The Effects of Weak Extremely Low Frequency Magnetic Fields on Calcium/Calmodulin Interactions

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ABSTRACT Mechanisms by which weak electromagnetic fields may affect biological systems are of current interest because of their potential health effects. Lednev has proposed an ion parametric resonance hypothesis (Lednev, 1991, *Bioelectromagnetics*, 12:71–75), which predicts that when the ac, frequency of a combined dc-ac magnetic field equals the cyclotron frequency of calcium, the affinity of calcium for calcium-binding proteins such as calmodulin will be markedly affected. The present study evaluated Lednev's theory using two independent systems, each sensitive to changes in the affinity of calcium for calmodulin. One of the systems used was the calcium/calmodulin-dependent activation of myosin light chain kinase, a system similar to that previously used by Lednev. The other system monitored optical changes in the binding of a fluorescent peptide to the calcium/calmodulin complex. Each system was exposed to a 20.9 μT static field superimposed on a 20.9 μT sinusoidal field over a narrow frequency range centered at 16 Hz, the cyclotron frequency of the unhydrated calcium ion. In contrast to Lednev's predictions, no significant effect of combined dc-ac magnetic fields on calcium/calmodulin interactions was indicated in either experimental system.

INTRODUCTION

The question of how, and indeed whether, weak low-frequency magnetic fields might affect living organisms has received a great deal of attention in recent years, particularly since weak extremely low frequency (ELF) magnetic fields have been implicated as having both potentially beneficial and adverse effects in humans. A number of laboratory studies have focused on the response of biological systems to a combination of alternating current (ac) and direct current (dc) magnetic fields. An interesting feature observed in many of these studies (Smith et al., 1987; Liboff et al., 1987; Lerchl et al., 1991; Yost and Liburdy, 1992; Deibert et al., 1994; Fitzsimmons et al., 1994; Jenrow et al., 1995) has been the response of biological systems to magnetic fields modulated at the cyclotron frequency of the unhydrated calcium ion, defined as:

$$f_c = qB_0/2\pi m \quad (1)$$

where f_c is the cyclotron frequency, q is the charge of the calcium ion, m is the mass of the calcium ion, and B_0 is the dc magnetic field magnitude. Because calcium is involved in a vast array of cellular phenomena, the effects of electromagnetic fields on calcium-dependent biological processes could have wide-ranging consequences (reviewed in Walleczek, 1992; Frey, 1993). In attempting to explain the resonance character of these responses, several investigators have focused on ion cyclotron resonance, ion vibrational precession, and ion parametric resonance as possible mechanisms (Liboff et al., 1987; Lednev, 1991; Male, 1992; Chiabrera et al., 1992; Blanchard and Blackman, 1994). As noted by several of these investigators, these resonance mechanisms might also be applied to other biologically important ions such as potassium, sodium, iron, magnesium, and lithium. All of these mechanisms predict a maximum effect of weak ac magnetic fields at a frequency determined from the ion's mass and charge as given in Eq. 1. Although a number of laboratories have not been able to replicate the effects of weak magnetic fields on cells under cyclotron resonance conditions (Prasad et al., 1991; Parkinson and Sulik, 1992; Coulton and Barker, 1993; Prasad et al., 1994), the cyclotron resonance mechanism has recently been suggested to be a potentially useful paradigm in predicting the risk of childhood leukemia from exposure to weak combined dc-ac magnetic fields (Bowman et al., 1995).

The molecular targets of ion cyclotron resonance effects are as yet unknown although several possibilities have been suggested, including cell membrane ion channels and specific ion-binding proteins. Lednev has proposed a specific molecular mechanism by which combined dc-ac magnetic fields might interact with biological systems through calcium-binding proteins such as calmodulin (Lednev, 1991). He postulated that a calcium ion in the binding domain of a calcium-binding protein acts as a charged spatial oscillator. The breaking and formation of coordination bonds between the calcium ion and chelating groups on the calcium-binding protein correspond to transitions between specific energy levels of the ion-protein complex. Lednev suggested

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Abbreviations: CaM, calmodulin; EGTA, ethylene glycol bis(aminoethyl ether) tetraacetic acid; ELF, extremely low frequency; MLCK, myosin light chain kinase; MOPS, morpholinopropanesulfonic acid; v, enzyme activity rate; V_{max}, maximal enzyme activity rate.

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that an applied magnetic field causes the energy difference between the bound and unbound states to split into two sublevels, altering the probability of a binding event taking place between a calcium ion and a calcium-binding protein. Lednev further explained that this effect has a quantum parametric resonance character, with maximum effect occurring when the frequency of the ac component of the applied magnetic field is equal to the cyclotron frequency of the unhydrated calcium ion. This implies that the applied magnetic fields might alter the equilibrium dissociation constant (K_D) between calcium and the corresponding calcium-binding protein, provided the cyclotron frequency is greater than the dissociation rate constant (k_{off}) of the calcium-binding reaction. According to Lednev's calculations, the calcium equilibrium dissociation constant (K_D) should change by approximately one order of magnitude when the chemical system is exposed to combined dc-ac fields that meet the cyclotron resonance criterion. Moreover, the frequency response of the calcium-binding reaction should follow the relationship described in Eq. 1. If weak ELF magnetic fields alter the calcium-binding properties of proteins such as calmodulin, this could have many cellular effects ranging from changes in cell motility and contraction to changes in cellular metabolism and growth control.

Although Lednev's hypothesis has been criticized (Adair, 1992; Bennett, 1994), Lednev and co-workers performed experiments that, in qualitative support of the theory, showed a strong resonance effect at 16 Hz, the cyclotron frequency of the unhydrated calcium ion (Shuvalova et al., 1991). They used the activation of the calcium/calmodulin-dependent enzyme, myosin light chain kinase (MLCK), to detect changes in the affinity of calcium for calmodulin (CaM). MLCK activity is completely dependent on the formation of a calcium/calmodulin complex (Blumenthal and Stull, 1980), and results from the two-step process indicated in the following scheme:

$$4\,Ca^{2+} + CaM \rightleftharpoons Ca_4^{2+} \cdot CaM \quad (2)$$

$$Ca_4^{2+} \cdot CaM + MLCK_{(inactive)} \rightleftharpoons Ca_4^{2+} \cdot CaM \cdot MLCK_{(active)} \quad (3)$$

When activated, MLCK catalyzes the phosphorylation of the P ("phosphorylatable") light chain subunit of myosin. Lednev and co-workers measured changes in the phosphorylation of the P-light chain of myosin in response to conditions of combined dc-ac magnetic fields of varying frequency where calmodulin was only partially saturated with calcium ion (Shuvalova et al., 1991). The magnetic field exposure conditions were based on the equation:

$$B = B_0 + B_1 \sin(2\pi ft) \quad (4)$$

where $B_0 = B_1 = 20.9\ \mu T$, and the modulation frequency of the ac magnetic field, f, was varied from approximately 8 to 18 Hz. In qualitative agreement with the proposed mechanism, they saw substantial changes in MLCK activity (three- to sixfold changes in the amount of phosphorylated substrate) of samples exposed to magnetic fields at specific frequencies near 16 Hz, the cyclotron frequency of unhydrated calcium ion. Two sample preparations were investigated, one containing "crude" MLCK and one containing purified MLCK from rabbit skeletal muscle. A decrease in activity was observed for the crude MLCK preparation, whereas the purified MLCK preparation exhibited an increase in activity at 16 Hz. Shuvalova et al. (1991) explained that the decrease in MLCK activity seen with the crude MLCK preparation was due to the possible presence of a competing calcium-binding protein such as troponin-C.

The data of Lednev and co-workers can be analyzed to determine the magnitude of change in the K_D of Ca^{2+} for calmodulin using an equation (Eq. 5) derived by Blumenthal and Stull (1980), which describes the fractional activation (v/V_{max}) of MLCK as a function of Ca^{2+} and calmodulin (CaM) concentration.

$$v/V_{max} = [CaM]/(K_{CaM}(1 + K_{Ca^{2+}}/[Ca^{2+}])^4 + [CaM]) \quad (5)$$

In Eq. 5, K_{CaM} is the activation constant of calcium-saturated calmodulin for MLCK and $K_{Ca^{2+}}$ is the average equilibrium dissociation constant (K_D) for calcium binding to calmodulin. In accordance with Lednev's theory, it is assumed that the observed effects on MLCK activity are due solely to effects on $K_{Ca^{2+}}$, and therefore all other parameters ([CaM], K_{CaM}, and [Ca^{2+}]) in Eq. 5 are kept fixed. When Lednev's purified MLCK preparation was exposed to combined dc-ac magnetic fields, the fractional MLCK activity increased from 0.1 to 0.7 at 14 Hz (Shuvalova et al., 1991), indicating $K_{Ca^{2+}}$ decreased by at least a factor of three. In contrast, analysis of the data from Lednev's several experiments with a crude MLCK preparation showed three- to sixfold decreases in MLCK fractional activity at an ac magnetic field frequency of 16 Hz (Shuvalova et al., 1991), indicative of a threefold increase in $K_{Ca^{2+}}$.

Because of the potential importance of Lednev's hypothesis with regard to its ability to predict the the effects of weak combined dc-ac magnetic fields on the behavior of a well-characterized biochemical system, we have attempted to replicate his observations using two separate experimental systems. One system is essentially identical to Lednev's MLCK activity assay system (Eqs. 2 and 3), whereas the other is a fluorescence-based assay system containing significantly fewer components.

MATERIALS AND METHODS

Magnetic field generation

Magnetic field exposure systems consisting of orthogonal coil pairs were used to generate the applied magnetic fields. The two coil systems were designed using techniques suggested by Montgomery (1969) to generate magnetic fields of maximum uniformity across the sample exposure regions. The coil apparatus used for the MLCK activity assay contains two coil pairs with a common vertical axis, and two coil pairs with a common horizontal axis. The two horizontal coil pairs were separately used to generate the ac and dc components of the applied magnetic field. One of the vertical coil pairs was used to cancel the vertical component of the ambient geomagnetic field; the other vertical coil pair was unused.

A smaller coil apparatus had to be designed specifically for the fluorescence experiments because of the spatial limitations of the spectrofluorometer sample chamber. The sample was contained in an optical cuvette, which determined the sample exposure region (10 mm (w) × 10 mm (h) × 4 mm (d)). The coil apparatus for the fluorescence system consists of one coil pair with a horizontal axis and one coil pair with a vertical axis. As in the MLCK experiments, the horizontal coil pair was used to generate the combined ac-dc magnetic fields and the vertical coil pair was used to cancel the vertical component of the ambient field.

The physical dimensions of the coils used in the two experimental setups are given in Fig. 1. The on-axis magnetic field profiles were calculated for the MLCK and fluorescence coil systems by considering each turn in the coil to be a separate loop, and summing their individual contributions at points along the coil pair axis. The calculated magnetic field profiles for the MLCK and fluorescence coil systems are shown in Fig. 2. In both the fluorescence and MLCK experimental setups, the horizontal coil axis was aligned parallel with magnetic north-south. Commercially available dc power supplies (Hewlett-Packard 6033A and 6203B, Palo Alto, CA) were used to generate the dc magnetic fields. For the MLCK activity assays, an audio amplifier (Bogen C100B, Paramus, NJ) driven by a function generator (Hewlett-Packard 3314A) was used to generate the ac magnetic fields. For the fluorescence-based assays, the function generator had sufficient current capacity to generate the ac magnetic field without amplification. A Bell 620 Gaussmeter configured with an STB4–0404 transverse probe (F.W. Bell Associates, Orlando, FL) was used to set the magnetic fields by adjusting the coil current while monitoring the Gaussmeter analog output with a digital multimeter (Hewlett-Packard 3438A). The combined dc-ac field is described by Eq. 4, where $B_0 = B_1 = 20.9\ \mu T$. For the MLCK activity assays, the ac field frequency was varied from 14 to 19 Hz in 1-Hz increments. For the fluorescence experiments, the field frequency was varied from 6 to 18 Hz in 0.5-Hz increments. Ambient geomagnetic field conditions were achieved by disconnecting the coils from the power supplies and amplifiers; the ambient magnetic field at the assay site (University of Utah, Salt Lake City, UT) was 37 μT (12 μT horizontal, 35 μT vertical).

According to the grant contractor's standard procedure, Dr. Martin Misakian of the National Institute of Standards and Technology (NIST) came to our laboratory and independently checked the accuracy of our magnetic field measurements. The fields were set according to the procedures described above, after which Dr. Misakian used an NIST ac magnetic field meter with a miniature coil probe to measure the ac fields and a fluxgate magnetometer to measure dc fields. All of Dr. Misakian's measurements were within 3–4% of the measurements made using the equipment described above. He found the ac field magnitude at the center of the MLCK activity assay coils to be 3–4% higher across the range of frequencies used in our experiments, 14 to 19 Hz, and the dc field magnitude at the coil center to be accurate within the precision limits of the NIST equipment

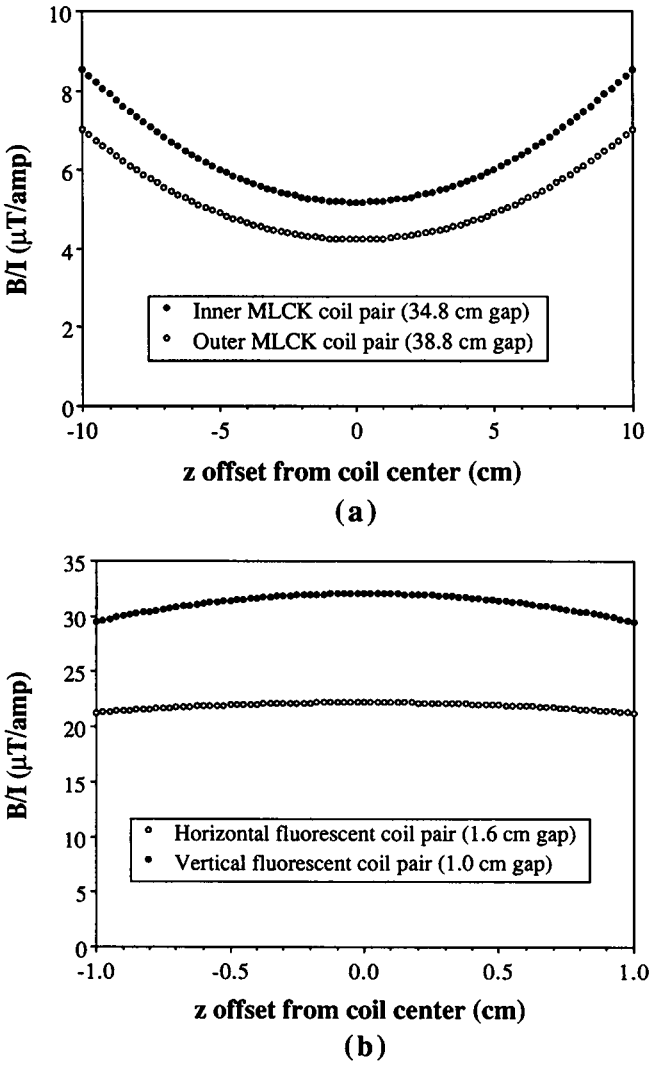

FIGURE 2 Calculated on-axis magnetic field profiles for the (*a*) MLCK and (*b*) fluorescence coil pairs. The indicated gap distance represents the space between inside edges of the coil windings.

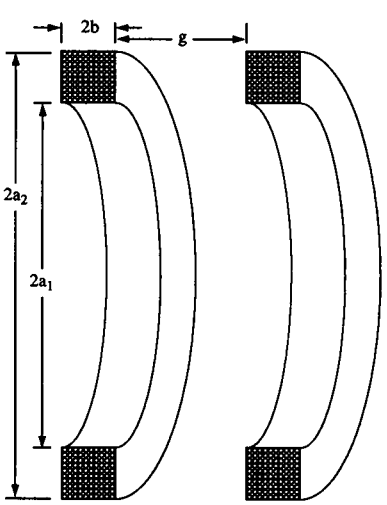

FIGURE 1 Dimensions of the coil apparatuses for the two experimental systems. *N* is the number of turns in each coil.

MLCK Coil Apparatus

Coil Pair	$2a_1$ (cm)	$2a_2$ (cm)	2b (cm)	g (cm)	N
Inner Horizontal	25.4	29.7	1.3	34.8	256
Outer Horizontal	25.4	29.7	1.3	38.8	256
Inner Vertical	25.4	29.7	1.3	34.8	256
Outer Vertical	25.4	29.7	1.3	38.8	256

Fluorescence Coil Apparatus

Coil Pair	$2a_1$ (cm)	$2a_2$ (cm)	2b (cm)	g (cm)	N
Horizontal	8.0	10.2	1.0	1.6	90
Vertical	5.0	7.1	1.0	1.0	90

(±2%). Dr. Misakian also checked the uniformity of the MLCK coil apparatus by measuring the magnetic field magnitude at several points within the 3.6 cm (w) × 3.6 cm (d) × 0.64 cm (h) MLCK sample exposure region. Consistent with the theoretical predictions of Fig. 2, all measured values were within 3% of the value measured at the center of the sample exposure region. For the fluorescence assay coils, the central ac field was 3–4% higher than the 20.9 μT target value (frequency range 9–18 Hz), and was uniform throughout the sample exposure region to within 2% of the central field value. The fluxgate magnetometer probe was too large to fit within the restricted exposure space of the fluorescence coil apparatus; consequently, the dc field magnitude of the fluorescence coils could not be verified with the NIST equipment. However, based on the good agreement between the NIST measurements and those made using our instrumentation, the dc fields within the fluorescence coil apparatus are likely to be accurate. These independent measurements of ac and dc fields indicate that the equipment and procedures used in our studies for setting field exposures are accurate and that the homogeneity of field exposure within both apparatuses is in excellent agreement with the calculated homogeneity.

MLCK activity assay

The calcium/calmodulin-dependent activation of MLCK was one method used to monitor changes in the binding of Ca^{2+} to calmodulin. MLCK was purified from rabbit skeletal muscle (Dasgupta et al., 1989) and assayed as described by Blumenthal and Stull (1980) using either myosin light chains or a synthetic peptide substrate, KKRPQRATSNVFS-amide, corresponding to the phosphorylation site in the chicken gizzard smooth muscle myosin light chain (Michnoff et al., 1986). The calmodulin used in the MLCK assays and for fluorescence experiments was purified from bovine testes (Dasgupta et al., 1989). All reactions contained 50 mM MOPS, pH 7.0, and 1 mM dithiothreitol in a final volume of 50 μl. Reactions were initiated by the addition of [γ-^{32}P]ATP and immediately placed in the energized coil apparatus. Aliquots of 20 μl were removed from each reaction after 5 and 10 min exposure times and spotted on 1-cm filter paper squares. A 3MM filter paper (Whatman, Maidstone, U.K.) procedure was used with the protein substrate (Blumenthal and Stull, 1980), whereas a P81 phosphocellulose filter paper (Whatman) procedure was used for the peptide substrate (Roskoski, 1983). After thorough washing, the filters were dried, placed in minivials with scintillation cocktail, and ^{32}P incorporation into substrate measured using a liquid scintillation counter. All reactions exhibited linear rates of incorporation of ^{32}P into substrate over the 10 min reaction time. In addition to the combined dc-ac magnetic field exposures, two ambient geomagnetic control exposure reactions were performed, one at the beginning and one at the end of each day's set of experiments.

The MLCK reactions using peptide substrate contained 173 μM synthetic peptide substrate and were started by the addition of [γ-^{32}P]ATP (~300 cpm/pmol) to a final concentration of 1 mM. Three different sets of reaction mixtures ($[Ca^{2+}]_{sat}$, $[Ca^{2+}]_{lim}$, blank) were prepared for each magnetic field exposure exposure condition and for the ambient controls. The $[Ca^{2+}]_{sat}$ reaction mixtures contained 10 mM magnesium acetate, 200 μM $CaCl_2$, 1 μM calmodulin, and 1 nM MLCK, and were used to determine the enzymatic rate of maximally activated MLCK (V_{max}). The free calcium concentration of the $[Ca^{2+}]_{lim}$ reaction mixtures was adjusted to a limiting concentration using a Ca^{2+}/EGTA buffer system so that MLCK activation was ~60% of V_{max}. In operating near the midpoint of the MLCK activation curve, these reaction mixtures were maximally sensitive to either an increase or a decrease in Ca^{2+} binding to calmodulin. The $[Ca^{2+}]_{lim}$ reaction mixtures contained 10 mM magnesium acetate, 2.1 mM $CaCl_2$, 3 mM EGTA, 200 nM calmodulin, and 1 nM MLCK. The free Ca^{2+} concentration in the $[Ca^{2+}]_{lim}$ reaction mixtures was estimated to be 1.17 μM using the SPECS program described by Fabiato (1988). Blank reaction mixtures were used to correct for nonspecific binding of ^{32}P and [γ-^{32}P]ATP to the filter papers and lacked MLCK but contained 20 mM magnesium acetate, 4 mM EGTA, and 5 nM calmodulin, in addition to the concentrations of MOPS, dithiothreitol, peptide substrate, and [γ-^{32}P]ATP used in the regular reaction mixtures. All reaction mixtures were prepared fresh the morning of an experiment and kept on ice until used later that day. Just before each experiment, samples were removed from the ice bath and preincubated at room temperature (~23 ± 0.5°C) for 5 min. Each set of reaction mixtures was run in triplicate for each exposure condition.

The MLCK reactions using myosin light chains contained 5 μM P-light chain, 1.8 μM calmodulin, 6 nM MLCK, 0.5 mg/ml bovine serum albumin, 10 mM magnesium acetate, and were started by adding [γ-^{32}P]ATP (~600 cpm/pmol) to a final concentration of 0.5 mM. Myosin light chains were purified from bovine cardiac muscle by the method of Blumenthal and Stull (1980). Four different sets of reaction conditions were used: $[Ca^{2+}]_{sat}$, $[Ca^{2+}]_{high}$, $[Ca^{2+}]_{low}$, and blank. The $[Ca^{2+}]_{sat}$ reactions contained 200 μM $[Ca^{2+}]$; the $[Ca^{2+}]_{high}$ reactions contained 1.6 mM Ca^{2+} and 3 mM EGTA (free $[Ca^{2+}]$ = 0.586 μM); the $[Ca^{2+}]_{low}$ reactions contained 1 mM Ca^{2+} and 3 mM EGTA (free $[Ca^{2+}]$ = 0.255 μM); and the blank reactions contained 2 mM EGTA and no added calcium. The $[Ca^{2+}]_{high}$ reactions were ~60% the rate of $[Ca^{2+}]_{sat}$ reactions and the $[Ca^{2+}]_{low}$ reactions were ~20% of the $[Ca^{2+}]_{sat}$ reactions. Reactions were run in duplicate at 30°C.

Fluorescence experiments

A synthetic fluorescent peptide corresponding to the calmodulin-binding domain of MLCK was used to monitor Ca^{2+}-dependent changes in calmodulin interactions resulting from magnetic field exposure (Persechini et al., 1989; Blumenthal, 1993). The fluorescence-based assay is a two-stage equilibrium system illustrated in the following scheme (Eqs. 6 and 7) where F-peptide is a fluorescently labeled peptide that corresponds to the calmodulin-binding domain of MLCK (Persechini et al., 1989; Blumenthal, 1993):

$$4\,Ca^{2+} + CaM \rightleftharpoons Ca_4^{2+} \cdot CaM \qquad (6)$$

$$Ca_4^{2+} \cdot CaM + \text{F-peptide} \rightleftharpoons Ca_4^{2+} \cdot CaM \cdot \text{F-peptide} \qquad (7)$$

The first stage of the equilibrium system, represented by Eq. 6, is identical to the first stage of the MLCK activity assay (Eq. 2), in which four calcium ions form a complex with calmodulin. The second part of the fluorescence equilibrium reaction, represented by Eq. 7, involves the binding of the calcium/calmodulin to the F-peptide. The binding of the F-peptide to the much larger calmodulin protein results in an increase in the fluorescence intensity and the fluorescence anisotropy of the F-peptide, which can be accurately monitored using a spectrofluorometer (Persechini et al., 1989; Blumenthal, 1993). Using the fluorescence system, data with a high signal-to-noise ratio can be collected in a very rapid manner, allowing for examination of the equilibrium reaction over a much broader range of experimental conditions than is possible with the MLCK assay system.

The peptide, KRRWKKAFIAVSAAARFKKC-amide, was labeled on the C-terminal cysteinyl residue with the sulfhydryl-reactive fluorescent reagent acrylodan. The fluorescent sample consisted of 3 M guanidine-HCl, 50 mM MOPS, pH 7.0, 80 nM fluorescent peptide, 330 nM calmodulin, and 140 μM $CaCl_2$. The concentration of Ca^{2+} used was such that the fluorescence anisotropy was positioned near the midpoint ($r \approx 0.130$ for total $[Ca^{2+}]$ = 140 μM) between the maximum anisotropy value ($r_{max} \approx 0.170$) and the minimum anisotropy value ($r_{min} \approx 0.085$). Operation of the fluorescence system near the fluorescence anisotropy midpoint made it maximally sensitive to either increases or decreases in the binding of calcium to calmodulin.

The sample was contained in an optical glass semi-microcuvette (Hellma 109.004F-OS, Jamaica, NY; interior dimensions 4 mm × 10 mm × 50 mm). The cuvette was positioned within the coil apparatus with the 10-mm path parallel to the excitation beam. The fluorescence signal was obtained from a volume (4 mm × 4 mm × 4 mm) at the center of the coil apparatus, formed by the overlap of the excitation beam and the collection beam. An ISS (Champaign, IL) model PC1 photon-counting spectrofluorometer, operated in the L configuration, was used to measure the fluorescence anisotropy of the sample. The sample was excited at 370 nm (bandwidth 16 nm) with a Schott (Duryea, PA) BG39 bandpass filter in the excitation path to eliminate higher order harmonics of the excitation

beam. The emitted light was collected through a Schott KV470 cut-on filter. Anisotropy values were calculated using software provided by ISS. Ten anisotropy measurements (each with a 5-s integration time) were taken at each exposure condition to determine statistical parameters. Measurements of fluorescence anisotropy under ambient exposure conditions were taken periodically throughout each experiment (approximately every fifth measurement). All measurements were taken at room temperature, which varied between 22 and 24°C. The sample temperature was recorded before measuring the sample anisotropy measurement at each exposure condition. The order of exposure frequencies was randomized to separate frequency-dependent effects from possible time-dependent effects (e.g., anisotropy changes due to photobleaching of the peptide or temperature changes in the sample).

RESULTS

MLCK activity assay

The results of three independent MLCK activity assays (performed on separate days) using the synthetic peptide substrate are shown in Fig. 3, where the data are plotted in the chronological order in which the data points were obtained. The $[Ca^{2+}]_{lim}$ reaction mixture, which contained a limiting concentration of free calcium (1.17 μM), was designed to be maximally sensitive to changes in the affinity of calcium for calmodulin. The $[Ca^{2+}]_{sat}$ reaction mixture was prepared with saturating concentrations of free calcium (200 μM) and calmodulin (1 μM) to determine the reaction rate of maximally activated MLCK. Comparison of the ambient geomagnetic control reactions before and after the dc-ac field exposure reactions indicated that, in general, there was a slight decrease ($\leq$15%) in the MLCK activity rate as a function of time for both the $[Ca^{2+}]_{lim}$ and the $[Ca^{2+}]_{sat}$ reactions. This gradual decrease in MLCK activity over the course of an experiment taking ~4 h to complete is most likely due to denaturation of a small portion of the enzyme, and is not considered to be significant to the results of this experiment. The data were not corrected for this drop in activity because it was only slightly greater than the typical experimental variation between identical reactions. The data from Fig. 3 are replotted in Figs. 4 and 5 as a function of magnetic field frequency. The individual experiments are plotted separately in Fig. 4, whereas Fig. 5 represents a composite of all three experiments. Although the MLCK activity appears to be slightly higher at 16 Hz in Fig. 4 *b*, this was not seen in the other two experiments (Fig. 4, *a* and *c*), nor is there evidence for any significant increase in activity at 16 Hz or any other frequency in the composite plot (Fig. 5).

The data shown in Fig. 6 are the results of dc-ac magnetic field exposure on MLCK activity using myosin light chain as substrate. In addition to $[Ca^{2+}]_{sat}$ reactions, MLCK activity was also assayed at two subsaturating concentrations of Ca^{2+}. The higher Ca^{2+} concentration (free $[Ca^{2+}]$ = 0.586 μM) resulted in ~60% activation of MLCK, whereas the lower concentration (free $[Ca^{2+}]$ = 0.255 μM) gave ~20% activation. The reactions performed at the higher concentration of Ca^{2+} are better suited for detecting decreases in calmodulin's affinity for Ca^{2+}, whereas the reactions performed at the low concentration of Ca^{2+} are optimal for monitoring increases in affinity for Ca^{2+}. However, the two subsaturating $[Ca^{2+}]$ reactions should respond in a parallel manner if there is any effect of magnetic field exposure on Ca^{2+} binding to calmodulin. The data shown in Fig. 6 indicate that combined dc-ac magnetic fields had no significant effect on MLCK activity at 16 Hz or any of the other frequencies tested.

Fluorescence experiments

Fluorescence anisotropy was used to measure the amount of fluorescently labeled calmodulin-binding peptide com-

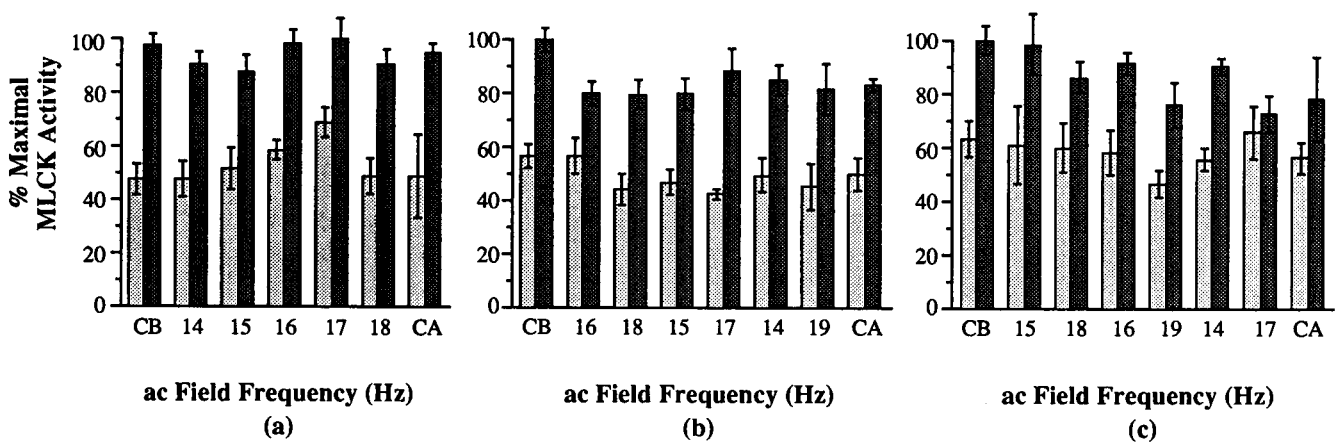

FIGURE 3 Myosin light chain kinase activity as a function of magnetic field frequency. Results of three independent MLCK activity assays are presented (*a*, *b*, and *c*). Reactions were exposed to combined dc-ac magnetic fields in the chronological order indicated (left to right along the horizontal axis). In experiments *b* and *c*, the order of frequency exposure was randomized to separate time-dependent effects from frequency-dependent effects. *CB* and *CA* represent MLCK activity of control samples exposed to the ambient geomagnetic field (37 μT) before (*CB*) and after (*CA*) magnetic field exposures. The MLCK activity rates represent the average of six determinations (three reactions, each sampled at 5 and 10 min). The data in each experiment are normalized to the highest MLCK activity rate among the $[Ca^{2+}]_{sat}$ reaction mixtures (darkly shaded bars). Lightly shaded bars represent data from $[Ca^{2+}]_{lim}$ reaction mixtures. Error bars represent the standard deviation of the six determinations.

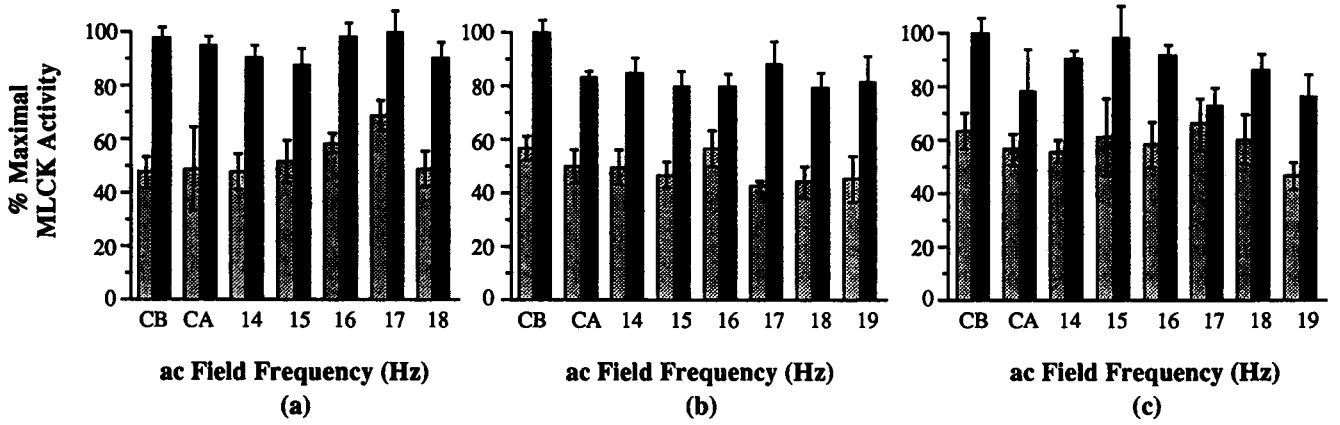

FIGURE 4 Myosin light chain kinase activity as a function of magnetic field frequency. Results of the three MLCK activity assays presented in Fig. 3, plotted as a function of the frequency of the ac magnetic field.

plexed to calcium/calmodulin as a function of magnetic field frequency. The results of one such experiment are shown in Fig. 7. The data from the various magnetic field exposure conditions are shown in the chronological order in which they were taken (Fig. 7 *a*). The gradual decrease in anisotropy was time-dependent, most likely attributable to photobleaching of the fluorescent peptide. This was verified by performing an experiment of similar duration in which the sample was exposed only to ambient geomagnetic fields, and changes in fluorescence anisotropy were measured as the experiment progressed. The data from this ambient experiment exhibited a similar gradual decrease in fluorescence anisotropy (data not shown). Because the time-dependent effect seen in Fig. 7 *a* was relatively large compared with the variation between replicate measurements and because it was mathematically predictable, the effect was compensated for by calculating the slope of a straight line fit to a plot of fluorescence anisotropy versus time, then adding a value to each measurement that corresponded to the calculated slope multiplied by the time elapsed since the start of the experiment. Fig. 7 *b* shows the data from Fig. 7 *a* corrected for the time-dependent effect and plotted versus field frequency. These data indicate that the binding of the fluorescent peptide to the calcium/calmodulin complex is not significantly affected by the applied ac magnetic fields at 16 Hz or by magnetic field exposure at any other frequency over the range of 6 to 18 Hz.

DISCUSSION

The results of our experiments using MLCK activity to monitor changes in the affinity of calcium for calmodulin indicate no biologically significant effect of weak combined dc-ac magnetic fields on the binding of calcium to calmodulin. In particular, the large changes in MLCK activity (three- to sixfold) reported by Lednev (Shuvalova et al., 1991) were not seen in our experiments, even though very similar exposure conditions and assay systems were used. Consistent with the results of our MLCK activity assays, fluorescence experiments using a fluorescently labeled calmodulin-binding peptide as reporter group showed no sig-

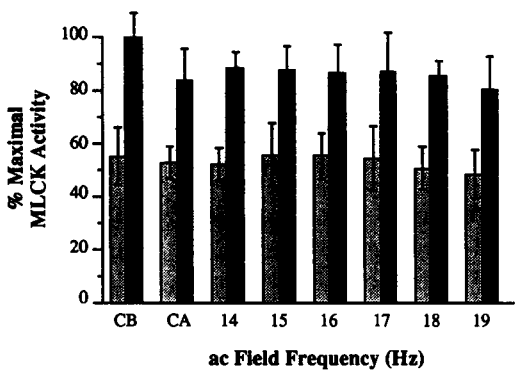

FIGURE 5 Myosin light chain kinase activity as a function of magnetic field frequency. Combined results from the three MLCK activity assays shown in Figs. 3 and 4.

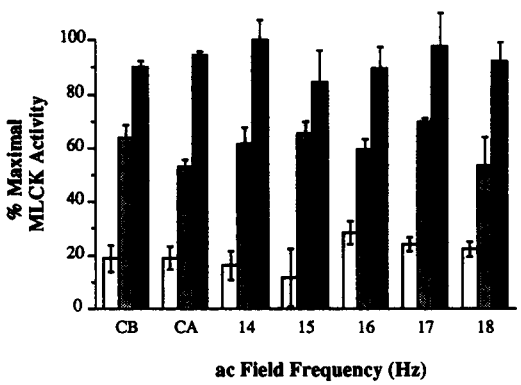

FIGURE 6 Myosin light chain kinase activity as a function of magnetic field frequency using myosin light chains as substrate. MLCK activity was assayed as described in Materials and Methods. $[Ca^{2+}]_{sat}$ reaction mixtures are represented by darkly shaded bars, $[Ca^{2+}]_{high}$ reaction mixtures by lightly shaded bars, and $[Ca^{2+}]_{low}$ reaction mixtures by unshaded bars. Each bar represents the mean of four activity measurements ± S.D.

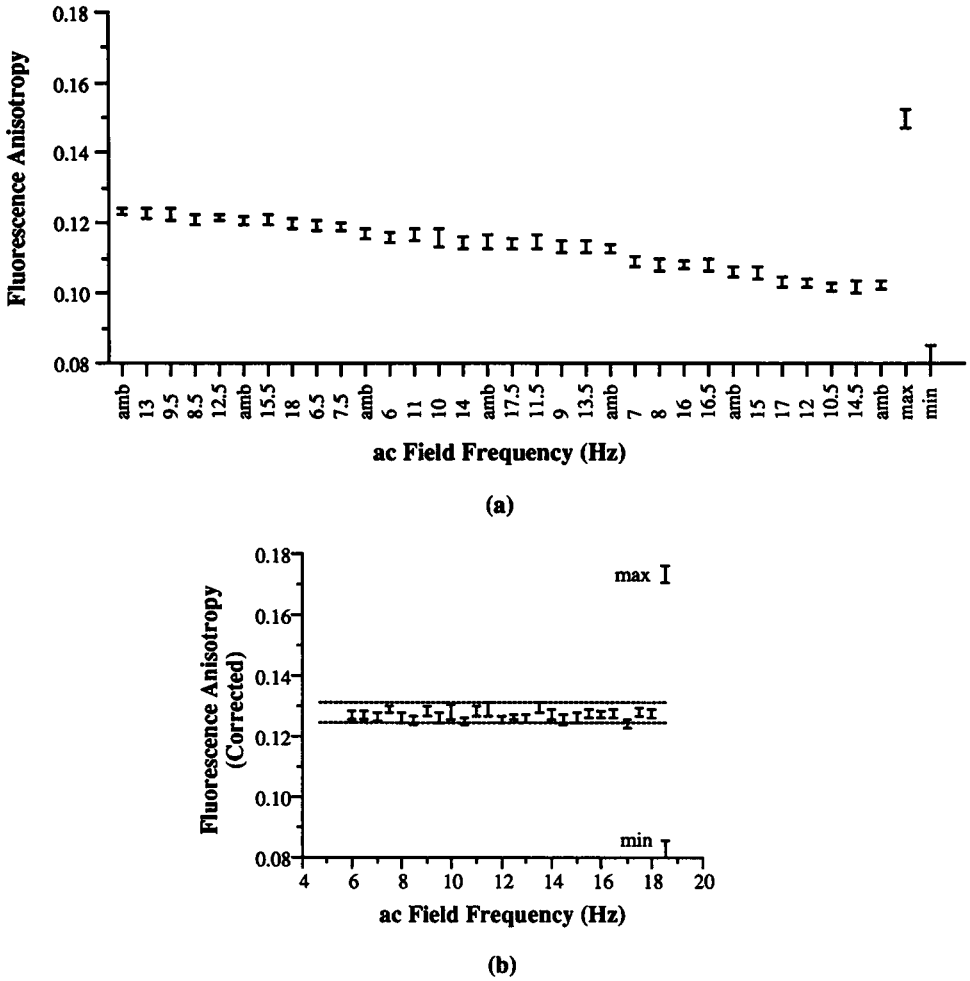

FIGURE 7 Fluorescence anisotropy of the Ca^{2+}/calmodulin-binding fluorescent peptide as a function of magnetic field frequency. In *a*, the data are presented in the chronological order in which they were taken. Where numerical values are indicated on the horizontal axis, the sample was exposed to combined dc-ac magnetic fields oscillating at the indicated frequency (Hz). Where *amb* is indicated, fluorescence anisotropy of the sample was measured under ambient geomagnetic field conditions (37 μT). *Max* and *min* represent the maximum (under conditions of saturating free [Ca^{2+}]) and minimum (no added [Ca^{2+}]) fluorescence anisotropy values of the sample. Error bars represent the standard deviation of the data (n = 10). The gradual time-dependent decrease in fluorescence is most likely due to photobleaching of the sample. In *b*, the data are corrected for the time-dependent decrease (see Results), and plotted as a function of increasing magnetic field frequency. The dashed lines indicate the range of mean anisotropy values for the ambient geomagetic field control condition.

nificant effect of combined dc-ac magnetic fields on the binding of calcium to calmodulin. The conditions in each of the two experimental systems used in this study were adjusted to make each system maximally sensitive to either an increase or decrease in the affinity of calmodulin for calcium. Neither system exhibited a significant response to combined dc-ac magnetic fields modulated at or near the cyclotron frequency of calcium (16 Hz). The results obtained from these two independent systems taken together provide compelling evidence that under the exposure conditions used in the present study, the binding of calcium to calmodulin is not significantly affected by weak, combined dc-ac magnetic fields at or near the cyclotron frequency of calcium.

We are unable to readily explain the discrepancies between our results and those of Lednev and co-workers (Shuvalova et al., 1991). Although there are minor differences between the MLCK assay system used in the present study and that used by Lednev, these are unlikely to account for the large differences in results. For instance, Lednev and co-workers used unlabeled ATP and a gel-electrophoretic method followed by densitometry to monitor changes in MLCK activity, whereas [γ-^{32}P]ATP was used to follow peptide or protein phosphorylation in the present study. The use of radiolabeled ATP to assay MLCK activity is more sensitive and can more accurately and precisely quantify MLCK activity than the gel electrophoretic/densitometry method, but both assay methods should yield results that are at least qualitatively similar. A second difference between the two MLCK assay systems was the phosphate-acceptor substrate used. Lednev and co-workers used myosin light chains purified from rabbit skeletal muscle, whereas a synthetic peptide substrate and myosin light chains purified from bovine cardiac muscle were used in the present study. In the present study, both the peptide and protein substrate gave identical results (Figs. 3–6). The synthetic peptide substrate, which is widely used to assay MLCK activity, was used in the present study because it can be obtained in highly purified form free from contaminating proteins such as calmodulin. However, the peptide substrate lacks the EF-hand calcium-binding domain regions found in the myosin P-light chain substrate and cannot bind Ca^{2+}. Thus if the effects of dc-ac magnetic field exposure seen by Lednev were primarily effects on Ca^{2+} binding to the myosin light chain, rather than on calmodulin, then the effects would not be seen with the peptide substrate. However, no effects of dc-ac magnetic fields were seen when myosin light chains were used instead of peptide substrate in the MLCK assay

(Fig. 6). It is possible that because Lednev used myosin light chains from rabbit skeletal muscle (Shuvalova et al., 1991) and bovine cardiac light chains were used in the present study, that this might account for the difference in results between the two laboratories. This is unlikely, however, because the P-light chains from rabbit skeletal muscle and bovine cardiac muscle are indistinguishable in their biochemical properties (Stull et al., 1986).

Markov and colleagues have also investigated the effects of applied magnetic fields on smooth muscle MLCK activity (Markov et al., 1992; Markov et al., 1993). The results of their studies indicate that MLCK activity is decreased not only by weak, combined dc-ac magnetic fields, but also by ac fields alone and dc fields alone (Markov et al., 1992). In further investigations of the effect of dc magnetic fields on MLCK activity, Markov et al. (1993) demonstrated a nearly linear relationship between the magnitude of the static field and the rate of substrate phosphorylation from 0 to 200 μT. For static field strengths below the ambient magnitude, MLCK activity was less than that of sham-exposed controls. When the field strength was increased above the ambient level, substrate phosphorylation exceeded that of controls. In a similar study, Bull et al. (1993) reported a 13% increase in calcium/calmodulin-dependent cyclic nucleotide phosphodiesterase activity in samples exposed to dc fields of 19.2 to 20.4 μT compared with controls maintained at zero field. Because the purpose of the present investigation was to test Lednev's hypothesis regarding the effects of weak ELF magnetic fields on calcium binding to calmodulin, the effect of dc magnetic field strength on MLCK activity was not investigated. However, the fluorescence system described herein was used to determine the effects of static magnetic fields on Ca^{2+}-dependent interactions of calmodulin with the fluorescent calmodulin-binding domain peptide. No effects of dc magnetic fields were seen between 0 and 200 μT (Hendee and Christensen, unpublished observations).

Other laboratories have also failed to see effects of weak low-frequency magnetic fields under cyclotron resonance conditions using other well-characterized biochemical systems including Ca^{2+} ion transport through patch-clamped β-cell membranes (Höjevik et al., 1995), conductance of monovalent cations through gramicidin A channels in lipid bilayers (Wang and Hladky, 1994a; Galt et al., 1993), and K^+ conductance through ATP-sensitive potassium channels (Wang and Hladky, 1994b). Experiments by Durney and co-workers also provided no evidence for cyclotron resonance effects of combined ac-dc magentic fields on calcium binding to calmodulin (Bruckner-Lea et al., 1992) or on current flux through phospholipid bilayers (Durney et al., 1992). Thus, even though there are many reports of cyclotron resonance effects in cells, such effects have not as yet been reproduced in well-characterized biochemical systems. Until cyclotron resonance effects can be reproducibly observed and characterized in a simple biochemical system, the potential effects of combined ac-dc magnetic fields on cellular functions and their role in disease processes must remain speculative.

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REFERENCES

Adair, R. K. 1992. Criticism of Lednev's mechanism for the influence of weak magnetic fields on biological systems. *Bioelectromagnetics.* 13: 231–235.

Bennett, W. R. 1994. Cancer and power lines. *Physics Today.* 47:23–29.

Blanchard, J. P., and C. F. Blackman. 1994. Clarification and application of an ion parametric resonance model for magnetic field interactions with biological systems. *Bioelectromagnetics.* 15:217–238.

Blumenthal, D. K. 1993. Development and characterization of fluorescently-labeled myosin light chain kinase calmodulin-binding domain peptides. *Mol. Cell. Biochem.* 127/128:45–50.

Blumenthal, D. K., and J. T. Stull. 1980. Activation of skeletal muscle myosin light chain kinase by Ca^{2+} and calmodulin. *Biochemistry.* 19: 5608–5614.

Bowman, J. D., D. C. Thomas, S. J. London, and J. M. Peters. 1995. Hypothesis: the risk of childhood leukemia is related to combinations of power-frequency and static magnetic fields. *Bioelectromagnetics.* 16: 48–59.

Bruckner-Lea, C., C. H. Durney, J. Janata, C. Rappaport, and M. Kaminski. 1992. Calcium binding to metallochromic dyes and calmodulin in the presence of combined, ac-dc magnetic fields. *Bioelectromagnetics.* 13: 147–162.

Bull, A. W., K. A. Cherng, K. A. Jenrow, and A. R. Liboff. 1993. Weak magnetostatic fields alter calmodulin-dependent cyclic nucleotide phosphodiesterase activity. *In* Electricity and Magnetism in Biology and Medicine. M. Blank, editor. San Francisco Press, Inc., San Francisco. 319–322.

Chiabrera, A., B. Bianco, J. J. Kaufman, and A. A. Pilla. 1992. Bioelectromagnetic resonance interactions: endogenous field and noise. *In* Interaction Mechanisms of Low-Level Electromagnetic Fields in Living Systems. B. Nordén and C. Ramel, editors. Oxford University Press, Oxford. 164–179.

Coulton, L. A., and A. T. Barker. 1993. Magnetic fields and intracellular calcium: effects on lymphocytes exposed to conditions for 'cyclotron resonance.' *Phys. Med. Biol.* 38:347–360.

Dasgupta, M., T. Honeycutt, and D. K. Blumenthal. 1989. The γ-subunit of skeletal muscle phosphorylase kinase contains two noncontiguous domains that act in concert to bind calmodulin. *J. Biol. Chem.* 264: 17156–17163.

Deibert, M. C., B. R. McLeod, S. D. Smith, and A. R. Liboff. 1994. Ion resonance electromagnetic field stimulation of fracture healing in rabbits with a fibular ostectomy. *J. Orthop. Res.* 12:878–885.

Durney, C. H., M. Kaminski, A. A. Anderson, C. Bruckner-Lea, J. Janata, and C. Rappaport. 1992. Investigation of ac-dc magnetic field effects in planar phospholipid bilayers. *Bioelectromagnetics.* 13:19–33.

Fabiato, A. 1988. Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Methods Enzymol.* 157: 378–417.

Fitzsimmons, R. J., J. T. Ryaby, F. P. Magee, and D. J. Baylink. 1994. Combined magnetic fields increased net calcium influx in bone cells. *Calcif. Tissue Int.* 55:376–380.

Frey, A. H. 1993. Electromagnetic field interactions with biological systems. *FASEB J.* 7:272–281.

Galt, S., J. Sandblom, Y. Hamnerius, P. Höjevik, E. Saalman, and B. Nordén. 1993. Experimental search for cyclotron resonance effects on ion channels. *Bioelectromagnetics.* 14:315–321.

Höjevik, P., J. Sandblom, S. Galt, and Y. Hamnerius. 1995. Ca^{2+} ion transport through patch-clamped cells exposed to magnetic fields. *Bioelectromagnetics.* 16:33–40.

Jenrow, K. A., C. H. Smith, and A. R. Liboff. 1995. Weak extremely-low-frequency magnetic fields and regeneration in the planarian Dugesia tigrina. *Bioelectromagnetics.* 16:106–112.

Lednev, V. V. 1991. Possible mechanism for the influence of weak magnetic fields on biological systems. *Bioelectromagnetics.* 12:71–75.

Lerchl, A., R. J. Reiter, K. A. Howes, K. O. Nonaka, and K. A. Stokkan. 1991. Evidence that extremely low frequency Ca(2+)-cyclotron resonance depresses pineal melatonin in vitro. *Neurosci. Lett.* 124:213–215.

Liboff, A. R., R. J. Rozek, M. L. Sherman, B. R. McLeod, and S. D. Smith. 1987. $^{45}Ca^{2+}$ cyclotron resonance in human lymphocytes. *J. Bioelectr.* 6:13–22.

Male, J. 1992. Biological effects of magnetic fields: a possible mechanism? *Biologist.* 39:87–89.

Markov, M. S., J. T. Ryaby, J. J. Kaufman, and A. A. Pilla. 1992. Extremely weak ac and dc magnetic fields significantly affect myosin phosphorylation. *In* Charge and Field Effects in Biosystems, Vol. 3. M. J. Allen, S. F. Cleary, A. A. Sawers, and D. D. Shilady, editors. Birkhauser, Boston. 225–230.

Markov, M. S., S. Wang, and A. A. Pilla. 1993. Effects of weak low-frequency sinusoidal and dc magnetic fields on myosin phosphorylation in a cell-free preparation. *Bioelectrochem. Bioenerg.* 30:119–125.

Michnoff, C. H., B. E. Kemp, and J. T. Stull. 1986. Phosphorylation of synthetic peptides by skeletal muscle myosin light chain kinases. *J. Biol. Chem.* 261:8320–8326.

Montgomery, D. B. 1969. Solenoid Magnet Design. Wiley-Interscience, New York.

Parkinson, W. C., and G. L. Sulik. 1992. Diatom response to extremely low-frequency magnetic fields. *Radiat. Res.* 130:319–330.

Persechini, A., D. K. Blumenthal, H. W. Jarrett, C. B. Klee, D. O. Hardy, and R. H. Kretsinger. 1989. The effects of deletions in the central helix of calmodulin on enzyme activation and peptide binding. *J. Biol. Chem.* 264:8052–8058.

Prasad, A. V., M. W. Miller, E. L. Carstensen, C. Cox, M. Azadniv, and A. A. Brayman. 1991. Failure to reproduce increased calcium uptake in human lymphocytes at purported cyclotron resonance exposure conditions. *Radiat. Environ. Biophys.* 30:305–320.

Prasad, A. V., M. W. Miller, C. Cox, E. L. Carstensen, H. Hoops, and A. A. Brayman. 1994. A test of the influence of cyclotron resonance exposures on diatom motility. *Health Phys.* 66:305–312.

Roskoski, R. 1983. Assays of protein kinase. *Methods Enzymol.* 99:3–6.

Shuvalova L. A., M. V. Ostrovskaia, E. A. Sosunov, and V. V. Lednev. 1991. [The effect of a weak magnetic field in the paramagnetic resonance mode on the rate of the calmodulin-dependent phosphorylation of myosin in solution]. *Dokl. Akad. Nauk. SSSR.* [*Proc. Natl. Acad. Science USSR (Biophysics).*] 317:227–230 (in Russian).

Smith, S. D., B. R. McLeod, K. Cooksey, and A. R. Liboff. 1987. Calcium cyclotron resonance and diatom mobility. *Bioelectromagnetics.* 8:215–227.

Stull, J. T., M. H. Nunnally, and C. H. Michnoff. 1986. Calmodulin-dependent protein kinases. *In* The Enzymes, Vol. XVII. P. D. Boyer and E. G. Krebs, editors. Academic Press, Orlando. 113–166.

Yost, M. G., and R. P. Liburdy. 1992. Time-varying and static magnetic fields act in combination to alter calcium signal transduction in the lymphocyte. *FEBS Lett.* 296:117–122.

Walleczek, J. 1992. Electromagnetic field effects on cells of the immune system: the role of calcium signaling. *FASEB J.* 6:3177–3185.

Wang, K.-W., and S. B. Hladky. 1994a. Absence of effects of low-frequency, low-amplitude magnetic fields on the properties of gramicidin A channels. *Biophys. J.* 67:1473–1483.

Wang, K.-W., and S. B. Hladky. 1994b. An upper limit for the effect of low frequency magnetic fields on ATP-sensitive potassium channels. *Biochim. Biophys. Acta.* 1195:218–222.