi(q/m)(B_{d.c.})$. The a.c. field intensity was set at 421 mGauss_{rms}, according to a.c./d.c. = 1.8 (Fig. 1). Ambient a.c. and d.c. fields present inside of the μ -metal shielded exposure chamber and the control chamber were ≤ 5 and 20 mGauss, respectively.

2.3. Statistics

Data from treatment groups were subjected to paired *t*-tests in which an assumption of normality for data was made. Raw data from paired treatment groups performed on different days was pooled for analysis.

3. RESULTS

3.1. Effect of a.c./d.c. field combination (a.c./d.c. = 1.8) on mitogen-activated calcium influx

Fig. 3 presents data from experiments in which calcium uptake was measured for resting and for mitogen-activated thymocytes in the absence or presence of the a.c./d.c. field combination. The two bar graphs on the left reveal that calcium uptake increased in a typical fashion for cells treated only with Con-A (no a.c./d.c. fields). The two bars on the right show resting and activated cells, exposed to a.c./d.c. fields. In resting thymocytes (no Con-A) that were exposed to a.c. fields, calcium uptake was identical to that observed for unexposed cells. Thus the a.c./d.c. field combination appears to have no effect on calcium uptake in resting thymocytes. In contrast, mitogen-activated thymocytes assayed for calcium uptake in the presence of the a.c./d.c. fields displayed a level of calcium uptake identical to that observed for resting cells. This data suggests that the a.c./d.c. field combination inhibits calcium influx during Con-A activated signal transduction. Taken together these results suggest that calcium signal transduction triggered by mitogen activation is a critical *biological constraint* that operates to mediate this field effect.

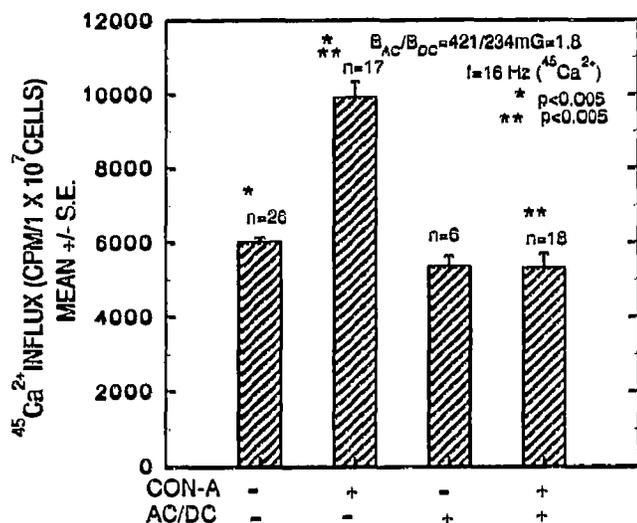


Fig. 3. Effect of a.c./d.c. magnetic field combination (a.c./d.c. = 1.8) on mitogen activated calcium influx in thymocytes. Treatment of cells for 60 min to the a.c./d.c. magnetic field combination or to the control waterbath were carried out simultaneously in time. Frequency = 16 Hz, a.c. = 421 mGauss_{ms}, d.c. = 234 mGauss. Temperature = $37 \pm 0.05^\circ\text{C}$.

3.2. Lack of effect of a 421 mGauss a.c. field on mitogen-activated calcium influx

Calcium uptake experiments were also performed using only a 421 mGauss a.c. field and results are presented in Fig. 4. In these experiments thymocytes were assayed simultaneously during treatment without Con-A, with Con-A, or with Con-A plus the a.c. field. Results indicate that thymocytes responded positively to mitogen by exhibiting increased calcium uptake over that for resting cells. When thymocytes were assayed for calcium influx during mitogen activation in the presence of the a.c. field no significant differences were observed compared to that for mitogen alone.

During construction of the μ -metal shielded chambers we exposed thymocytes to only a static d.c. magnetic field of several hundred mGauss (approximately equivalent to the background geomagnetic field in our laboratory) and determined that this does not alter calcium uptake in resting or in activated thymocytes.

4. DISCUSSION

Previous experimental studies suggest that a.c./d.c. field combinations which follow a ' q/m ' relation act to influence calcium transport of human peripheral blood lymphocytes [3,4]. These lymphocytes were not treated with mitogen, as done here. Other laboratories have not found that a ' q/m ' relation influences calcium transport in human peripheral lymphocytes [10] or influences $[\text{Ca}^{2+}]_i$ in three established cell lines [11]. On theoretical grounds the concept of ' q/m ' resonance based on the cyclotron equation as it applies in free space has been challenged, because, in addition to other problems, ions such as calcium are hydrated and experience heavy

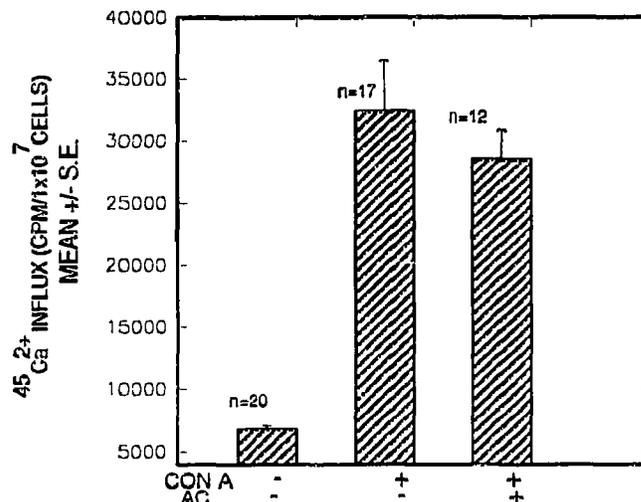


Fig. 4. Effect of a 421 mGauss a.c. magnetic field on mitogen-activated calcium influx in thymocytes. Conditions as in Fig. 3, with the d.c. magnetic field component nulled.

viscous damping in biological systems: such ions are unlikely to experience cyclotron motion in situ [7,12-14]. Some of these issues may be eliminated or minimized if special circumstances are invoked such as restricting a.c./d.c. field interactions to the interior of membrane channels [15], where water may be stripped from ions, or by restricting interaction sites to the cell surface where hydrophobic pockets may exist that strip the hydration shell from ions [5].

Results we report here for *resting* thymocytes exposed to a.c./d.c. fields are consistent with the negative report of Prasad [10] who employed human peripheral blood lymphocytes exposed to a.c./d.c. fields in the absence of mitogen. Our results are also consistent with the negative results of Parkinson [11] who employed three cultured cell lines that were exposed to a.c./d.c. fields in the absence of growth factors or mitogen. The exposure conditions used by these investigators, however, correspond to an a.c./d.c. field intensity ratio of approximately 1.0, not 1.8 (Fig. 1), and they followed the same ' q/m ' equation for calcium ions as we did. Our positive results for *mitogen-activated* thymocytes are different from the positive results reported by Liboff [3] and Rozek [4]. These investigators did not treat their peripheral blood lymphocytes with mitogen; the possibility exists that they stressed or activated the lymphocytes during handling.

An important finding in the series of experiments presented here is that *biological constraints* must be considered in cellular experiments. Work from our laboratory [16], and others referenced therein, have shown that mitogen-activation facilitates responses of lymphocyte cells to electromagnetic fields. Moreover, we have recently reported that the *level* of mitogen activation achieved by thymocytes, which varies inversely with animal age, is a predictor of cellular response to a 60 Hz magnetic field [8,9]. Maximal field responses are

elicited in thymocytes from older animals which are suboptimally activated by mitogen. Our findings that mitogen-activated thymocytes, in contrast to resting cells, are responsive to fields is consistent with and supports the idea that electromagnetic fields can interact with biological systems that are far from equilibrium [17].

Signal transduction plays a critical role in our findings. Calcium influx in the lymphocyte is an early event in signal transduction and calcium is an important second messenger for a wide variety of important cellular processes such as RNA, DNA and protein synthesis; modulation of calcium signalling by electromagnetic fields has the potential to influence these cell functions. Using the fluorescent probe FURA-2 we have recently shown that $[Ca^{2+}]_i$ is altered in Con A-stimulated thymocytes during direct application of 60 Hz current [8]; real-time alterations in $[Ca^{2+}]_i$ were detected within 100 s of field exposure and resting cells were unresponsive. Importantly, other researchers have reported changes in DNA, RNA and protein synthesis, which are dependent on calcium signalling, in cells exposed to time-varying magnetic fields [18–20]. In our studies mitogen activation is assessed by monitoring the early event of calcium influx and it would be of interest to assess in the same cell sample total DNA synthesis or cellular proliferation, which are important indices of cellular activation distal to calcium signalling.

Calcium influx during mitogen-activated signal transduction in the lymphocyte is triggered by ligand binding to the T-cell receptor complex on the cell surface which activates the phosphatidylinositol (PI) pathway [21]. Results in Fig. 3 indicate that thymocytes in the presence of Con-A plus a.c./d.c. fields fail to exhibit enhanced calcium influx: signal transduction is inhibited. Perhaps the simplest explanation for this is an inhibition of ligand (Con-A) binding, which is the first event in the signal transduction process, and this can be directly tested in binding experiments. Alternatively, down-regulation of components of the PI pathway, such as production of inositol 1,4,5-trisphosphate (IP_3), which facilitates opening of a calcium channel to permit extracellular calcium to enter the cell, could be involved. In this case failure of the calcium channel to open effectively in response to successful ligand gating (Con A binding) in the presence of the fields could involve a direct effect on channel structure or conformation. Studies are underway in our laboratory to monitor $[Ca^{2+}]_i$ in real time during a.c./d.c. field exposures which will provide important information on early changes in intracellular calcium before and after Con-A binding. In addition, perhaps the best characterized model system for investigating cell signalling pathways is the Jurkat cell line, a human T-cell leukemia cell line, which has a variety of available subcloned somatic mutants [22]. The Jurkat can be used to address questions about field interactions on specific pathway elements of the

signalling process; for example, the J.RT3T.5 subclone is a β -chain deficient mutant that is T cell receptor negative.

In summary we emphasise that the findings presented here are merely consistent with a '*q/m*' or parametric resonance interaction for a.c./d.c. fields with thymocytes. Our results only indicate that the field combination, in contrast to the a.c. or the d.c. field itself, is effective in influencing calcium signalling in activated thymocytes. Recently calcium uptake in two lymphocytic cell lines and in murine splenic lymphocytes in the presence of mitogenic serum growth factors was reported to be altered by an a.c./d.c. field combination (a.c./d.c. = 1.2) that followed a '*q/m*' relation for calcium [23]; however, field separation exposures were not performed. As in our experiments only one a.c./d.c. field combination was employed. Further research is required to substantiate that a '*q/m*' relation is followed in such experiments. These tests will need to (1) vary a.c. frequency away from that required for the '*q/m*' relation, and (2) vary the a.c./d.c. field intensity ratios according to the Bessel function curve shown in Fig. 1.

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