Cellular Responses to Low-Frequency Electromagnetic Fields: Resonant Effects on Calcium Binding to Calcium-Binding Proteins

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Cellular Responses to Low-Frequency Electromagnetic Fields: Resonant Effects on Calcium Binding to Calcium-Binding Proteins

Over the past several decades, scientists have evaluated the effects of low-frequency electric and magnetic fields on biological systems. Studies with conflicting results have caused a high degree of uncertainty regarding the effects of such fields. A lack of a viable mechanism of interaction for weak fields has added to this uncertainty. The purpose of this project was to test the hypothesis of V.V. Lednev, who proposed that certain combinations of dc-ac magnetic fields have a frequency-dependent effect on the binding of calcium ions to calmodulin, a protein that regulates a variety of cellular processes.

INTEREST CATEGORIES

Electric and magnetic fields

KEYWORDS

Magnetic fields
Electric fields
Biological effects
Biological models
Health effects

BACKGROUND Biophysical interaction mechanisms can potentially explain how electric and magnetic fields (EMF) cause biological events and usually involve processes at or below the cellular level. Excepting certain specialized electric field sensory systems, a valid biophysical mechanism has not been identified that adequately explains reported or postulated effects of weak, environmental level, field effects in animals or humans. Identification of valid mechanisms is crucial in determining whether environmental EMF can cause health effects.

Lednev proposed a mechanism in which certain combinations of direct current (dc) and alternating current (ac) fields result in frequency-dependent (resonant) transfer of energy to macromolecules causing significant changes in cellular function (Lednev, 1991, *Bioelectromagnetics* 12:71-75). Furthermore, Lednev and coworkers reported supportive experiments in which predicted combinations of dc-ac magnetic fields produced changes in the phosphorylation of myosin, a molecular component of muscle tissue (Shuvalova et al., 1991, Dokl. Akad. Nauk. *SSSR* 317:227-230). According to Lednev's biophysical model, these changes arose due to field-induced alteration of the binding of calcium to calmodulin, a protein that regulates the phosphorylation of myosin, along with other cell activities. Lednev's model stimulated the development of the related ion parametric resonance (IPR) model by Blackman, Blanchard, and their colleagues (Blanchard and Blackman, 1994, *Bioelectromagnetics* 15:217-238; Blackman et al., 1994, *Bioelectromagnetics* 15:239-260).

OBJECTIVES To test Lednev's hypothesis that combined dc-ac magnetic fields have a frequency-dependent effect on the binding of calcium ions to calmodulin by conducting experiments, using several types of fluorescent molecular probes, to look for field effects on calmodulin directly, and by replicating reported experiments on myosin phosphorylation.

APPROACH The investigators conducted a high quality study with well documented exposure conditions. They used a broad range of magnetic field exposure conditions, including but not restricted to parameters predicted by the Lednev's model. They also examined several different, but related biological endpoints. Experiments were also performed to replicate work by Markov and coworkers (Markov et al., 1993, *Bioelectrochem. Bioenerg.* 30:119-125), who reported that weak dc magnetic fields alone, also affect myosin phosphorylation.

RESULTS The investigators did not observe any effects of magnetic field exposure for a broad range of dc-ac exposure conditions on any of the molecular/biochemical endpoints measured. Specifically, the measurements using fluorescent probes were negative as were the replication experiments on myosin phosphorylation, even though the investigators used exposure conditions and assay systems that were very similar to those used by Lednev and coworkers. These results are compelling evidence that the binding of calcium (or terbium) to calmodulin is not significantly affected by the magnetic field conditions specified by the Lednev resonance model. The experiments to replicate work by Markov and coworkers were also negative.

EPRI PERSPECTIVE The results of this study do not support the Lednev resonance model as a basis for weak EMF biological effects. If, in fact, the biological effects predicted and reported by Lednev and colleagues exist, it is unlikely that they could have been missed in this study. A number of other ongoing EPRI-sponsored studies address weak-field EMF mechanisms, including magnetic field interactions with magnetite (WO4307-03) and electroreception in rays and sharks (WO8021-18). An interim report was published on the magnetite research (TR-102008). Completed EPRI-sponsored research examined the "cyclotron resonance" mechanism in diatoms and calcium efflux in human lymphocytes with negative results (WO2965-02). EPRI Report TR-104800 provides a thorough review of EMF mechanisms. An EPRI-sponsored workshop on EMF neural effects also addressed mechanistic issues (TR-104327).

PROJECT
8911-16, 2965-29, 4308-04
EPRI Project Manager: Charles N. Rafferty
Environment Group
Strategic R&D
Contractor: University of Utah

ABSTRACT

The purpose of this project was to test Lednev's hypothesis that combined dc-ac magnetic fields have a parametric resonance effect on Ca^{2+} ions in the Ca^{2+}-binding sites of calmodulin. He postulated that the probability of a binding event taking place is altered when the ac magnetic field frequency is equal to the cyclotron frequency of the Ca^{2+} ion, and at subharmonics of the cyclotron frequency. His laboratory published experimental data showing that the Ca^{2+}/calmodulin-dependent activation of myosin light chain kinase (MLCK) is affected by combined dc-ac magnetic fields.

To test Lednev's hypothesis, we performed two series of experiments**:** (1) experiments using fluorescent reagents to determine the effects of low-frequency dc-ac magnetic fields on the binding of calcium or terbium to calmodulin in a direct way with a minimum number of constituents present, and (2) a replication of Lednev's semi-quantitative measurements of the effects of resonant low-frequency dc-ac magnetic fields on the Ca^{2+}/calmodulin-dependent activation of MLCK.

We performed three different kinds of fluorescence experiments to study the effects of low-frequency dc-ac magnetic fields on binding events. In the first, we used the fluorescent reagent, Calcium Green, which is sensitive to changes in calcium concentration, to study the effects of the magnetic fields on the binding of calcium to calmodulin. In the second set, we used a fluorescently labeled peptide that binds to calmodulin (at the same site as MLCK) in the presence of calcium to study the effects of the magnetic fields on the binding of calcium to calmodulin. In the third set, we used a fluorescent energy-transfer technique to determine the effects of the magnetic fields on the binding of terbium and calcium to calmodulin. Tb^{3+} has an ionic radius nearly identical to that of Ca^{2+} so it binds to calmodulin in the same binding sites as calcium and is often used in metal-binding studies.

In the MLCK experiments, we first replicated Lednev's work with two minor modifications: (1) we used a synthetic peptide substrate in place of the myosin light chains, and (2) instead of using gel electrophoresis, we used radioactive [γ-^{32}P] ATP in the phosphorylation of the peptide substrate, and a liquid scintillation counter to measure the amount of ^{32}P incorporated into the substrate. The synthetic peptide substrate is widely used for assays of MLCK because it is well-defined, highly purified, and convenient. In this study, the synthetic substrate had the added advantage of containing none of the Ca^{2+}-binding domains present in the protein substrate, which eliminates a potentially confounding variable from the assay system. In addition to the

experiments with the synthetic peptide substrate, we did MLCK experiments using a myosin light chain substrate, as Lednev and his colleagues did.

In other MLCK experiments, we attempted to replicate the experiments of Markov and Pilla (Markov et al., 1992, 1993), who reported that weak dc magnetic fields can affect the activity of smooth muscle MLCK. These investigators had used unusually low concentrations of the MLCK substrates, myosin light chains and ATP in their assays. Even though our calculations indicated that their assay conditions would result in negligible ^{32}P incorporation, we performed one experiment using conditions as close to theirs as possible. Consistent with our calculations based on MLCK enzyme kinetics, we could not detect any significant ^{32}P incorporation above background levels. Moreover, in our fluorescence experiments we saw no change in calcium-calmodulin interactions over the range of dc magnetic field strengths used by Markov and Pilla.

In all of the experiments we performed, there was no biologically significant effect (sufficiently large to alter in some significant way the function of a biochemical, cellular, or organismal process) of weak combined dc-ac magnetic fields on the binding of calcium or terbium to calmodulin. In particular, the results of our experiments did not show the changes in MLCK activity (three- to six-fold with a linewidth of about 1 Hz) reported by Lednev (Lednev, 1990; Shuvalova et al., 1991), even though very similar exposure conditions and assay systems were used. These results provide compelling evidence that under the exposure conditions used in this study, the binding of calcium (or terbium) to calmodulin is not significantly affected by weak, combined dc-ac magnetic fields at or near the cyclotron frequency of calcium (or terbium).

SUMMARY

The purpose of this project was to test Lednev's hypothesis that combined dc-ac magnetic fields have a parametric resonance effect on Ca^{2+} ions in the Ca^{2+}-binding sites of calmodulin. He postulated that the probability of a binding event taking place is altered when the ac magnetic field frequency is equal to the cyclotron frequency of the Ca^{2+} ion, and at subharmonics of the cyclotron frequency. His laboratory has published experimental data showing that the Ca^{2+}/calmodulin-dependent activation of myosin light chain kinase (MLCK) is affected by combined dc-ac magnetic fields. We tested Lednev's hypothesis in two ways, in fluorescence experiments and in replication of his phosphorylation experiments.

Validation of magnetic field measurements. Dr. Martin Misakian of the National Institute of Standards and Technology (NIST) checked the accuracy of our magnetic field measurements. He summarized the results of his measurements by stating in the cover letter of his report: "Very briefly, the ac and dc field levels which were examined agreed with or were close to the NIST measured values."

Fluorescence experiments. To test Lednev's hypothesis in a direct way, we performed three different kinds of fluorescence experiments to study the effects of low-frequency dc-ac magnetic fields on binding events in a system containing a minimum number of constituents. In the first, we used the fluorescent reagent, Calcium Green, which is sensitive to changes in calcium concentration, to study the effects of the magnetic fields on the binding of calcium to calmodulin. In the second set, we used a fluorescently labeled peptide that binds to calmodulin (at the same site as MLCK) in the presence of calcium to study the effects of the magnetic fields on the binding of calcium to calmodulin. In the third set, we used a fluorescent energy-transfer technique to determine the effects of the magnetic fields on the binding of terbium and calcium to calmodulin. Tb^{3+} has an ionic radius nearly identical to that of Ca^{2+} so it binds to calmodulin in the same binding sites as calcium and is often used in metal-binding studies. When bound, terbium will accept energy from nearby excited tyrosine molecules in the calmodulin, and will in turn emit fluorescent radiation.

These fluorescence systems offer a number of advantages. Since they are equilibrium systems, there is no depletion of any component (except for gradual photobleaching), which allows a single sample to be used for a large number of measurements. Since the detection means is optical, a single data point can be obtained in less than 30 seconds, and a ten-replicate average reading can be obtained in less than five minutes. Thus a large range of experimental conditions can be covered in the course of one day, using

the same solution sample. This eliminates possible sample-to-sample variations which could complicate data interpretation. The signal-to-noise ratios of the optical results are very high, and the fluorescence solutions contain the minimum number of constituents needed to test the calcium/calmodulin binding theory, as described earlier.

Phosphorylation experiments. We did two sets of phosphorylation experiments to replicate the work reported by Lednev and his colleagues and to test Lednev's cyclotron frequency theory. The experiments are based on a biochemical reaction in which the activation of the enzyme, myosin light chain kinase (MLCK), is dependent on the binding of four calcium ions to calmodulin. Once activated, MLCK catalyzes the phosphorylation of one of the light chain subunits of myosin. According to Lednev's theory, the binding of ionic calcium to calmodulin in this reaction is sensitive to combined dc-ac magnetic fields. The MLCK enzyme is activated by calmodulin only when all four Ca^{2+} binding sites in calmodulin are occupied. The rate of myosin light chain phosphorylation, determined by the amount of active enzyme, is therefore directly affected by any change in the affinity of calmodulin for Ca^{2+}.

We first replicated Lednev's work with two minor modifications in experimental techniques: (1) we used a synthetic peptide substrate in place of the myosin light chains, and (2) instead of using gel electrophoresis, we used radioactive [γ-^{32}P] ATP in the phosphorylation of the peptide substrate, and a liquid scintillation counter to measure the amount of ^{32}P incorporated into the substrate. The synthetic peptide substrate is widely used for assays of MLCK because it is well-defined, highly purified, and convenient. In this study, the synthetic substrate had the added advantage of containing none of the Ca^{2+}-binding domains present in the protein substrate, which eliminates a potentially confounding variable from the assay system.

The synthetic substrate has many advantages over the protein substrate, but may not behave like the protein substrate in all instances. For instance, the myosin light chains contain functional Ca^{2+}-binding sites, as in calmodulin, but these are absent in the synthetic peptide substrate. If electromagnetic-field (EMF) exposure acts on the myosin light chain substrate to alter its Ca^{2+}-binding sites, this may in turn alter its properties as a substrate for MLCK. To account for this possibility, we performed another set of experiments using a purified myosin light chain substrate. We also used an additional reaction mixture that contained a low concentration of calcium (20% of saturation) to test the possibility that the observed effects of the EMF exposure might occur more readily at low concentrations of free calcium.

In other MLCK experiments, we attempted to replicate the experiments of Markov and Pilla (Markov et al., 1992, 1993), who reported that weak dc magnetic fields can affect the activity of smooth muscle MLCK. These investigators had used unusually low concentrations of the MLCK substrates, myosin light chains and ATP in their assays. Even though our calculations indicated that their assay conditions would result in negligible ^{32}P incorporation, we performed one experiment using conditions as close to

theirs as possible. Consistent with our calculations based on MLCK enzyme kinetics, we could not detect any significant ^{32}P incorporation above background levels. Moreover, in our fluorescence experiments we saw no change in calcium-calmodulin interactions over the range of dc magnetic field strengths used by Markov and Pilla.

Results. We found no biologically significant effect (sufficiently large to alter in some significant way the function of a biochemical, cellular, or organismal process) of weak combined dc-ac magnetic fields on the binding of calcium or terbium to calmodulin in any of the experiments we performed. Specifically, we did not observe the changes in MLCK activity (three- to six-fold with a linewidth of about 1 Hz) reported by Lednev (Lednev, 1990; Shuvalova et al., 1991), even though we used very similar exposure conditions and assay systems. Our results provide compelling evidence that under the exposure conditions used in this study, the binding of calcium (or terbium) to calmodulin is not significantly affected by weak, combined dc-ac magnetic fields at or near the cyclotron frequency of calcium (or terbium).

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INTRODUCTION

1.1 Background

Over the past several decades, scientists have evaluated the effects of low-frequency electromagnetic radiation on biological systems. While some studies have indicated a significant biological response to such field exposure, other studies have indicated no response at all. These conflicting results have caused a high degree of uncertainty with regard to the effect of low-frequency magnetic fields on biological systems. The lack of a viable mechanism of interaction has added to this uncertainty. Recent work performed by Lednev (1990, 1991), however, has indicated a possible mechanism.

Lednev proposed that combined dc-ac magnetic fields have a parametric resonance effect on Ca^{2+} ions in the Ca^{2+}-binding sites of calmodulin. He has postulated that the probability of a binding event taking place is altered when the ac magnetic field frequency is equal to the cyclotron frequency of the Ca^{2+} ion, and at subharmonics of the cyclotron frequency. Lednev performed experiments in which he exposed a well-characterized Ca^{2+}/calmodulin-dependent system, the activation of MLCK, to combined dc-ac fields. The experimental results were largely consistent with the theory, indicating that perhaps a mechanism of interaction between dc-ac magnetic fields and biological systems had been identified.

More recently, Blackman, Blanchard and their colleagues (Blanchard and Blackman, 1994; Blackman et al., 1994) have elaborated upon Lednev's model by applying it to other ions and calling it the ion parametric resonance (IPR) model, and have related measured magnetic-field effects on neurite outgrowth stimulated by nerve growth factor to IPR model calculations.

Markov and colleagues (Markov et al., 1992, 1993) have also investigated the effects of applied magnetic fields on purified smooth muscle MLCK activity. They found that MLCK activity is decreased not only by weak, combined dc-ac magnetic fields, but also by ac fields alone and dc fields alone. For dc fields alone, their results showed a nearly linear relationship between the magnitude of the static field and the rate of substrate phosphorylation. For static field strengths below the ambient level, substrate

phosphorylation was less than that of sham-exposed controls. For levels above ambient, substrate phosphorylation was above that of controls.

1.2 Purpose of this Work

The purpose of this study has been to test Lednev's cyclotron resonance model. In the first year of the project, we exposed two separate experimental systems to the magnetic field conditions that Lednev used in his experiments. The first system is a replicate of the system used by Lednev (Ca^{2+}/calmodulin-dependent activation of MLCK), with two minor modifications. The first change is that the substrate used in the modified reaction was a synthetic peptide instead of purified myosin light chain that Lednev used. The second change lies in the technique used to monitor the degree of substrate phosphorylation. The second system employs a fluorescently-labeled peptide that simulates the Ca^{2+}/calmodulin binding domain of MLCK. The response of each system is sensitive to changes in the amount of Ca^{2+} bound to the calmodulin protein.

As stated in the original proposal, the objectives of the work during the first year were:

1. To replicate Lednev's (1990) semi-quantitative measurements of the effects of resonant low-frequency dc-ac magnetic fields on the Ca^{2+}/calmodulin activation of MLCK.

2. In addition to replicating Lednev's work, improve on it by: (1) using a fluorescent calmodulin-binding peptide in place of the MLCK enzyme in order to reduce the experimental system to its essential components and thereby identify specific points in the activation pathway that may be sensitive to magnetic fields; (2) rigorously testing his model by comparing experimentally determined curves of MLCK activation effects with uniquely shaped curves calculated from the model.

At the end of the first year's work we concluded the following:

1. Based on data from experiments done using both of the experimental systems we have developed, the cyclotron resonance theory does not apply to the specific case of calcium binding to calmodulin. Therefore the effects observed by Lednev and by Markov et al. must be due to some other site of interaction in the system. Further experiments should be done to identify these sites of interaction.

2. The experimental systems we have developed, in particular the modified fluorescence spectrometer, have great potential for investigating electromagnetic field effects in a variety of biochemical and cellular systems.

The work we have accomplished during the first year of the project had some limitations, including the following:

1. All the MLCK assay experiments used a synthetic peptide substrate in place of purified myosin light chain that Lednev used in his experiments. The synthetic peptide has many advantages over the protein substrate, but may not behave like the protein substrate in all instances.

2. The experiments above used a concentration of calcium that allows maximum sensitivity to changes in the binding of calcium to calmodulin, but it did not vary the concentration of calcium to monitor the effects of low frequency EMF exposure on MLCK activity at low concentration of free calcium.

The purpose of the work for the remainder of the project has been to extend the previous experiments. In addition, we utilized the fluorescent system that we developed during the first year of the project to further monitor the effects of magnetic fields on the binding of ions to calmodulin and myosin light chain. The tasks proposed for the remainder of the project were:

1. To purify myosin light chains from several different sources, including bovine heart, rabbit skeletal muscle, and chicken gizzard (smooth muscle) and determine whether a substantial effect of weak low-frequency dc-ac magnetic fields can be observed on this preparation. These experiments are to be done under a variety of calcium and magnesium concentrations since the myosin light chains are known to bind both of these physiological divalent cations, and differences in the response of the experimental system to ac or dc magnetic fields could depend upon the relative concentrations of the two ions.

2. To attempt to replicate Markov's experiments in which he and his colleagues measure the phosphorylation of myosin light chains as affected by dc magnetic fields. The main difference between this work and that in Task #1 will be that Markov's experiments were done at 37°C. Doing the experiments at 37°C will require the construction of a thermostatted water bath to maintain the temperature of the samples while they are inside the magnetic-field generating coils.

3. To develop a more efficient means of monitoring magnetic field effects on the binding of divalent metal ions to myosin light chains using the fluorescence system developed during the present project period, by using three different kinds of fluorescence-based assays: (1) fluorescently label purified light chains and determine whether there is a change in fluorescence due to changes in divalent metal ion binding to the labeled light chains; (2) employ a fluorescent calcium or magnesium ion indicator to monitor changes in free divalent ion concentration that result from magnetic field effects on divalent ion binding to purified light chains; (3) use the luminescent lanthanide, terbium, to monitor changes in the binding of this ion to purified myosin light chains. The terbium ion has an ionic radius very similar to calcium but is trivalent, so this ion typically binds with higher affinity than divalent ions to calcium-binding proteins such as the myosin light chains and calmodulin. These three different assay systems should give similar results even though the underlying principle behind each assay is quite different. In addition, the magnetic field-fluorescence assay system has already been built and thoroughly tested, so assay development time should be minimal.

2

WORK ACCOMPLISHED

2.1 Publications

The following abstracts and papers have resulted from our work:

1. S. P. Hendee, D. A. Christensen, D. K. Blumenthal, and C. H. Durney, "Do Weak Low-Frequency Electromagnetic Fields Have Cyclotron Resonance Effects on Calcium/Calmodulin Interactions with Myosin Light Chain Kinase?" Fifteenth Annual Meeting, Bioelectromagnetics Society, Los Angeles, June 13-17, 1993.

2. S. P. Hendee, D. A. Christensen, D. K. Blumenthal, and C. H. Durney, "Calcium/Calmodulin Interactions are not Affected by Various Weak ELF Magnetic Field Conditions," 1994 Annual Bioeffects Review, Albuquerque, NM November 6-10, 1994.

3. F. A. Faour, D. K. Blumenthal, C. H. Durney, and D. A. Christensen." A Test of the Ion Parametric Resonance Hypothesis Using a Terbium-Calmodulin Fluorescent System." Seventeenth annual meeting, Bioelectromagnetic Society, Boston, June 1995.

4. Hendee, S.P., Faour, F.A., Christensen, D.A., Patrick, B., Durney, C.H., and Blumenthal, D.K. "The Effects of Weak Extremely Low Frequency MagneticFields on Calcium/Calmodulin Interactions" Biophysical Journal, Vol. 70 (June) 1996, pp. 2915-2923.

5. F. A. Faour, S. P. Hendee, D. A. Christensen, C. H. Durney, and D. K. Blumenthal, "The Effects of Weak ELF Magnetic Fields on the Binding of Ions to Calmodulin," In preparation, to be submitted to Bioelectromagnetics.

2.2 Validation of Magnetic-Field Measurements

Dr. Martin Misakian of the National Institute of Standards and Technology (NIST) came to our laboratory on April 12, 1993 and checked the accuracy of our magnetic field measurements (this was after we had taken some of the data reported here). His report is included in Appendix A. He found the ac field magnitude at the center of the MLCK activity assay coils to be slightly higher than our measurements by 3-4% across the range of frequencies used in the experiments (14 to 19 Hz). He found the dc field magnitude at the coil center to be accurate within the precision limits of the NIST

equipment (± 2%). Consistent with our calculations, Dr. Misakian found the magnetic field to be uniform within 3% over the MLCK sample region. For the fluorescence assay coils, he found the central ac field to be 3-4% higher than the 20.9 μT target value (frequency range 9-18Hz). According to his measurements, the ac field magnitude throughout the sample exposure region was uniform to within 2% of the central field value. Because his fluxgate magnetometer probe was too large to fit within the restricted exposure space of the fluorescence coil apparatus, he could not verify the dc field strengths of the fluorescence coils.

Dr. Misakian summarized his results by stating in his cover letter (see Appendix A): "Very briefly, the ac and dc field levels which were examined agreed with or were close to the NIST measured values." Because the differences in his measured values and ours were small and consistent over the range of exposure conditions used in our experiments, we felt it not necessary to redo the earlier experiments.

2.3 Fluorescence Experiments

2.3.1 Rationale.

In addition to the MLCK phosphorylation experiments described below, which were intended to closely replicate Lednev's experimental conditions, we also performed a series of experiments using two types of fluorescent markers that are sensitive to calcium concentration. The first experiment used Calcium Green (Haugland, 1992-1994) as an indicator in a solution of calcium and calmodulin, as detailed below. The second set of experiments used a fluorescently labeled peptide which binds to calmodulin (at the same site as MLCK) in the presence of calcium. We also used a third fluorescent system that is sensitive to the binding of terbium to calmodulin. The solutions employed in the three fluorescent systems above were much more elementary than those used in the phosphorylation experiments. They contained only calcium or terbium, calmodulin, and the fluorescent indicator or labeled peptide (plus buffer). They did not contain MLCK, ATP, or any substrate. Thus the system was reduced to just the essential components implicated by Lednev's parametric resonance theory (plus the fluorescent indicator). These experiments, then, were intended to test the hypothesis of magnetic field modulation of the binding of calcium or terbium to calmodulin in a direct way with the minimum constituents necessary.

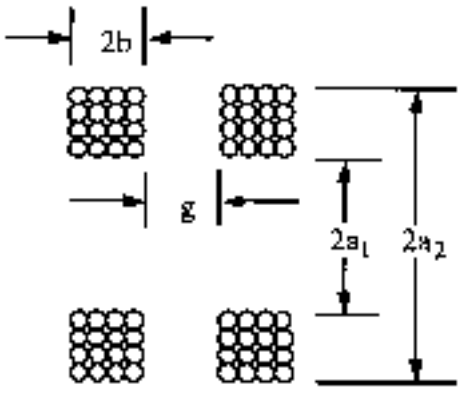
2.3.2 Experimental Configuration.

An ISS model PC1 fluorimeter was used for all of the fluorescence experiments. Its exposure chamber was modified to eliminate the presence of any paramagnetic metals within the chamber itself and to provide room for the placement of two pairs of exposure coils, one pair aligned with its axis in the horizontal direction and one in the vertical direction. These coils were designed by the procedure of Montgomery (1969)

with a geometry that maximized the volume of magnetic field uniformity inside the coils. As tested with a Bell model 620 Hall-effect gaussmeter equipped with an STB4-0404 transverse probe, the magnetic field was uniform to within 3% over a 1x1x1 cm^3 volume at the center of the coil pairs. The accuracy of the field measurements was independently confirmed by Dr. Misakian, whose visit is described in Section 2.2. Figure 2-1 shows the dimensions of the coil pairs; details of coil construction were given in a previous report (Durney et al., 1992).

The fluorimeter was used in a "T" configuration such that the excitation beam and the emission beam were at right angles. A quartz micro-cuvette containing the test solution with an excitation path length of 1 cm and an emission path length of 4 mm was placed inside the coils. A quartz lens focused the excitation beam to the center of the cuvette, and a second quartz lens collected the emission. Openings were provided in the coil forms to allow passage of the excitation and collection beams into and out of the cuvette.

The entire fluorimeter was rotated such that the axis of the horizontal coil pair was oriented magnetic north-south at its particular location in the lab. A dc current was then passed through the vertical coil pair by a Hewlett-Packard Model 6033B dc power supply until the vertical component of the earth's magnetic field was canceled as measured by the Bell gaussmeter probe. The horizontal coil pair was driven by a Hewlett-Packard Model 3314A function generator. The dc component was adjusted by varying the offset voltage, and the ac component was adjusted by varying the signal amplitude and frequency; various ac and dc magnetic field parameters were employed for the exposure experiments, as described later.



Cross section of coils with dimensions labeled

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

Figure 2-1
Dimensions of coils used to produce magnetic fields for the fluorescence experiments.

2.3.3 Advantages of Fluorescence Experiments.

The fluorescent systems used in this study are equilibrium systems. As a result, there is no depletion of any of the components (except for gradual photobleaching). This allows a single sample to be used for a large number of measurements. Since the detection means is optical, the readings at each experimental data point are rapid. A single reading can be obtained in less than 30 seconds, and a ten-replicate average reading can be obtained in less than five minutes. Thus a large range of experimental conditions can be covered in the course of one day, using the same solution sample. This eliminates possible sample-to-sample variations which could complicate data interpretation. The signal-to-noise ratios of the optical results are very high, as evidenced by a low variance in the output data points. Finally, the fluorescence solutions contain the minimum constituents needed to test the calcium/calmodulin binding theory, as explained earlier.

2.3.4 Calcium Green /Calmodulin Experiments.

This set of experiments was designed to measure the concentration of free calcium in a solution of calcium and calmodulin under both exposure and sham (ambient) conditions. First, the optical response of Calcium Green was investigated while titrating the concentration of calcium to determine an optimum operating point. These results are shown in Fig. 2-2. A solution of approximately 40 nM Calcium Green was illuminated with 475 nm light in the fluorimeter, and the intensity of the fluorescent emission at 530 nm (bandwidth = 16 nm) was detected as a function of the concentration of calcium. As can be seen in the figure, fluorescence intensity increases with free calcium concentration. In order to be at the steepest part of the response curve, allowing for maximum sensitivity to either increasing or decreasing calcium concentrations, we chose to fix the exposure conditions at a concentration of free calcium of 35 μM.

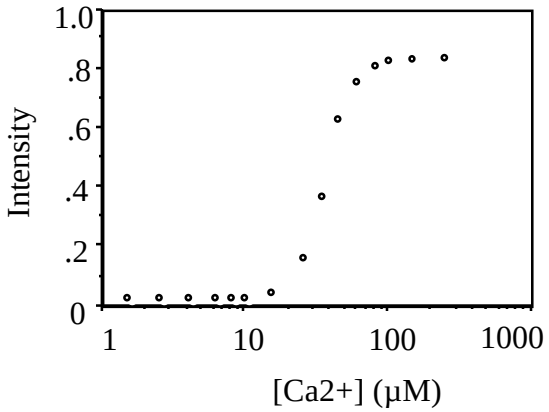


Figure 2-2
Intensity of Calcium Green fluorescence (530 nm) as a function of total calcium concentration. For subsequent exposure experiments, the total calcium concentration was fixed near the midpoint of this curve (35 µM).

The exposed solution contained 35 µM calcium, 5 µM calmodulin and 40 nM Calcium Green in a buffer containing 7 mM MOPS (4-morpholinepropanesulfonic acid), pH=7.0. In Fig. 2-2, the x axis indicates the total concentration of Ca^{2+} in the sample. All of the buffers (MOPS and Calcium Green) were treated to remove contaminating divalent metal ions by passing them through a Chelex-100 column. Since the concentration of Calcium Green in the sample is low (40 nM) relative to even the lowest indicated concentration of Ca^{2+}, the free Ca^{2+} concentration is essentially equal to the total Ca^{2+} concentration. (This curve can therefore be considered to be a calibration curve.)

The magnetic field in the horizontal coil pair was set to $20.9 (1 + \sin 2\pi ft)$ µT, and the frequency f of the ac portion was varied between 7 Hz and 19 Hz in a random order. Fluorescence intensity was monitored and recorded for each data point; every fifth measurement was an exposure to ambient conditions (i.e., the applied magnetic fields were turned off and the solution was therefore in the earth's ambient magnetic field) in order to provide some interspersed control points.

The results of this experiment are shown in Fig. 2-3. Here the fluorescence intensity is plotted as a function of the exposure frequency along with the range of intensity for ambient exposures. Also shown in Fig. 2-3 are the "min" and "max" intensity points, each determined at the end of the entire experiment. The "max" point was obtained by saturating the solution with an excess (500 µM) of calcium; this concentration of calcium is necessary to saturate the Calcium Green which has a K_D for calcium of approximately 30 µM. The "min" point was then obtained by adding an excess (1 mM) of EDTA, which effectively reduced the free calcium concentration to a negligibly small value. These two points were valuable in verifying that the experiment was indeed performed

in a calcium concentration range that was sensitive to any changes in its level, as seen by the fact that all data points fell about midway between the "max" and "min" values.

Examination of the data points for all exposure frequencies plotted in Fig. 2-3 indicates the lack of any effect of the exposing magnetic fields on the level of fluorescence and therefore on the concentration of free calcium, which is in turn related to the binding equilibrium of calcium to calmodulin. We therefore conclude that there is no evidence in this experiment for parametric resonance effects of calcium binding to calmodulin as predicted by Lednev.

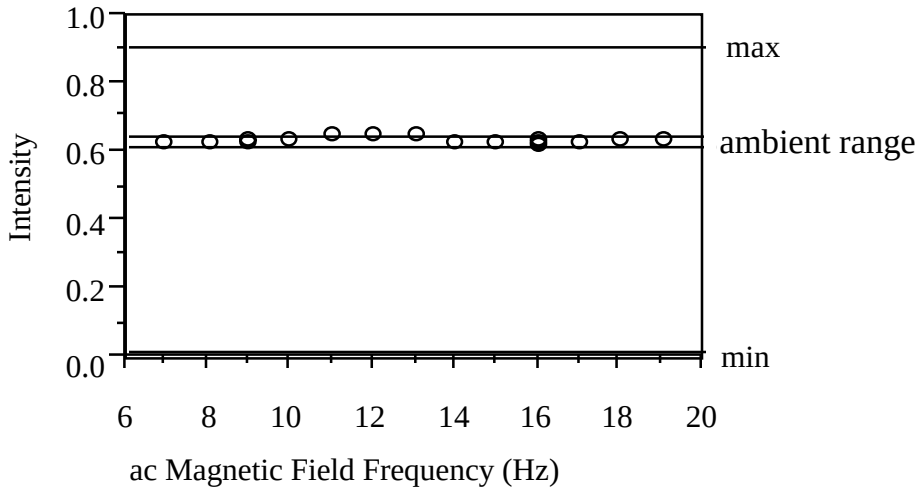


Figure 2-3
Calcium Green fluorescence intensity for various ac magnetic field frequencies. The applied magnetic field is given by B = 20.9 (1+sin2πft) μT. "max" and "min" represent the intensities observed with 500μM Ca2+ and 1mM EDTA, respectively. The range of intensities observed under the ambient field condition is indicated by the horizontal lines marked "ambient range." The total calcium concentration was 35 μM.

2.3.5 Calcium Green/Myosin Light Chain Experiments.

This set of experiments contained purified myosin light chain instead of calmodulin in the Calcium Green/calmodulin experiments described above. The experiments were done to study the effects of various magnetic exposure conditions on the binding of calcium to myosin light chain.

The assay contained 40 nM Calcium Green, 5 μM myosin light chains in a buffer containing 7 mM MOPS. Myosin light chains were purified from bovine cardiac muscle by the method of Blumenthal and Stull (1980). The fluorescence response of Calcium

Green in this assay was investigated by titrating with calcium. We chose to use a concentration of 7 μM for calcium in the assay because, as Fig. 2-4 shows, this concentration is near the midpoint of the calcium titration curve and therefore allows for maximum changes in Calcium Green fluorescence intensity as a function of changes in the free calcium concentration.

The Calcium Green/myosin light chain assay system was exposed to the following magnetic field conditions:

1. The vertical magnetic field was set to zero, while both the dc and ac components of the horizontal field were set to 20.9 μT amplitude. The frequency of the ac component varied between 14 Hz and 19 Hz in one Hz increments. Thus,

$$B_v = 0$$

$$B_h = 20.9\,[1+\sin(2\pi ft)] \quad (\mu T)$$

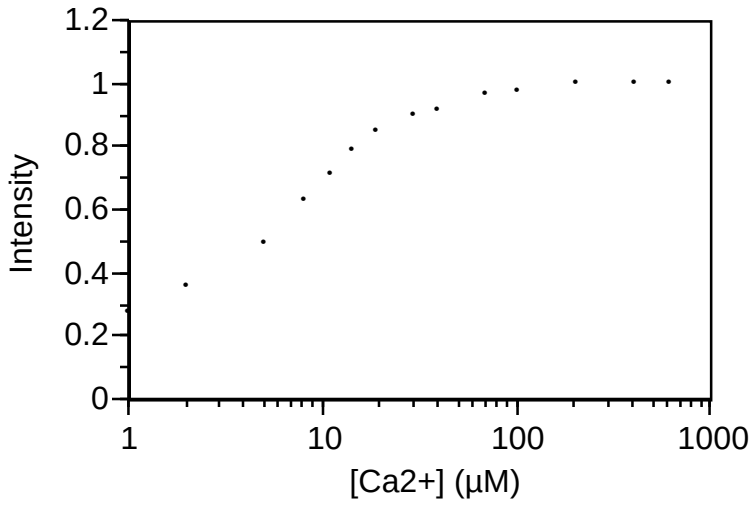


Figure 2-4
Fluorescence intensity of Calcium Green as a function of free calcium concentration. [Calcium Green] = 40 nM, and [myosin light chains] = 5 μM.

2. The vertical magnetic field was set to zero, while the horizontal field was set with a dc component of 20.9 μT amplitude and 100% amplitude modulated ac component. The modulation frequency (f_c) was varied between 14 Hz and 19 Hz in one Hz increments while the frequency (f) of the modulated ac component was set to 4 kHz. Thus,

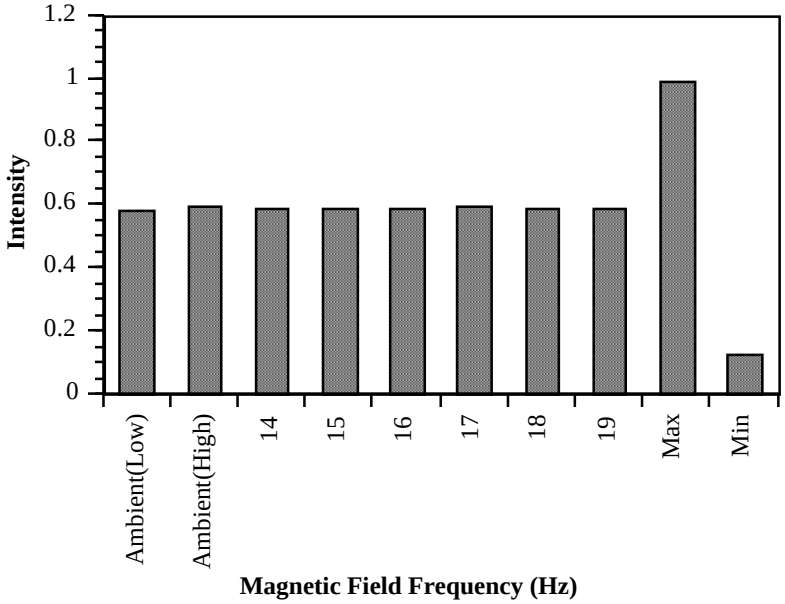
$$B_v = 0$$

$$B_h = 20.9\,\sin(2\pi f_c t)[1+\sin(2\pi ft)] \quad (\mu T)$$

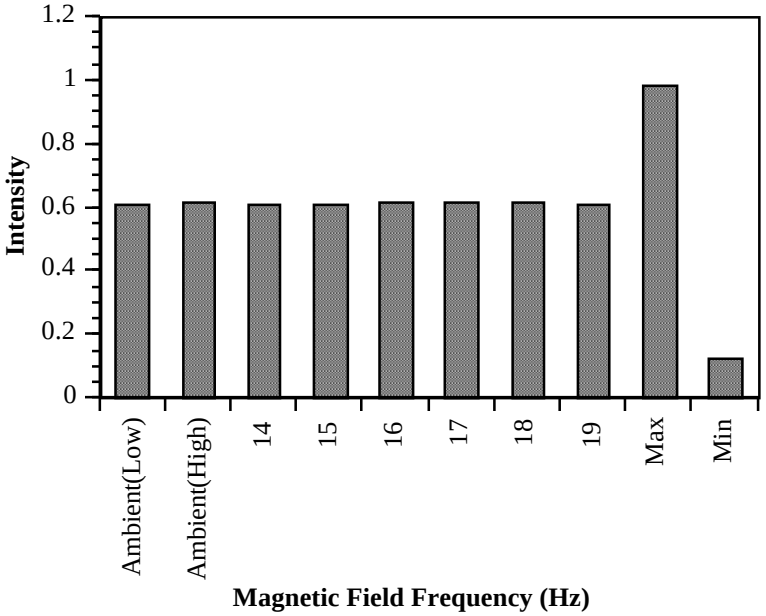
3. Same as (2) above but with the carrier frequency set to 200 kHz.

The last two conditions were chosen to explore the possibility that binding events might be sensitive to modulation signals instead of carrier signals.

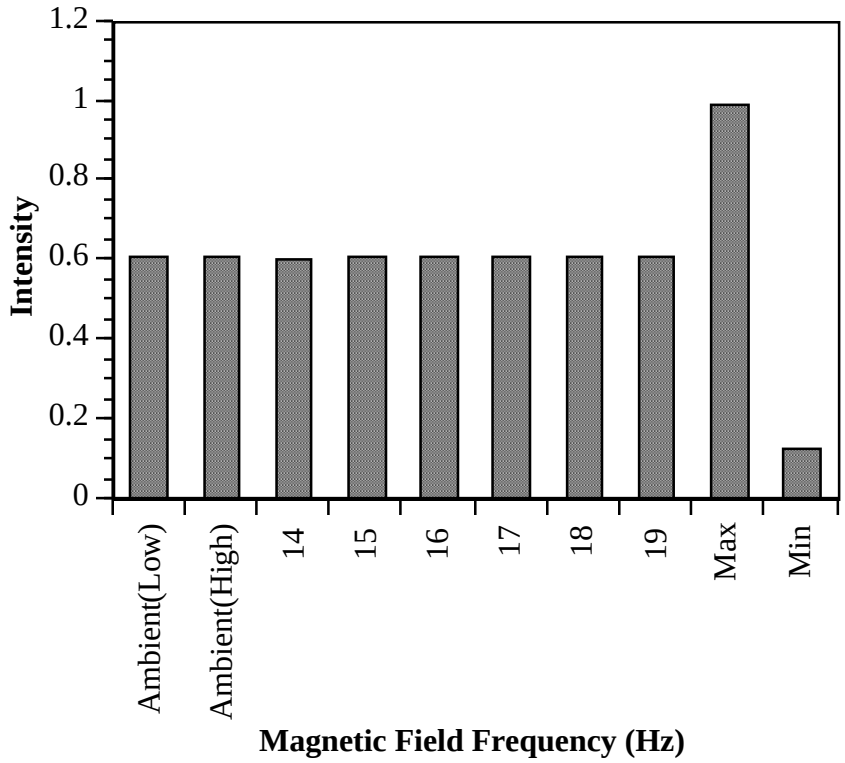
Examination of the results of the experiment in Fig. 2-5 shows that none of the magnetic field exposure conditions used had any measurable effect on the fluorescence intensity of Calcium Green. Thus, none of the conditions caused a measurable effect on the binding of calcium to myosin light chains.



(a)



(b)



(c)

Figure 2-5
Changes in Calcium Green fluorescence intensity as a function of changes in the frequency of the ac component of the horizontal magnetic fields. Frequencies were randomized. Standard deviation is negligible. (a) No amplitude modulation. (b) Carrier frequency is 4 kHz. (c) Carrier frequency is 200 kHz.

2.3.6 Anisotropy Experiments.

As an additional test of the possible effects of low-level magnetic effects on the binding of calcium to calmodulin, we performed an extensive series of fluorescence anisotropy measurements under various exposure (including ambient) conditions. The test solution for these experiments consisted of 140 µM calcium, 130 nM calmodulin, and 80 nM fluorescently labeled synthetic peptide which binds to calmodulin. These components were placed in a buffer solution containing 3 M Guanidine-HCl and 50 mM MOPS, pH=7.0. The stock buffers were purified of contaminating Ca^{2+} by passing them through a Chelex-100 column. The peptide (synthesized in Dr. Blumenthal's laboratory) was labeled at its C-terminus with the fluorescent dye acrylodan (Blumenthal, 1994). Acrylodan is excited at a wavelength of 370 nm, and has its peak emission between 470 and 520 mm, depending on the environment (Blumenthal, 1994).

The anisotropy measurements are based upon the following phenomenon: The peptide (in a similar fashion to MLCK) will only bind calmodulin with significant probability when four calcium ions have already been bound to the calmodulin, as follows:

$$4Ca^{2+} + \text{calmodulin} \longleftrightarrow Ca^{2+}{}_4 \bullet \text{calmodulin}$$

$$Ca^{2+}{}_4 \bullet \text{calmodulin} + \text{fluorescent peptide} \longleftrightarrow Ca^{2+}{}_4 \bullet \text{calmodulin} \bullet \text{fluorescent peptide}$$

Thus, changes in calcium binding to calmodulin will change the peptide binding equilibrium, which can be measured by fluorescence anisotropy.

The rotation in space of the acrylodan-labeled peptide (which is a relatively small molecule of low molecular weight) is significantly hampered when it binds to the much larger calmodulin molecule. The reduction in rotational mobility is manifested by a reduction in the anisotropy of the emitted fluorescence. In a sense, the binding of the peptide to the high-inertia calmodulin causes the fluorescent dipole to remain fixed in the direction of polarization of the exciting light, thus producing more linearly polarized fluorescent radiation than when the dipole is unbound and free to rotate during its emission lifetime. The lack of rotation (anisotropy) can be measured accurately and quickly by measuring the amount of radiation passing through an analyzer plate in each of two orthogonal orientations. The measurements take only a few seconds; in our experiments we averaged the readings from ten measurements to obtain an excellent signal-to-noise ratio in about five minutes.

Since the measurements could be taken quickly, it was possible to go through a broad range of experimental conditions in one session. We first ascertained the range of sensitivity of the anisotropy technique by titrating the calcium concentration in a solution of 130 nM calmodulin and 80 nM peptide, and measuring the fluorescence anisotropy as a function of calcium concentration. These data are shown in Fig. 2-6. As before, we wanted to position our experimental conditions such that we were at the midpoint of the Ca^{2+} titration so the system could be maximally sensitive to either an increase or decrease in the calcium binding constant. Therefore, we chose a calcium concentration of 140 µM for all further tests, as detailed below.

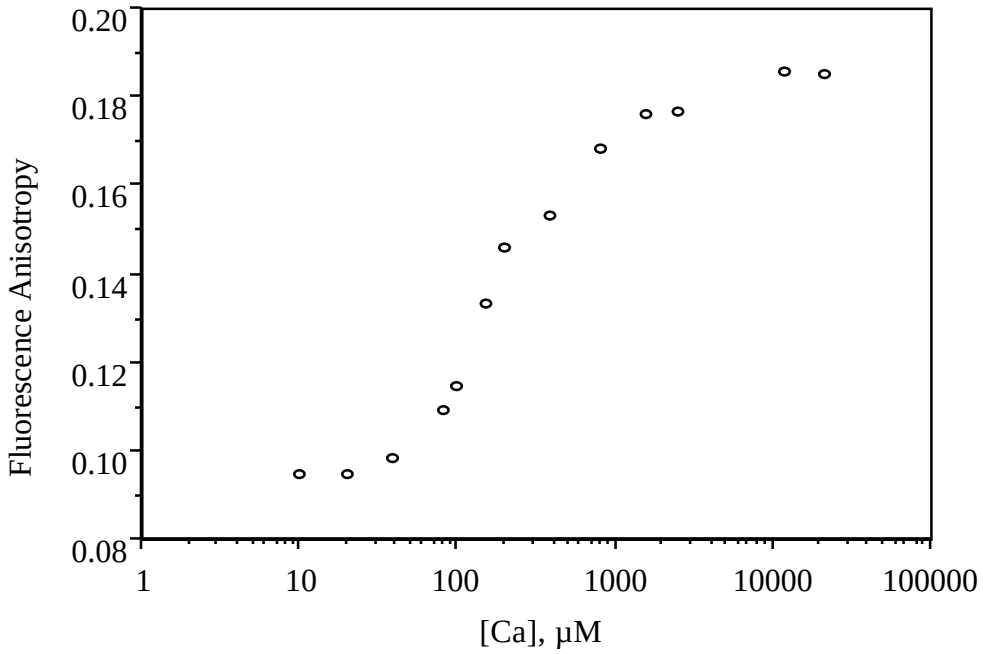


Figure 2-6
Fluorescence anisotropy as a function of calcium concentration in a solution of calcium, calmodulin and acrylodan labeled calmodulin-binding peptide. For maximum sensitivity to changes in calcium binding, the calcium concentration was chosen near the midpoint (140 µM) for subsequent exposure experiments.

The following test parameters were selected to investigate the validity of Lednev's theory over a broader range of conditions than was examined with the MLCK phosphorylation or Calcium Green experiments. These conditions are summarized in Table I and consisted of:

1. 60 Hz test with horizontal fields - For this test, the magnetic field was kept horizontal but increased in magnitude to $B=78.4(1 + \sin 2\pi ft)$ µT, and the frequency points were increased to 56, 60, and 64 Hz.

2. 60 Hz test with vertical fields - The parameters were kept the same as in test 1, but the field was applied in the vertical direction.

3. Ac amplitude doubled - In this test, the magnitude of the ac component was twice that of the dc component, $B=20.9(1 + 2\sin 2\pi ft)$ µT, and the frequency was varied around 16 Hz.

4. Ac amplitude halved - The parameters were the same as in test 3, except the ac component was one-half the dc component, with $B=20.9(1 + 0.5\sin 2\pi ft)$ µT.

5. Fixed frequency, variable dc amplitude - For this test, the frequency was held at 16 Hz and the dc amplitude was varied from 0 to 31 µT in 5 steps. The ac amplitude was held at 20.9 µT.

6. Noncollinear fields - The dc component of magnetic field was oriented in the vertical direction with an amplitude of 20.9 μT, while the ac component was horizontal with a value of 20.9sin2πft μT. The frequency was varied around 16 Hz.

7. Dc fields only - This experiment tested exposure to purely dc magnetic fields. The horizontal field was varied from 0 to 200 μT in steps of 25 μT. The vertical field was zero.

Table 2-1
Summary of exposure conditions for experiments using the acrylodan labeled calmodulin-binding peptide to detect changes in the binding of calcium to calmodulin. Experiments 1-6 are variations on the ion parametric resonance conditions used by Lednev, where f_r is the ion parametric resonance frequency.

Exp.	Exp. Description	B_{horiz} (μT)	B_{vert} (μT)	Result
1	IPR Evaluation, f_r = 60 Hz; Horizontal field orientation	78.4[1 + sin(2πft)]; f = 56, 60, 64 Hz	0	No effect
2	IPR Evaluation; f_r = 60 Hz; Vertical field orientation	0	78.4[1 + sin(2πft)]; f = 56, 60, 64 Hz	" "
3	IPR Evaluation, f_r = 16 Hz; Bac = 2Bdc	20.9[1 + 2sin(2πft)]; f = 14, 16, 18 Hz	0	" "
4	IPR Evaluation, f_r = 16 Hz; Bac = 0.5Bdc	20.9[1 + 0.5sin(2πft)]; f = 14, 16, 18 Hz	0	" "
5	IPR Evaluation, f_r = 16 Hz; Vary Bdc, hold f constant	Bdc + 20.9sin(2πft); Bdc = 0, 15.9, 20.9, 25.9, 30.9	0	" "
6	IPR Evaluation, f_r = 16 Hz; Non-collinear fields	20.9sin(2πft); f = 14, 16, 18 Hz	20.9	" "
7	dc Fields Only	Bdc = 0, 25, 50, 75, 100, 125, 150, 175, 200	0	" "

The results of all of the above tests grouped in blocks are shown in Fig. 2-7, where the data are arranged in blocks in chronological order. The magnitude of the measured

anisotropy is plotted for each of the tests as a function of the elapsed time since the beginning of the experiments. Interspersed with the exposure data points are ambient data points, which were taken at the beginning and ending of each test block. In the raw data, there was a gradual decrease in anisotropy during the course of the session, which we attributed to gradual photobleaching of the acrylodan-labeled MLCK peptide. This was consistent with the fact that a plot of only the ambient data points showed the same decrease, and the fact that the decrease was linear with the number of data points and therefore with the accumulated time of exposure of the dye to the excitation beam. (We minimized the effect of photobleaching by shuttering the excitation beam during all nonmeasurement times). Therefore the data shown in Fig. 2-7 have been corrected to cancel the time-dependent effect of the photobleaching.

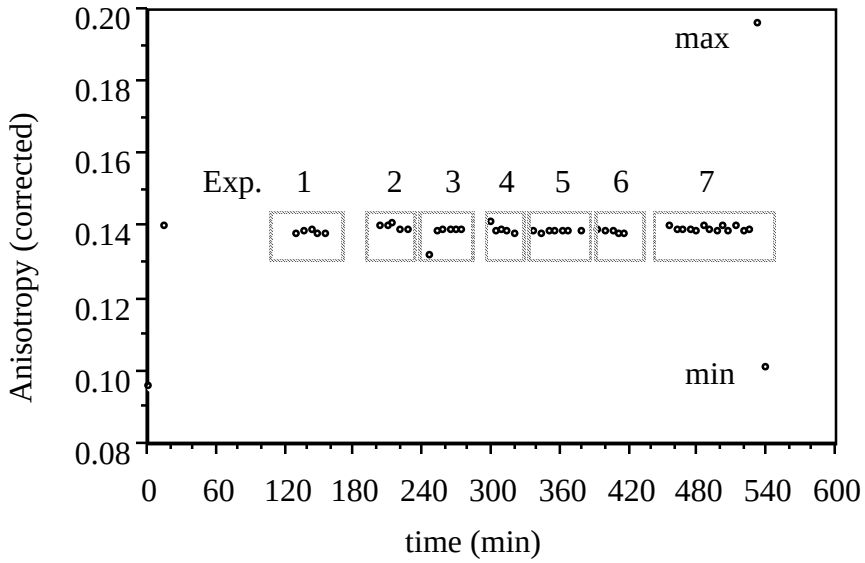
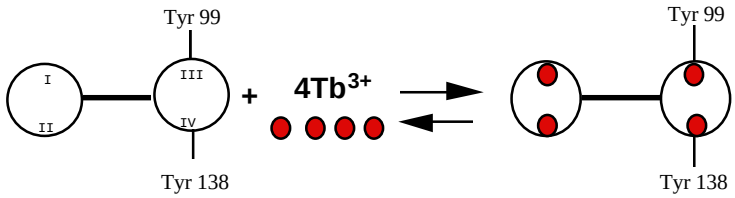


Figure 2-7
Fluorescence anisotropy values for the seven experimental conditions listed in Table 2-1, plotted in the order they were taken. "max" and "min" represent results for 500µM calcium and 1mM EDTA, respectively.

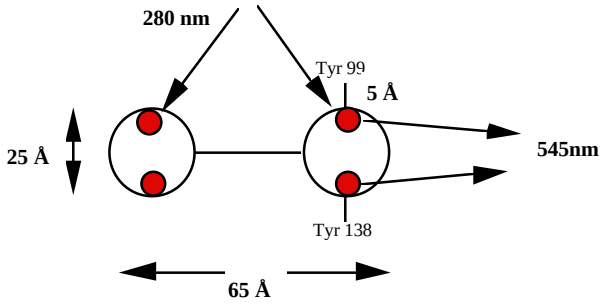
It is evident from the data points shown in Fig. 2-7 that there was no measurable change in the anisotropy values, and therefore no measurable change in the calcium/calmodulin binding constant, for any of the test conditions in this experimental series. This is consistent with the findings of the Calcium Green experiment.

2.3.7 Terbium/calmodulin Binding Experiments.

To further test by fluorescence means whether weak magnetic field exposure affects the binding of ions to calmodulin, we carried out experiments using terbium as the calmodulin-binding ion instead of calcium. Tb^{3+} has an ionic radius nearly identical to that of Ca^{2+} so it binds to calmodulin in the same binding sites as calcium and is often used in calmodulin-binding studies. When terbium binds to sites III and IV of calmodulin it is in close proximity to Tyr-99 and Tyr-138, respectively. Excitation of these tyrosines at 280 nm results in fluorescence energy transfer to the bound terbium which can be monitored by emission of light at 545 nm (Fig. 2-8). The intensity of the emitted light at 545 nm is proportional to the amount of bound terbium and can thus be used to monitor changes in calcium ion binding under various magnetic field exposure conditions.



(a)



(b)

Figure 2-8
(a) Four ions of terbium bind to the four ion binding sites of calmodulin.
(b) Terbium ions bound to sites III and IV of calmodulin emit 545 nm light when the system is excited with 280 nm light. The terbium in sites I and II are not excited because they are too far from Tyr 99 and Tyr 138.

Three different terbium assays where used in this experiment. The first assay monitored the binding of only terbium to calmodulin, while the second assay contained both terbium and calcium and was used to simultaneously monitor the binding of both terbium and calcium to the ion binding sites of calmodulin. The third assay contained

terbium and a synthetic peptide that corresponds to the calmodulin binding domain of MLCK. The fluorescence response of terbium in each of the three assays was monitored, and the optimal concentrations of the assay components was chosen to allow maximum changes in the fluorescence intensity as a function of changes in the amount of terbium bound to calmodulin. In assay 1 and assay 3 we chose a concentration of terbium that places the assays on the steepest part of the terbium titration curve (Fig. 2-9). In assay 2, enough terbium was added to nearly saturate calmodulin; then calcium was titrated to a concentration that places the assay on the steepest part of the terbium/calcium back-titration curve (Fig. 2-10). Table II lists the components of each assay.

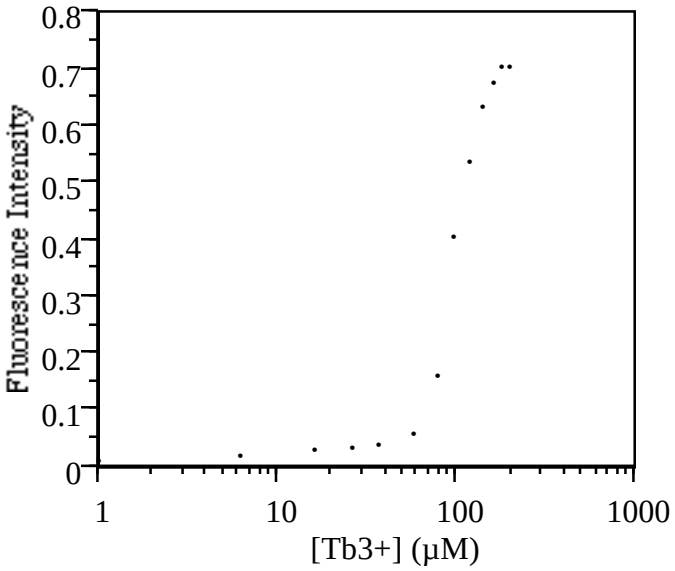


Figure 2-9
Terbium titration curve. Fluorescence response of terbium as a function of terbium concentration. Excitation was at 280nm (8nm bandpass) and emission at 545nm (8nm bandpass). A Schott UG11 bandpass filter was used in the excitation path and a Schott KV470 longpass filter in the emission path to minimize the scattering artifacts.

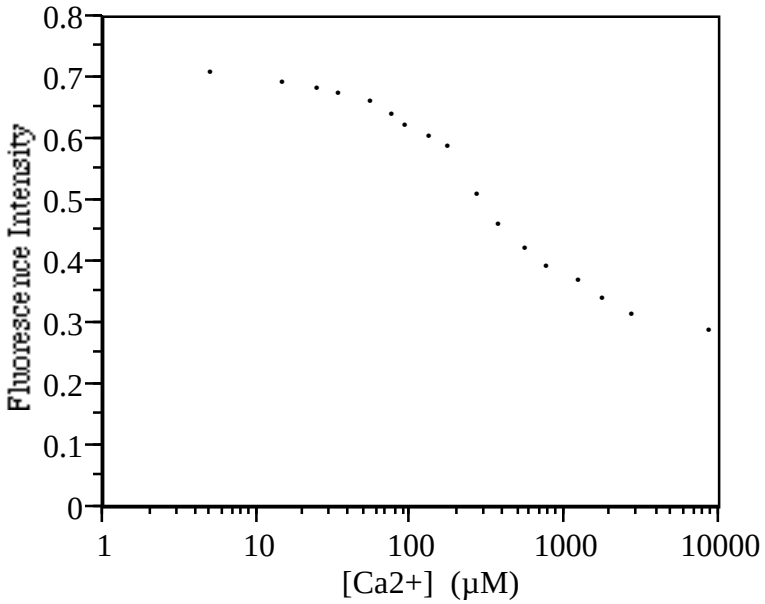


Figure 2-10
Terbium/calcium back titration curve. Fluorescence response of terbium as a function of calcium concentration. The assay contains a saturating concentration of terbium (120 µM).

Table 2-2
Chemical components of the three terbium assays used in the terbium fluorescence experiment.

Component	**Assay 1** (Fig. 2-9)	**Assay 2** (Fig. 2-10)	**Assay 3**
MOPS	7 mM	7 mM	7 mM
Calmodulin	30 µM	30 µM	30 µM
Terbium Chloride	80 µM	120 µM	100 µM
Calcium Chloride	0 µM	120 µM	0 µM
Peptide	0 µM	0 µM	30 µM

Two filters were used to reduce the effect of light scattering on the fluorescence measurements. One filter, a Schott UG11 280-370 nm band-pass, was placed in the path of the excitation light, while the second filter, 470 nm long-pass Schott KV470, was placed in the path of the emission light. The excitation monochrometer was set to 280 nm (bandwidth = 8 nm) and the emission monochrometer to 545 nm

(bandwidth = 8 nm). Each assay was exposed to the following magnetic field conditions:

1. The vertical magnetic field was set to zero, while the horizontal magnetic field was set to the sum of 20.9 μT dc component and 20.9 μT maximum amplitude sinusoidal ac component. The frequency (f) of the ac component was varied between 2 Hz and 20 Hz in 1 Hz increments. Under these magnetic exposure conditions, the cyclotron frequency of terbium is 6 Hz while that of calcium is 16 Hz. Therefore, for this case:

$$B_v = 0$$

$$B_h = 20.9\,[1+\sin(2\pi ft)] \quad (\mu T)$$

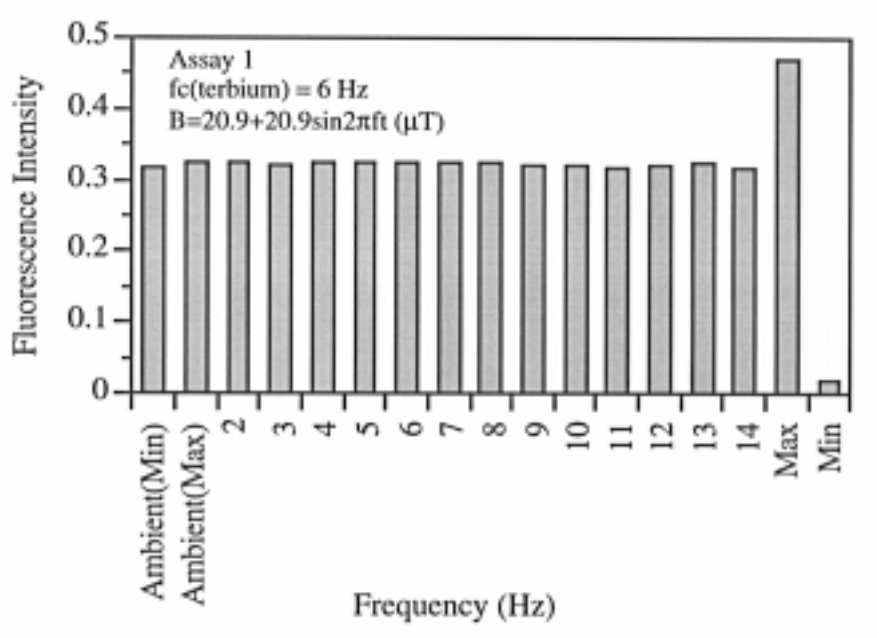
2. Same configuration as above, but using 55.6 μT amplitude instead of 20.9 μT. The frequency was varied between 14 Hz and 20 Hz in 1 Hz increments. Under these magnetic exposure conditions, the cyclotron frequency of terbium is 16 Hz. For this exposure:

$$B_v = 0$$

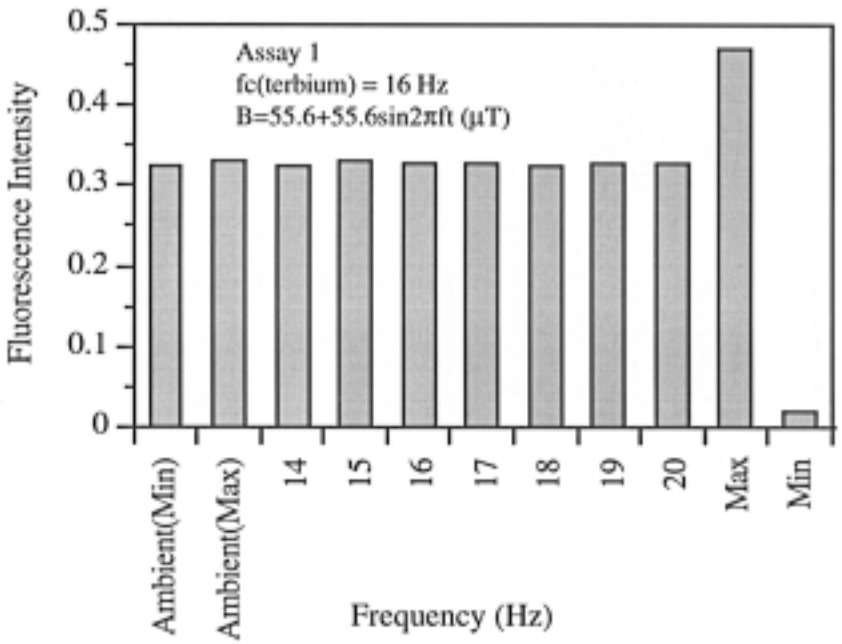
$$B_h = 55.6\,[1+\sin(2\pi ft)] \quad (\mu T)$$

3. The vertical magnetic field was set to zero, while the horizontal magnetic field was set to a dc signal. The dc amplitude was varied between 20 μT and 200 μT in 20 μT increments.

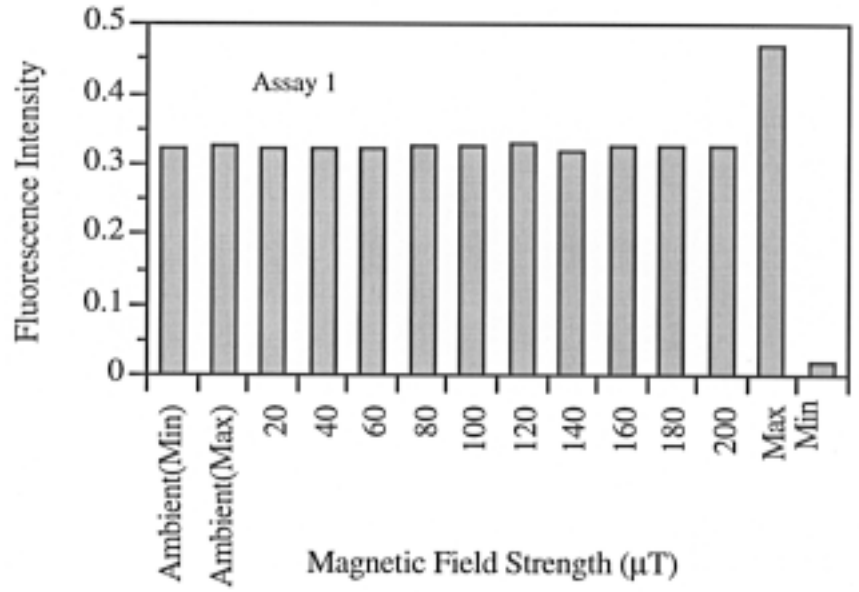
The results of the experiment are shown in Fig. 2-11. Every bar represents the average of ten measurements. The standard deviation from average is less than 1% for all exposure conditions. Ambient measurements were taken by disconnecting the power supply to the magnetic coils. One ambient measurement was taken every third exposure condition. The high and low values of fluorescent intensity for all ambient measurements are plotted. The maximum fluorescence intensity value, “Max”, was obtained by saturating calmodulin with terbium (500 μM terbium chloride), while the minimum fluorescence intensity, “Min”, was obtained by adding excess EGTA (3mM). The results show no effect of low-frequency, weak magnetic fields on the binding of either terbium or calcium to calmodulin (Fig. 2-11a, b, d, e, f, h, i). Also, dc magnetic fields between 20 μT and 200 μT did not affect the binding of terbium or calcium to calmodulin (Fig. 2-11c, g, j).


Assay 1
fc(terbium) = 6 Hz
B=20.9+20.9sin2πft (μT)
Fluorescence Intensity
0.5
0.4
0.3
0.2
0.1
0
Ambient(Min)
Ambient(Max)
2
3
4
5
6
7
8
9
10
11
12
13
14
Max
Min
Frequency (Hz)

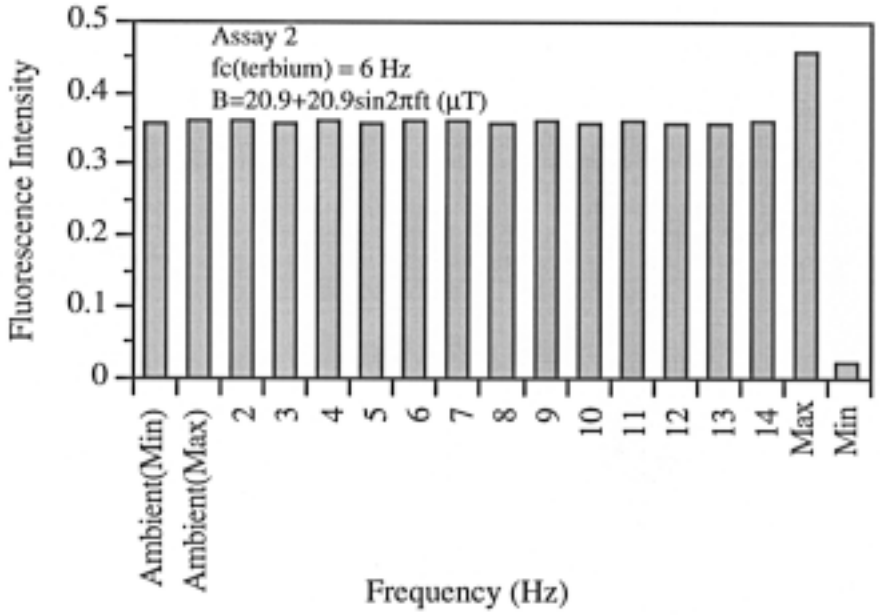
(a)


Assay 1
fc(terbium) = 16 Hz
B=55.6+55.6sin2πft (μT)
Fluorescence Intensity
0.5
0.4
0.3
0.2
0.1
0
Ambient(Min)
Ambient(Max)
14
15
16
17
18
19
20
Max
Min
Frequency (Hz)

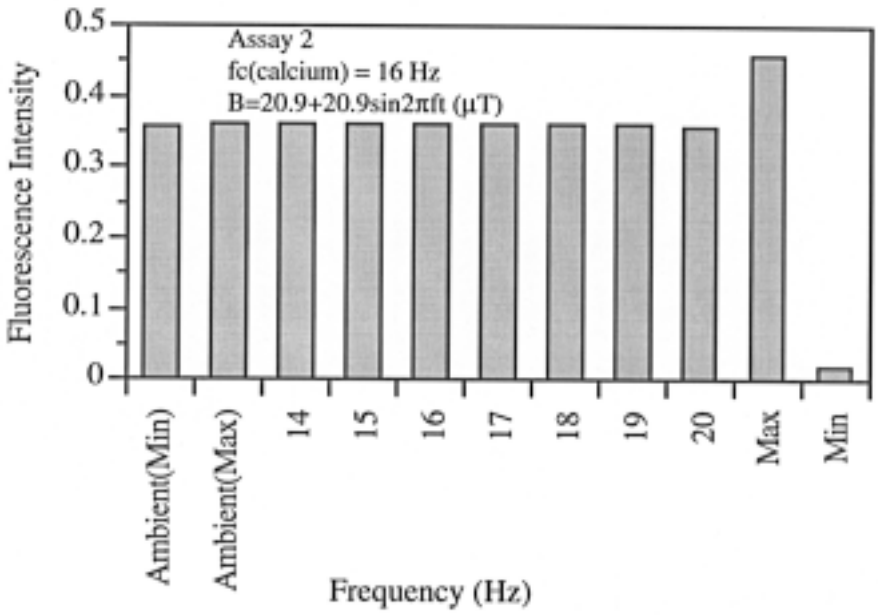
(b)


Assay 1
Fluorescence Intensity
0.5
0.4
0.3
0.2
0.1
0
Ambient(Min)
Ambient(Max)
20
40
60
80
100
120
140
160
180
200
Max
Min
Magnetic Field Strength (μT)

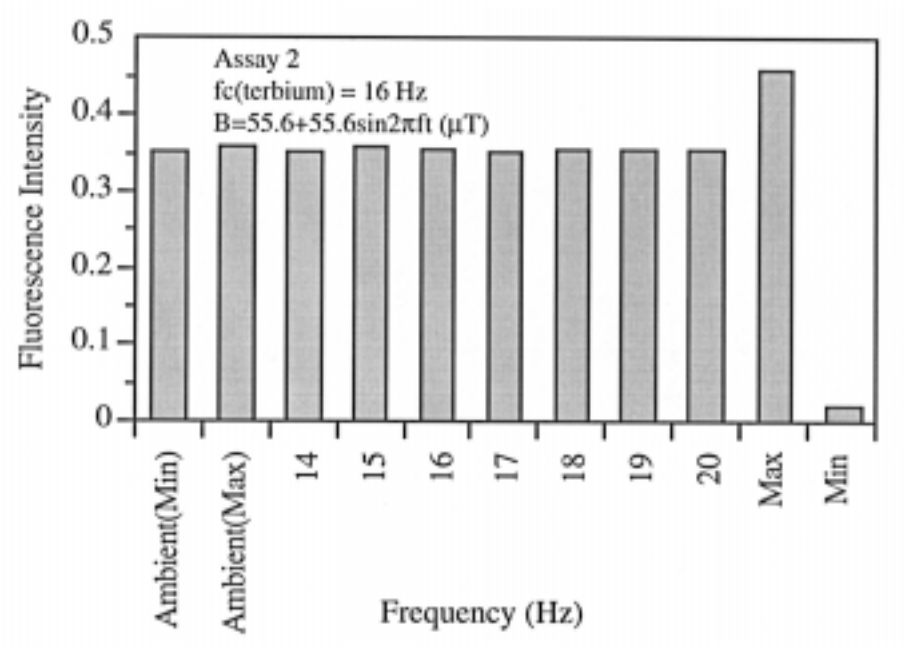
(c)


Assay 2
fc(terbium) = 6 Hz
B=20.9+20.9sin2πft (μT)
Fluorescence Intensity
0.5
0.4
0.3
0.2
0.1
0
Ambient(Min)
Ambient(Max)
2
3
4
5
6
7
8
9
10
11
12
13
14
Max
Min
Frequency (Hz)

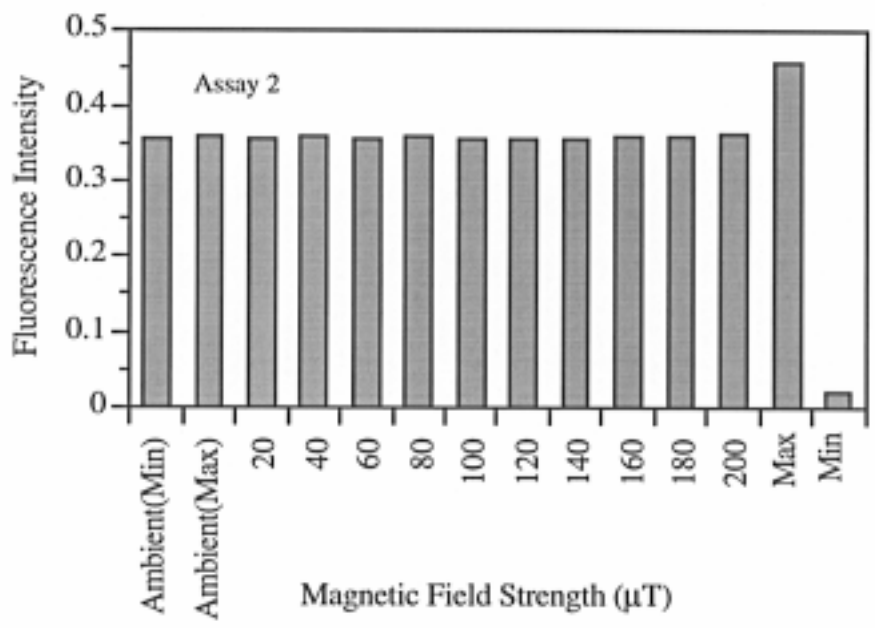
(d)


Assay 2
fc(calcium) = 16 Hz
B=20.9+20.9sin2πft (μT)
Fluorescence Intensity
0.5
0.4
0.3
0.2
0.1
0
Ambient(Min)
Ambient(Max)
14
15
16
17
18
19
20
Max
Min
Frequency (Hz)

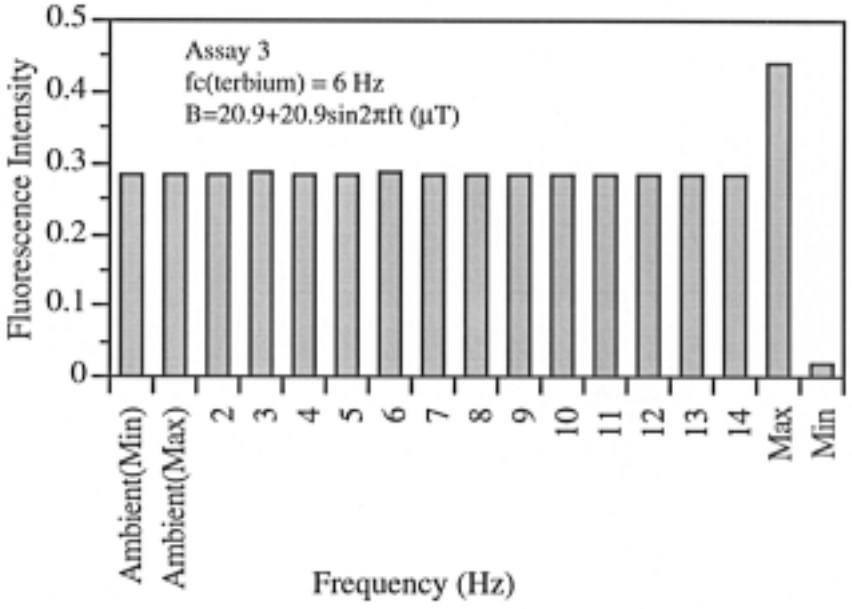
(e)


Assay 2
fc(terbium) = 16 Hz
B=55.6+55.6sin2πft (μT)
Fluorescence Intensity
0.5
0.4
0.3
0.2
0.1
0
Ambient(Min)
Ambient(Max)
14
15
16
17
18
19
20
Max
Min
Frequency (Hz)

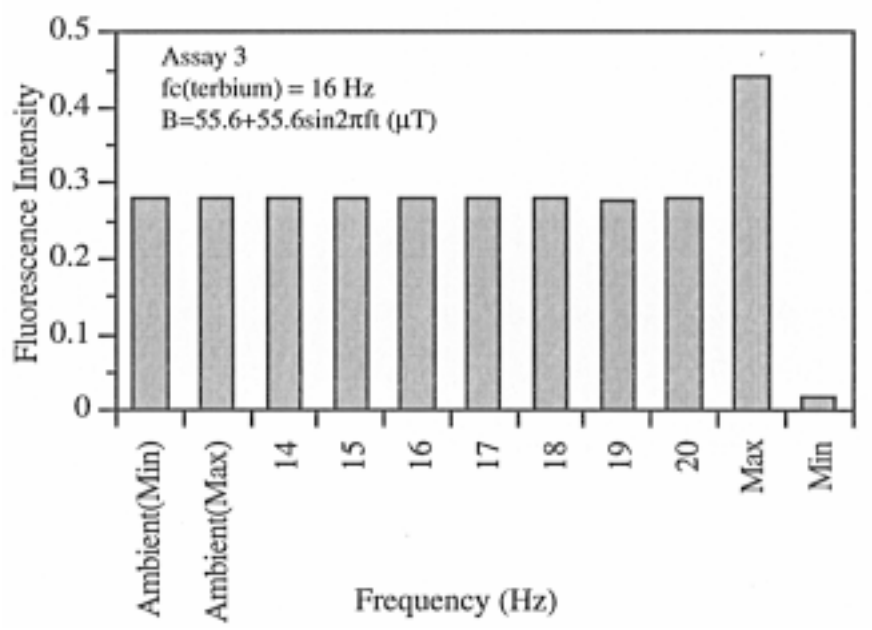
(f)


Assay 2
Fluorescence Intensity
0.5
0.4
0.3
0.2
0.1
0
Ambient(Min)
Ambient(Max)
20
40
60
80
100
120
140
160
180
200
Max
Min
Magnetic Field Strength (μT)

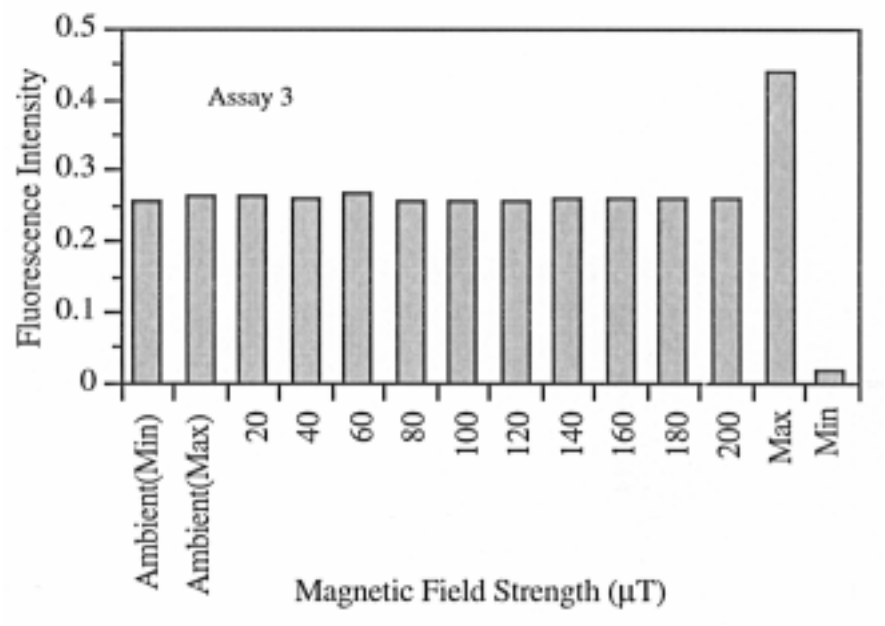
(g)


Assay 3
fc(terbium) = 6 Hz
B=20.9+20.9sin2πft (μT)
Fluorescence Intensity
0.5
0.4
0.3
0.2
0.1
0
Ambient(Min)
Ambient(Max)
2
3
4
5
6
7
8
9
10
11
12
13
14
Max
Min
Frequency (Hz)

(h)



(i)



(j)

Figure 2-11
Results of terbium fluorescence experiments. Standard deviations are negligible. fc is the cyclotron frequency. Panels a, b, d, e, f, h, and i are the results of exposure to combined ac and dc magnetic fields. Panels c, g, and j are the results of exposure to only a dc magnetic field.

2.4 Phosphorylation Experiments

2.4.1 Rationale.

This set of experiments was done to replicate the work reported by Lednev and to test Lednev's cyclotron frequency theory. The experiments utilize a biochemical reaction in which the activation of the enzyme, myosin light chain kinase (MLCK), is dependent on the binding of four calcium ions to calmodulin. Once activated, MLCK catalyzes the phosphorylation of one of the light chain subunits of myosin. A schematic of the biochemical reactions involved is given below:

$$4Ca^{2+} + \text{calmodulin} \longleftrightarrow Ca^{2+}{}_4 \bullet \text{calmodulin}$$

$$Ca^{2+}{}_4 \bullet \text{calmodulin} + MLCK_{(\text{inactive})} \longleftrightarrow Ca^{2+}{}_4 \bullet \text{calmodulin} \bullet MLCK_{(\text{active})}$$

According to Lednev's theory, the binding of ionic calcium to calmodulin (step 1 above) is sensitive to combined dc-ac magnetic fields. The MLCK enzyme is activated by calmodulin only when all four Ca^{2+} binding sites in calmodulin are occupied (step 2 above). The rate of myosin light chain phosphorylation, determined by the amount of active enzyme, is therefore directly affected by any change in the affinity of calmodulin for Ca^{2+}. Lednev exposed this system to combined dc-ac magnetic fields for a fixed period of time (10 min) and evaluated the effect on MLCK activity by comparing the ratio of phosphorylated myosin light chain to that of non-phosphorylated myosin light chain as measured by gel electrophoresis.

2.4.2 Magnetic Field Exposure System.

Control of the magnetic fields to which the reaction mixtures were exposed was achieved through the use of a coil apparatus containing four pairs of coils: two pairs oriented along a vertical axis and two pairs oriented along a horizontal axis. The coil apparatus was aligned north-south, so that the horizontal coil axis was parallel to the horizontal component of the geomagnetic field. One of the coil pairs along the vertical axis was driven with an HP6033B dc power supply to control the vertical component of the dc magnetic field in the center of the apparatus. The other pair along the vertical axis was not connected. One of the horizontally oriented pairs was driven with an HP6203B dc power supply to control the horizontal component of the dc magnetic field at the center of the apparatus. The other horizontally oriented coil pair was used to control the ac component of the magnetic field, and was driven by a Bogen amplifier (Model C 100B) connected to an HP3314A function generator. A block diagram of the field exposure system is shown in Fig. 2-12.

The magnetic fields were set by positioning the probe of a Bell 620 gaussmeter in the center of the apparatus, aligned to measure the vertical field component, and adjusting

the HP6033B power supply output to completely cancel the vertical component of the geomagnetic field (B_v = 0.0 μT ± 0.1 μT). The gaussmeter probe was then positioned to measure the horizontal field component along the axes of the horizontally oriented pairs. The HP6203B power supply was adjusted to set the desired dc component of the horizontal fields. The frequency of the HP3314A function generator was set to the desired exposure frequency (f) and verified with a counter, and the amplitude was adjusted to the desired maximum amplitude. The magnetic fields were measured several times during an experiment, and there was no indication of significant drift.

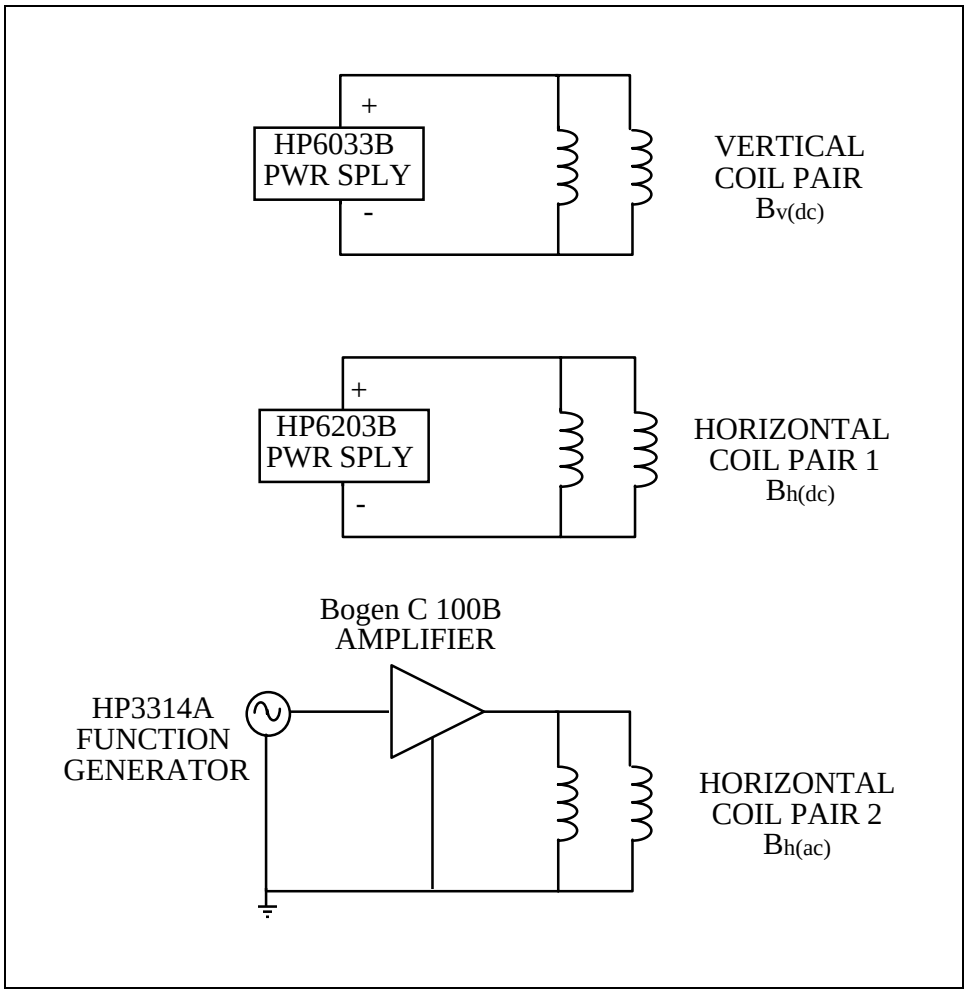


Figure 2-12
Block diagram of the magnetic field exposure system for the phosphorylation experiments.

2.4.3 Synthetic Peptide Substrate Experiments.

This set of experiments replicated Lednev's work using the calcium/calmodulin dependent activation of MLCK, but with two minor modifications. As in Lednev's

experiments, we used MLCK activity to monitor changes in the binding between Ca^{2+} and calmodulin [MLCK purified from rabbit skeletal muscle; calmodulin purified from bovine testes (Dasgupta et al., 1989)]. The first change is that the substrate used in the modified reaction was a synthetic peptide in place of myosin light chain. The synthetic peptide substrate that was used is the sequence of 14 amino acids that corresponds to the phosphorylation site in smooth muscle myosin P-light chain. This substitute is widely used for assays of MLCK because it is well-defined, highly purified, and convenient. In this study, the synthetic substrate had the added advantage of containing none of the Ca^{2+}-binding domains present in the protein substrate, which eliminates a potentially confounding variable from the assay system. The second modification lies in the technique used to monitor the degree of substrate phosphorylation. Radioactive [γ-^{32}P] ATP was used in the phosphorylation of the peptide substrate. Instead of using gel electrophoresis, we used a liquid scintillation counter to measure the amount of ^{32}P incorporated into the substrate.

In each assay, reactions were exposed to magnetic fields of $B=20.9[1+\sin(2\pi ft)]\mu T$, where f (representing the ac field frequency) was varied from 14 to 19 Hz in 1 Hz increments. Two ambient exposures ($B \approx 45\mu T$) were also performed, one each at the beginning and end of the experiment. Nine reactions, each having 50µl total volume, were prepared for each exposure condition: three samples of primary reaction mixture, three samples of "V_{max}" control reaction mixture, and three samples of "blank" control reaction mixture. The composition of these reaction mixtures is described in Table III.

Table 2-3
Reaction mixtures for phosphorylation assays.

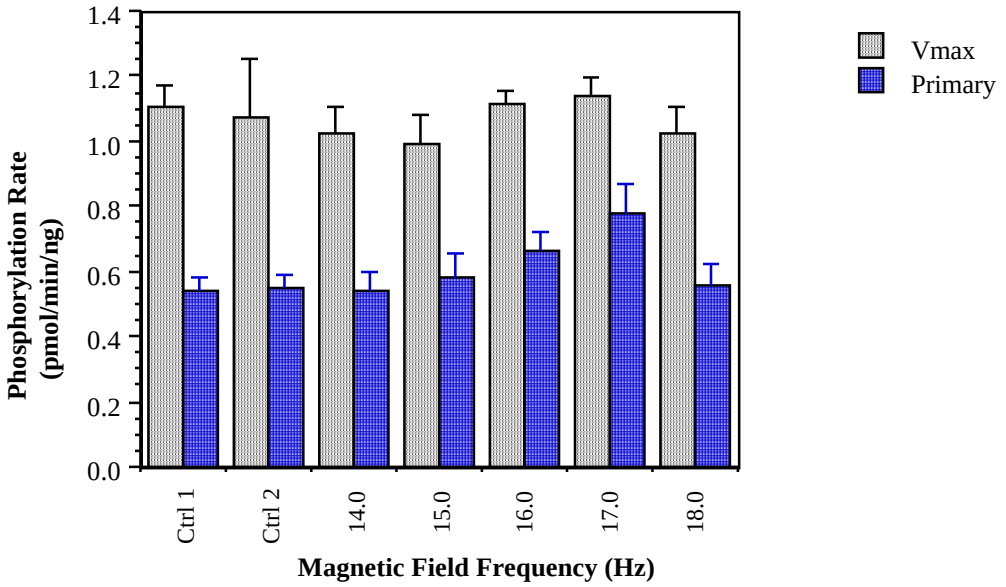
Reaction Mixture:	***primary***	***V_{max}***	***blank***
[MOPS]	50mM	50mM	50mM
pH	7.0	7.0	7.0
[dithiothreitol]	1mM	1mM	1mM
[magnesium acetate]	10mM	10mM	20mM
[$CaCl_2$]	2.1mM	200µM	0
[EGTA]	3mM	0	4mM
[calmodulin]	200nM	1µM	5nM
[MLCK]	1nM	1nM	0
[synthetic substrate]	173µM	173µM	173µM

The first control, V_{max}, differed from the primary reaction mixture in that there was a high concentration of calmodulin, saturating Ca^{2+}, and no EGTA. These conditions allowed the phosphorylation of the substrate to proceed at a maximum rate. The

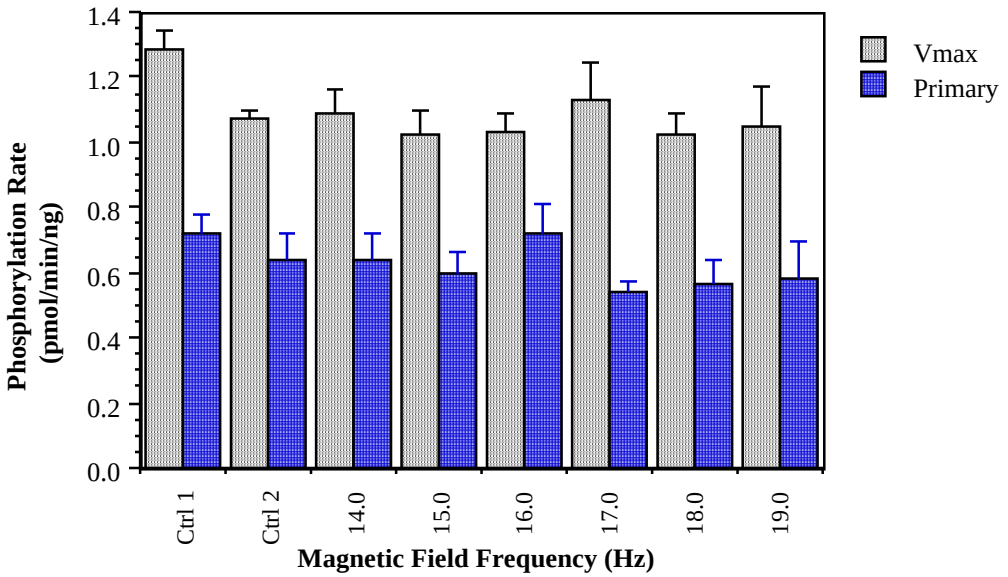
second control, a "blank" mixture, differed from the primary reaction mixture in that there was no enzyme present to catalyze the phosphorylation of the substrate. The blank reactions received the same amount of [γ-^{32}P] ATP as the other reactions and were used to correct for nonspecific ^{32}P adsorption to the filter.

For each exposure condition, the nine samples were taken from an ice bath and incubated at room temperature for 5 min. Reactions were then initiated by the addition of [γ-^{32}P]ATP to a final concentration of 1mM, and the samples were placed into the field exposure apparatus. The reactions were thermally insulated through the use of a Plexiglas block which had been drilled to accommodate the sample tubes. Aliquots of 20µl were taken from each of the nine samples after 5 and 10 min exposure times. These aliquots were spotted on Whatman P81 phosphocellulose filter papers, which were immediately immersed in a beaker containing 75mM phosphoric acid to quench the reaction. The phosphoric acid was exchanged at least five times to wash away all of the unreacted [γ-^{32}P] ATP. The filters were then dried, placed in a vial containing liquid scintillant, and counted in a liquid scintillation counter.

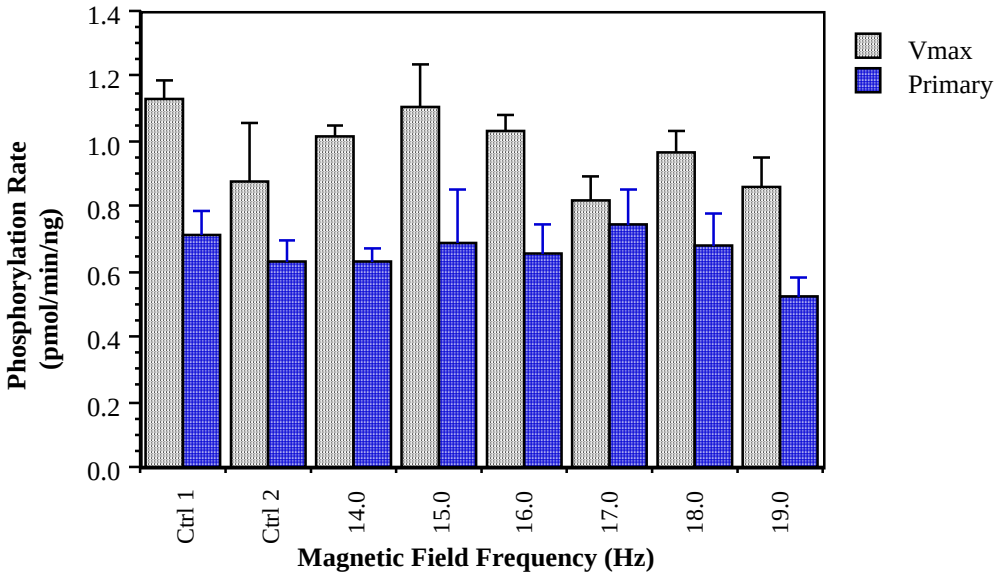
Results of three independent phosphorylation assays are shown in Fig. 2-13. The values indicated on the graphs represent the average phosphorylation rate of the exposed samples (n=6 at each frequency or control condition). The error bars in the data represent the standard deviations of the phosphorylation rates for the associated measurement point. Each reaction condition shown in Fig. 2-13 (data in-toto) was analyzed by 1-way analysis of variance (ANOVA) using the StatWorks program (v1.2, Cricket Software). No significant differences were observed between any of the magnetic field exposure conditions (criterion of significance $p<0.05$).



(a)



(b)



(c)

Figure 2-13
Results of phosphorylation assays. Ctrl 1 and Ctrl 2 represent ambient controls, taken at the beginning and end of the experiment, respectively. The phosphorylation rates shown combine the results of three samples at 5 and 10 min exposure times (n=6), with the error bars representing the standard deviation. The phosphorylation rates are given in pmol of substrate phosphorylated per min per ng of enzyme present in the sample.

(a) Data taken on August 11, 1992. (b) Data taken on August 26, 1992. (c) Data taken on August 27, 1992.

2.4.4 Purified Myosin Light Chain Experiments.

The phosphorylation experiments described above used a synthetic peptide substrate which is based on the sequence of the phosphorylation site of the myosin light chain substrate. This substrate has many advantages over the protein substrate, but may not behave like the protein substrate in all instances. For instance, the myosin light chains contain functional Ca^{2+}-binding sites, as in calmodulin, but these are absent in the synthetic peptide substrate. If EMF exposure acts on the myosin light chain substrate to alter its Ca^{2+}-binding sites, this may in turn alter its properties as a substrate for MLCK. To account for this possibility, we performed this set of experiments using a purified myosin light chain substrate. We also used an additional reaction mixture that contained a low concentration of calcium (20% of saturation) to test the possibility that the observed effects of the EMF exposure might occur more readily at low concentrations of free calcium.

The MLCK reactions using purified myosin light chain 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+}, 3 mM EGTA (free $[Ca^{2+}]$ = 0.586 µM); the $[Ca^{2+}]_{low}$ reactions contained 1mM Ca^{2+} , 3 mM EGTA (free $[Ca^{2+}]$ = 0.255 µM); the blank reactions contained 2 mM EGTA and no added calcium. The $[Ca^{2+}]_{high}$ reactions were approximately 60% the rate of $[Ca^{2+}]_{sat}$ reactions and the $[Ca^{2+}]_{low}$ reactions were approximately 20% the rate of $[Ca^{2+}]_{sat}$. Reactions were run in duplicate at 30°C. The indicated free calcium concentrations were calculated using the computer program developed by Fabiato (1988). This program is widely used by muscle physiologists using calcium/EGTA buffering systems to study the calcium dependence of muscle contraction.

The data shown in Fig. 2-14 are the results of dc-ac magnetic field exposure on MLCK activity using myosin light chains as substrate. The reactions performed at the higher concentration of Ca^{2+} are better suited for detecting decreases in calmodulin's affinity for Ca^{2+} while the reactions done at low concentrations 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 results indicate that combined dc-ac magnetic fields had no significant effect on MLCK activity at 16 Hz or any of the other frequencies tested. Statistical analysis for each reaction condition was performed by 1-way ANOVA using StatWorks (criterion of significance $p<0.01$). Thus, use of myosin

light chains as a substrate gave identical results to those obtained using a synthetic peptide substrate.

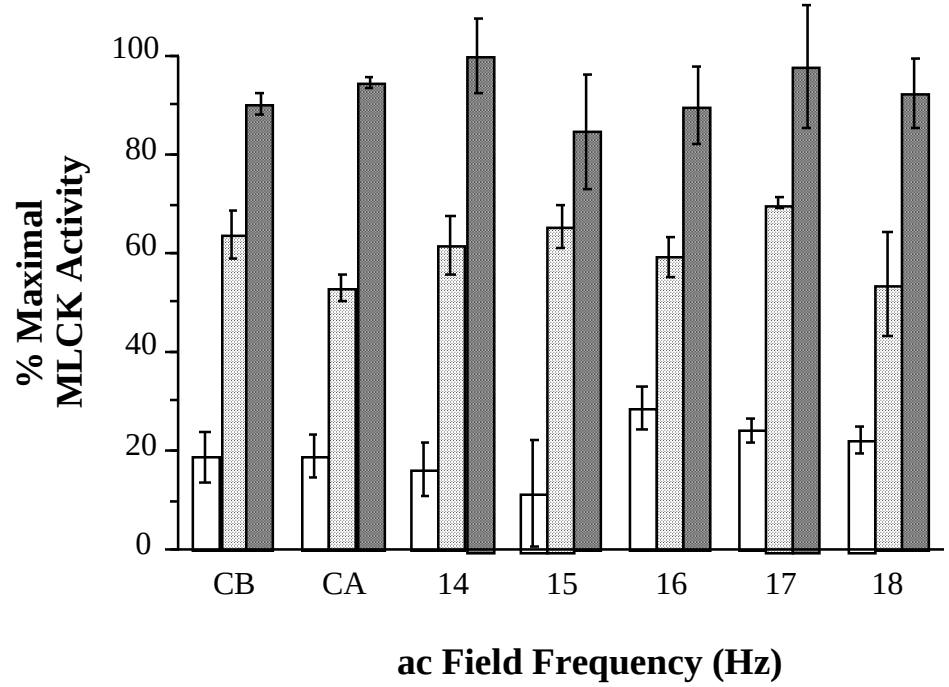


Figure 2-14
Myosin light chain kinase activity as a function of magnetic field frequency using myosin light chains as substrate. $[Ca^{2+}]_{sat}$ reaction mixtures are represented by darkly shaded bars, $[Ca^{2+}]_{high}$ reaction mixtures by lightly shaded bars, and $[Ca^{2+}]_{low}$ reaction mixtures by unshaded bars. CB and CA represent MLCK activity of control samples exposed to the ambient geomagnetic field before (CB) and after (CA) magnetic field exposures.

Our original intention was also to perform phosphorylation experiments using purified light chains from rabbit skeletal muscle and chicken gizzards. However, the yields of light chain from rabbit skeletal muscle were so low that obtaining enough purified material for the intended studies would have been too costly. We decided, therefore, to use bovine light chains, which we could purify in large quantity at relatively moderate cost, in the extensive experiments that we did. This strategy is also justified because the biochemical properties of the bovine cardiac light chains and rabbit skeletal muscle are identical. However, because no effects of magnetic fields were observed in any of the studies with bovine light chains (both MLCK and Calcium Green), further expensive studies with other light chains were not justified.

2.5 Lednev's Visit to our Laboratories

We arranged for Dr. V. V. Lednev to visit our laboratories, and he spent three days, February 9 - 11, 1994, with us. In addition to the time he spent in our laboratory in detailed discussions about our work, he also gave a formal lecture to which the University community was invited, and spent a morning in the laboratories of Dr. Chuck Grissom in the Chemistry Department. Dr. Grissom is also investigating the effects of magnetic fields on biological systems, but with much stronger fields than used in our work.

Dr. Lednev's visit was very fruitful. It afforded ample opportunity to discuss in depth our experiments as related to his, and to explore the meaning of his model of ion parametric resonance. After lengthy discussions about why we did not see the same kind of effects of magnetic fields on phosphorylation that he did, he gave his opinion that the difference was probably due to our use of a synthetic peptide substrate instead of the native myosin light chains. Lednev thought that there might be differences in calmodulin-MLCK interactions when different substrates were used in the MLCK reaction due to the effects of free-energy coupling. He encouraged us to use a protein substrate in our experiments (which we did, as reported herein)because he felt that this would allow us to see the kinds of effects of magnetic fields on MLCK activity that he had seen**.**

During discussions about his model of ion parametric resonance, Dr. Lednev explained that his model was a classical model, even though his published analysis was couched in quantum mechanical terms. He dismissed Adair's criticisms of his work as being invalid. Since his model consists essentially of a harmonic oscillator exposed to the forces of magnetic fields, we discussed with him how that model is related to effects on phosphorylation.

While Dr. Lednev obviously had very strong feelings about the validity of his model, we found that we could not share that enthusiasm, even after in-depth discussions with him. We wrote out the equations for the classical version of his model and discussed them with him while he was here. Our main question is how very weak, very low-frequency magnetic field forces can have a significant effect on the very high-frequency (in the infra-red region) oscillator. Both the classical equations and Lednev's equations show that the magnetic field forces have an entirely negligible effect unless some sort of probability of binding is included. In Lednev's model, he integrates over the lifetime of the binding, after calculating the transition probability, and it is only that integration that results in an effect; without it, there is no effect. Rather than affecting the very high-frequency oscillation of the oscillator, it seems more likely to us that the magnetic fields would affect some much slower process, perhaps related to the lifetime of the binding, which is within the time frame of the slowly varying magnetic fields. It does not make good physical sense to expect that a magnetic field varying at less than 100 Hz

could measurably affect an oscillation occurring at more than 10 orders of magnitude higher in frequency, no matter what the model.

2.6 Measurement of MLCK Activity Using the Conditions of Markov & Pilla

2.6.1 Rationale.

Because of recent reports from the laboratory of Markov & Pilla (Markov et al., 1992, 1993) that weak dc magnetic fields can affect the activity of smooth muscle MLCK, we have reviewed the assay conditions used by these investigators to determine if there were significant differences that might account for our inability to observe substantial effects in any of our experiments.

2.6.2 Approach.

The conditions used in the MLCK assays reported by Markov and Pilla were very different than those used in our experiments. The assay conditions used in our laboratory are essentially the same as those used by most other laboratories studying the biochemical properties of MLCK. We contacted Markov and Pilla before trying to replicate their work to be sure that we used assay conditions that were as similar as possible to theirs. The assay conditions used by Markov are as follows:

Reaction mixture (Markov & Pilla):
- 40 mM HEPES, pH 7.0
- 0.5 mM Magnesium acetate
- 0.5 mM Dithiothreitol
- 1mg/ml Bovine serum albumin
- 5 nM Myosin light chains (gizzard)
- 2 nM MLCK (gizzard)
- 70 nM Calmodulin
- 0.9 µM Calcium chloride
- 2.5 µM [γ-^{32}P]ATP (100-2000 cpm/pmol)

The reaction (w/o ATP) was pre-incubated for 1 min at 37° before adding ATP to start the reaction. The reaction was allowed to proceed for 2-15 min before quenching the reaction.

These reaction conditions are unusual in that the concentrations of the substrates, myosin light chains and ATP, are both well below their respective K_m values. The K_m for myosin light chains is approximately 15 µM and the K_m for ATP is approximately 100 µM. The rate of an enzyme reaction that follows Michaelis-Menten kinetics (as does MLCK) is described by the well-known Michaelis-Menten equation:

$$v/V_{max} = [S] / (K_m + [S]),$$

where V_{max} is the enzymatic rate at saturating concentrations of substrate, [S] represents the concentration of substrate, and K_m is the Michaelis-Menten constant. Thus, the rate of the reaction under the conditions used by Markov and Pilla will be approximately 120,000 lower than V_{max}. Using the published value for the V_{max} of gizzard MLCK (20 pmol phosphate incorporated per second per pmol enzyme) we estimate that using the highest specific activity ATP (2000 cpm/pmol) under the reaction conditions indicated above, there would only be 60 cpm of ^{32}P incorporated in a 0.1 ml reaction after 15 min. Since the background of the MLCK assay is normally several hundred cpm (using an ATP specific activity of 2000 cpm/pmol) incorporation of only 60 cpm would be difficult to quantitate accurately.

We discussed our concerns with Markov and Pilla, but they maintain that they are able to obtain meaningful data from their experiments. We have also contacted Drs. Robert Adelstein and James Sellers from NIH who provided Markov and Pilla with the MLCK and myosin light chains for their experiments. Adelstein and Sellers use assay conditions almost identical to ours in their laboratory and have spent considerable amounts of time trying to persuade Markov and Pilla to use more conventional assay conditions in their experiments.

2.6.3 Results.

Even though our calculations indicate that the levels of ^{32}P incorporation would be very close to background using the Markov and Pilla protocol, we performed one experiment using conditions as close to theirs as possible. The only difference from the conditions indicated above were that we used a peptide substrate instead of the light chain as substrate and we used rabbit skeletal muscle MLCK instead of gizzard MLCK. After a 15 min reaction, we could not detect any significant ^{32}P incorporation (262 ± 96 cpm) above background (258 ± 62 cpm) using an ATP specific activity of 1990 cpm/pmol. Because of the extremely low levels of incorporation using the conditions of Markov and Pilla we do not feel that their assay conditions can be used to collect meaningful data.

3

CONCLUSIONS

We have used both fluorescence and phosphorylation systems to monitor the affinity of calcium to calmodulin under various conditions of ac/dc magnetic field exposure. We also used the fluorescence properties of terbium to monitor changes in the binding of terbium to calmodulin. The results of our experiments indicate no biologically significant effect (sufficiently large to alter in some significant way the function of a biochemical, cellular, or organismal process) of weak combined dc-ac magnetic fields on the binding of calcium or terbium to calmodulin. In particular, the results of our experiments did not show the changes in MLCK activity (three- to six-fold with a linewidth of about 1 Hz) reported by Lednev (Lednev, 1990; Shuvalova et al., 1991), even though very similar exposure conditions and assay systems were used. The conditions in each of the 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 or terbium. The results obtained from our experiments provide compelling evidence that under the exposure conditions used in this study, the binding of calcium (or terbium) to calmodulin is not significantly affected by weak, combined dc-ac magnetic fields at or near the cyclotron frequency of calcium (or terbium).

4

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Appendix A

NIST REPORT OF MAGNETIC-FIELD VALIDATION

NIST | UNITED STATES DEPARTMENT OF COMMERCE
National Institute of Standards and Technology
[illegible]
Telephone: (301) 975-2426
Fax No. (301) 975-4091
Mail Room B344, Building 220

April 21, 1993

Dr. Charles Rafferty
Environmental Risk
and Health Science Department
Electric Power Research Institute
3412 Hillview Avenue
P.O. Box 10412
Palo Alto, CA 94303

Dear Chuck:

Enclosed is the measurement report for my recent site visit to the University of Utah. Very briefly, the ac and dc field levels which were examined agreed with or were close to the NIST measured values. A copy of the report is being sent to Dr. Durney. If there are any questions regarding the enclosed report, please call.

Sincerely,

Martin Misakian, Physicist
Applied Electrical Measurements
Electricity Division

Enclosure

cc: C.H. Durney

Measurement of ELF Magnetic Fields
at the University of Utah
Salt Lake City, UT
April 12, 1993

Applied Electrical Measurements
Report No. 811-3-74

Prepared by

Martin Misakian
Electricity Division
National Institute of Standards and Technology

Reviewed by: _____
William E. Anderson, Leader
Applied Electrical Measurements Group
Electricity Division

Approved by: _____
Oskars Petersons, Chief
Electricity Division

Measurements of ELF Magnetic Fields at the University of Utah

This report describes a visit in April 1993 to the University of Utah (UU) during which extremely low frequency magnetic fields were characterized in biological exposure systems. Measurements were performed in two exposure systems that are being used to test the theoretical predictions of V.V. Lednev. Briefly the larger of the two systems, referred to below as the MLCK system, consists of in part two pairs of circular coils with common horizontal axes aligned with the (laboratory) magnetic north-south direction and two pairs of coils with a common vertical axis. One of the vertical pairs is not used, while the other pair is used to null the vertical component of the earth's field. One coil-pair along the north-south direction modifies the earth's horizontal magnetic field so that it is 20.9 μT in magnitude. The remaining coil is used to generate magnetic fields from 14 Hz to 19 Hz with an rms value of 14.8 μT (20.9 μT peak). The smaller exposure system, referred to as the fluorescence system below, generates fields of the same magnitude and direction as the larger system over the frequency range 6 Hz to 18 Hz. Present during the measurements were Carl Durney (PI), Doug Christensen (CPI), Don Blumenthal (CPI), and Shonn Hendee from the University of Utah and Martin Misakian from NIST. Shonn Hendee participated in performing the measurements.

Magnetic Flux Density

The field levels are set by the UU by measuring the ac and dc magnetic fields with a Hall effect magnetic field meter. This procedure is followed prior to each experimental run. With the horizontal ac and dc fields set to 14.8 μT and 20.9 μT, respectively in the exposure systems, measurements of the fields were performed using a NIST ac magnetic field meter with a miniature coil probe (4.4 mm dia. x 5.7 mm; 8000 turns) and a NIST owned fluxgate magnetometer. The NIST measurements showed ac field values in both exposure systems that were consistently too high by about 5%. The NIST dc magnetic field measurement was in good agreement with the UU determined value.

Because NIST field meter calibrations are normally performed at 60 Hz and higher frequencies, the ac calibration procedure from 18 Hz to 9 Hz was reexamined following the site visit. It was determined, using a NIST standard ac voltage source, that the precision voltmeter (uncertainty specifications in the parts-per-million range) used for determining current for producing known magnetic fields must be operated in a special mode in order to obtain the small uncertainties specified by the manufacturer. The correct NIST magnetic field measurements are given in the tables below and the difference between the NIST and UU ac field values are between 2.7% and 4%.

MLCK System		
Frequency (Hz)	**ac Field (μT)**	**dc Field (μT)**
18	15.4	20.5-21.0
15	15.3	-

Fluorescence System		
Frequency (Hz)	**ac Field (μT)**	**dc Field (μT)**
18	15.3	-
15	15.3	-
9	15.2	-

Measurements with the NIST dc field meter indicated that the vertical magnetic field was near zero tesla. This ‘‘zero” measurement was difficult to perform because small misalignments of the probe led to non zero field readings. The uncertainty in the NIST field measurements is estimated to be less than ±2%.

The differences between the NIST and UU ac field values may be due, in part, to the calibration of the UU voltmeter which is used as a readout device. The jitter in the NIST dc field value is due to positioning the probe in the exposure system and the difference between the NIST and UU values is not considered significant.

Field Uniformity

MLCK System Measurements of the axial ac magnetic field were made at representative points enclosing the spatial volume normally occupied by the test samples using the NIST miniature coil probe. All field values were within 2% of the central value. Relative values of the dc axial field were also measured using the UU Hall effect probe. All but one field value were within 2% of the central field value. The ‘‘outlier” measurement was within 3% of the central value.

Fluorescence System Measurements of the axial ac magnetic field were made at the center of the exposure system (location of incident light beam from monochromator) and at locations about 7 mm above and below this point using the NIST miniature coil probe. The field values were all within 1% of one another. Precise positioning of the probe was made difficult during these measurements because of the inaccessibility of the measurement location (for the NIST probe). Lateral measurements of the ac field were not made because of the limited spatial resolution of the coil probe (4.4 mm dia.) and the width of the test sample region (1 cm). Because the same coils are used to produce the horizontal ac and dc magnetic fields in the fluorescence system, the vertical profile for the dc magnetic field should be the same as the ac field.

Lateral measurements of the dc magnetic field about 5 mm on either side of the central point, along a line perpendicular to the coil axis using the UU Hall effect probe, indicated field values within about 1% of the central value. For the reason given above (same coil for ac and dc field generation), the ac field values should have comparable uniformity.

Thus, at representative points normally occupied by the liquid in the test chamber, the field was uniform to within about 1%. It is noted that the liquid region intercepted by the monochromatic light beam is smaller than the liquid volume (~1.8 cm x 1 cm x 0.4 cm) in the test chamber.

Harmonic Content

During measurements of the ac magnetic field with the NIST field meter, the waveform of the field was also observed using an oscilloscope provided by the UU. No waveform distortion was discernible in the MLCK system at 18 Hz. At 15 Hz, some low frequency distortion was just discernible. This small distortion is attributed by the UU to the amplifier which is used to energize the coils.

Oscilloscope observations of the magnetic field waveform in fluorescence system indicated no discernible distortion at 18 Hz and 15 Hz, and some low level distortion at 9 Hz. The power amplifier used to energize the MLCK system is not used for the smaller coil system and therefore the origin of this distortion is unclear.

Background Magnetic Fields

Measurements of the ambient (coils de-energized) ac and dc magnetic fields were made for both exposure systems using NIST field meters. The resultant (square root of the sum-of-the-square spatial components) ac and dc field values in the MLCK system are near 0.25 μT and 36.8 μT, respectively. The ac ambient field waveform appeared to consist mainly of power-frequency harmonics. The NIST probe used for these ac

measurements is approximately 7 cm in diameter and thus measures an average magnetic field over its cross sectional area.

The resultant ac and dc background field values near the coils of the fluorescence exposure system are near 0.1 μT and 37.3 μT, respectively. Power frequency harmonics were again evident during the ac measurements.

The background ac fields indicated above were measured using the more sensitive scales of the NIST field meter. The scales have not been calibrated in conjunction with the 7 cm probe, but they have been checked with a voltage injection technique and found to have linearity and accuracy of indication for a given input voltage equivalent to that of the other scales, i.e., the measurement uncertainty is estimated to be less than ±2%.

Other Items

Vibrations Vibrations could not be sensed by touch when the MLCK coil system was energized. Similarly, vibrations could not be felt due to the energization of the coils in the fluorescence system. However, vibrations could be felt due to the operation of a cooling fan for the light source in the fluorescence system.

Test of Lednev Theory It was noted that the studies at the UU were tests of the Lednev theory rather than replication of the Lednev experiment. For example, the UU progress report of October 1992 discusses the reasons that a different substrate was used in the UU MLCK study and its possible implications. Further, the induced electric fields and current densities in the liquid medium due to the ac magnetic field are probably not being replicated (these quantities are not parameters in the Lednev theory). Because of the low frequency and very small sample volume in the UU experiment, the induced electric fields are expected to be very weak. It would be necessary to know details of the liquid/magnetic field geometry used in the Lednev experiments in order to match the experimental conditions at UU. For example, if much larger sample volumes were used in the Lednev experiment, larger induced electric fields would be present. Dr. Durney noted that to date Dr. Lednev has not responded to his inquiries regarding further details of the Lednev experimental conditions.

Blind Operation of Experiment It was noted that the experiments are not conducted in a blind fashion because the data analysis is not considered subjective in nature.

Continuous Monitoring of Exposure Fields The ''on'' condition for the magnetic fields is not continuously monitored because of the short durations of the exposure periods and the frequent resetting of the field levels.