Bruton’s Tyrosine Kinase Activity and Inositol 1,4,5-Trisphosphate Production Are Not Altered in DT40 Lymphoma B Cells Exposed to Power Line Frequency Magnetic Fields*

(Received for publication, August 28, 1998, and in revised form, September 29, 1998)

Steven C. Miller‡ and Michael J. Furniss
From the Signal Transduction Laboratory, Pharmaceutical Discovery Division, SRI International, Menlo Park, California 94025

Exposure of wild-type DT40 lymphoma B cells or Bruton’s tyrosine kinase (BTK)-deficient DT40 cells reconstituted with the human btk gene to a 1-gauss 60-Hz electromagnetic field (EMF) has been reported to rapidly increase inositol 1,4,5-trisphosphate (Ins 1,4,5-P₃) production (1, 2). Here we have used BTK-deficient DT40 B cells reconstituted with the human btk gene to evaluate the reproducibility of these findings. An experimental design with blinded exposures and anti-IgM treatment to induce Ins 1,4,5-P₃ production as a positive control, showed no significant effect of a 1-gauss 60-Hz EMF on Ins 1,4,5-P₃ production. Because recent work has shown that the activation of BTK was required for EMF-responsiveness (2), we also evaluated the reproducibility of this finding in wild-type DT40 cells. BTK was activated in a dose- and time-dependent manner by treatment with the tyrosine phosphatase inhibitor pervanadate. However, the ability to detect BTK activation, as measured by increased autophosphorylation by immune complex kinase assay, was dependent on the kinase buffer. Using cells from the original investigators, no evidence was obtained to support the hypothesis that exposure to a 1-gauss 60-Hz EMF had a causal effect on protein-tyrosine kinase activities affecting Ins 1,4,5-P₃ production.

Epidemiological studies suggesting a causal link between magnetic field exposure from 60-Hz power lines and childhood leukemia have focused attention on the possible molecular mechanisms by which 60-Hz electric and magnetic fields (EMFs)³ may contribute to childhood leukemia. However, mechanistic research is complicated by the lack of established biomarkers of exposure or response. Although various biophysical mechanisms have been proposed, there is no generally accepted mechanism for the biological effects of EMFs (3). Because of the uncertainty of biologically meaningful exposure metrics (4), it is essential that in vitro exposure studies provide a precise, known, consistent condition of exposure to EMF with as much control over environmental factors as possible. However, our understanding of the interaction of weak magnetic fields with biological systems is severely limited by the lack of well understood model systems that have been shown to respond to EMF exposure.

Exposure of human B-lineage leukemia cells to a 1-gauss 60-Hz EMF resulted in an enhanced tyrosine phosphorylation profile of substrate proteins and in LYN and SYK protein-tyrosine kinase (PTK) activation, followed by protein kinase C stimulation (5). The activation of protein kinase C was dependent on the activation of LYN kinase, a PTK of the Src family that is known to be involved in signal cascades affecting proliferation and survival of B-lymphoid cells. More recently, the DT40 chicken lymphoma B-cell model was used to demonstrate that LYN is essential for increased inositol 1,4,5-trisphosphate (Ins 1,4,5-P₃) production that increased within seconds of EMF exposure through a mechanism involving phospholipase Cβ2 activation (1). Further, in DT40 cells, BTK activity was shown to be required for EMF responsiveness (2). The results presented in these three publications provided multiple levels of evidence that support and strengthen the plausibility of the hypothesis that a delicate grow regulatory balance in B-lineage lymphoid cells might be altered by EMF exposure (1, 2, 5). These findings are significant because they show that exposure of DT40 cells to a magnetic field with an intensity of 1 gauss affects a well understood signal transduction pathway involved in signaling through the B cell receptor (BCR). Although these studies provide evidence for an EMF-induced signaling pathway and suggest a mechanism by which electromagnetic radiation could cause health problems, these results are from a single laboratory. Because our understanding of the biophysical process responsible for these effects is uncertain (3), replication of the evidence for an EMF-induced signaling pathway is important.

In the experiments reported here, DT40 wild-type and transfected lines were obtained from the original investigators to determine whether the previously reported results that exposure to a 60-Hz EMF may amplify the PTK-dependent signal transduction pathway affecting Ins 1,4,5-P₃ (1, 2, 5) were reproducible by an independent laboratory. Emphasis was placed on using cells and experimental protocols as close as possible to the original investigators. Our first studies evaluating the effect of a 1-gauss 60-Hz field on Ins 1,4,5-P₃ production were conducted in a blinded fashion to conceal the identity of the EMF- or sham-exposed samples until the data were obtained. In our second studies, DT40 cells were EMF- or sham-exposed, and we examined the enzymatic activity of BTK, as reflected by increased autophosphorylation using immune complex kinase assays. In addition, we used the tyrosine phosphatase inhibitor pervanadate as a positive control to stimulate protein tyrosine phosphorylation and BTK activation, and to show the sensitiv-

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*This work was supported by NIEHS Grant ES07127, in part by National Institutes of Health Grant GM48229, and by SRI International investment funds (to S. C. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Signal Transduction Laboratory, SRI International, 333 Ravenswood Ave., Menlo Park, CA 94025. Tel.: 650-859-5788; Fax: 650-859-3342; E-mail: smiller@unix.sri.com.

† The abbreviations used are: EMF, electromagnetic field; BTK, Bruton’s tyrosine kinase; Ins 1,4,5-P₃, inositol 1,4,5-trisphosphate; PTK, protein-tyrosine kinase; FTP, protein-tyrosine phosphatase; BCR, B cell receptor; SI, stimulation indices; PBS, phosphate-buffered saline; mG, milligauss; α-MEM, minimal essential medium α; µT, microteslas(§).
ity of the assays. Using well defined experimental and exposure conditions, our results do not support a causal relationship between EMF exposure and biological effects. The failure to replicate the original findings may be due to a number of reasons (6). Thus, emphasis was placed on defining the biological and exposure conditions used in our laboratory in an rigor- ous a fashion as practical.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents—**Wild-type DT40, BTK-deficient cells, BTK-deficient cells reconstituted with a human wild-type BTK gene, LYN-deficient cells, and LYN-deficient cells reconstituted with the human wild-type Btk gene were obtained from the original investigators’ laboratory (Wayne Hughes Institute, Roseville, MN), expanded, and frozen stocks prepared.

For our first studies on Ins 1,4,5-P3, the stock cultures were maintained at 37 °C in 5% CO2 in the top chamber of a humidified dual chamber incubator (Queue, model 2220) in a mu-metal box (reduces AC and DC fields) to provide a low EMF environment of less than 1 milligauss (mG). DT40 and mutant stocks were grown in RPMI 1640 containing 10% fetal bovine serum, 1% chicken serum, 1 mM pyruvate, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. 

For our second studies on protein tyrosine phosphorylation and BTK activation, new stock cultures were initiated from the original frozen stocks and the cells were maintained in the top chamber of the Que incubator with the lower chamber off and without the mu-metal box as shielded. To simulate the incubator environment used by the original investigators (1, 2), shielding sheets of metal alloy (CEDCO, Portland, OR) were placed at the top and the bottom of the top incubator chamber. The AC and DC fields within this incubator have been measured (Fred Dietrich, Electric Research and Management, Inc., State College, PA). The exposure environment (4) was simulated by placing into the sample chamber of the EMF facility. The exposure environment (5) was simulated by placing into the sham-energized or 1-gauss 60-Hz field in a blinded strength in each chamber during the EMF exposure period. 

**EMF Exposure and Anti-phosphotyrosine Immunoblot and Immune Complex Kinase Assays—**Exponentially growing cells were pelleted, washed, and resuspended (7.5 × 106 cells/ml) in 1% Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, and 3% 125 mM β-mercaptoethanol) containing 100 μg/ml leupeptin, and 1 mM EDTA, 3% 125 mM β-mercaptoethanol, 2 mM pyruvate, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, as described (1, 2).

The sample was added to the sample chamber of the EMF facility. The tubes were opened and the sample was placed into the sample chamber of the EMF facility. The caps were tightened, washed, and resuspended (7.5 × 106 cells/ml) in 1% Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, and 3% 125 mM β-mercaptoethanol) containing 100 μg/ml leupeptin, and 1 mM EDTA, 3% 125 mM β-mercaptoethanol, 2 mM pyruvate, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, as described (1, 2).

**EMF Exposure System—**All exposure studies were done using a computer controlled exposure system (model LES-002-1A-DC, Electric Research and Management, Inc., State College, PA) that generated vertical 60-Hz sinusoidal (AC) fields (linear polarization). The system consists of three chambers labeled A, B, and S maintained at 37 °C in a 5% CO2 environment. Chambers A or B are surrounded by bifilar wound coils in a Merritt’s four-square coil arrangement that can be either sham- or EMF-energized; chamber S is the sample chamber for unexposed samples. This system has been shown to produce a harmonic distortion of not more than 3.0% at 60 Hz and a field uniformity to within 5% over the exposure chamber volume (Electric Research and Management, Inc., State College, PA). The exposure environment (4) was simulated by placing into the sample chamber of the EMF facility. The caps were opened, and the samples were placed into the sample chamber. After a 30-min preincubation period, the zero time point sample was taken and the appropriate tubes were placed into separate racks designated for the A or B exposure chambers. The coils were energized before the sample tube was immediately placed into the A and B exposure chambers. At the indicated times of 15 and 30 s, and 1, 3, and 5 min, samples in the A and B exposure chamber were simultaneously removed, ice-cold perchloric acid was added, and the tubes were vortexed for 6 s and placed into an ice-bath. After the last 5-min time point, the field was turned off and samples in the sample chamber were immediately stimulated with the anti-IgM antibody. Zero time points were mock-stimulated with PBS and immediately acid-extracted. After the last sample, all tubes were vortexed for 10 s, kept on ice for 20 min, and then sedimented in a swinging bucket-holder at 2,000 × g for 15 min at 4 °C. The supernatant was collected, and the pH was neutralized to 7.5 as described (1). The samples were kept in an ice-bath throughout the neutralization, then sedimented by centrifugation at 2,000 × g for 15 min at 4 °C. The supernatant was collected, frozen on dry ice, and stored at −20 °C for subsequent measurement of Ins 1,4,5-P3.

**Analysis of Ins 1,4,5-P3—**A 100-μl portion of each extract was assayed (typically in duplicate, but some studies were done in triplicate) for Ins 1,4,5-P3, using a radioisogand competition assay system (catalog no. TRK0100) purchased from Amersham Pharmacia Biotech, as reported (1). The Ins 1,4,5-P3 assay was done according to the manufacturer’s protocol. Because of the blinded experimental design, we used a fixed assay format where each time point from the samples in chamber A or B were placed together. The percent bound ([%BB]) was calculated by: (sample cpm − nonspecific binding (NSB) cpm)/Bo cpm − NSB cpm) × 100. The assay result was considered as the [%BB] of the [Bo] levels that were undetectable or equal to Bo, (a value of 100/%BB, equals 0 pmol of Ins 1,4,5-P3). In some experiments, the results are presented as picomoles of Ins 1,4,5-P3 normalized to 106 cells. Student’s t test for paired and unpaired samples was used to test for significant differences between samples. Values of p < 0.05 were accepted as significantly different.

**EMF Exposure and Anti-phosphotyrosine Immunoblot and Immune Complex Kinase Assays of BTK—**Exponentially growing cells were pelleted, washed, and resuspended at 5.0 × 106 cells/ml in warm (37 °C) α-MEM, in 1.5-mL capacity microcentrifuge tubes, as described (2). The tubes were capped tightly and placed in a mu-metal box (<1 mG) for transport to the sample chamber of the EMF facility. The caps were opened, and the samples were placed into the sample chamber. After a 30-min preincubation period, the zero time point sample was taken and the appropriate tubes were placed into separate racks designated for the A or B exposure chambers. The coils were energized before the sample tube was simultaneously placed into the A and B exposure chamber. At the indicated times of 15 and 30 s, and 1, 3, and 5 min, samples in the A and B exposure chamber were simultaneously removed, ice-cold perchloric acid was added, and the tubes were vortexed for 6 s and placed into an ice-bath. After the last 5-min time point, the field was turned off and samples in the sample chamber were immediately stimulated with the anti-IgM antibody. Zero time points were mock-stimulated with PBS and immediately acid-extracted. After the last sample, all tubes were vortexed for 10 s, kept on ice for 20 min, and then sedimented in a swinging bucket-holder at 2,000 × g for 15 min at 4 °C. The supernatant was collected, frozen on dry ice, and stored at −20 °C for subsequent measurement of Ins 1,4,5-P3.

**No Effect of 60-Hz EMF on BTK or Ins 1,4,5-P3 Production**
phenylmethylsulfonyl fluoride as protease inhibitors). Lysates were vortexed for 30 s, incubated on ice for 10 min, and centrifuged at 12,000 \times g for 10 min at 4 °C prior to immunoprecipitation. Cell lysates (4.5 \times 10^6 cell equivalents) were immunoprecipitated with a polyclonal rabbit anti-BTK antibody (3 \mu l added to 450 \mu l of lysate). After a 1-h incubation on ice, 50 \mu l of protein A-agarose beads (Roche Applied Science, Indianapolis, IN) were added and samples were rotated for 1 h at 4 °C. The immune complexes were washed three times with 1% Nonidet P-40 lysis buffer supplemented with inhibitors, and washed once in kinase buffer, and immune complex kinase assays were performed in 25 \mu l of kinase buffer supplemented with 5 \mu M ATP and 10 \mu l of [γ-32P]-ATP (6,000 Ci/mmol, Amersham) for 10 min at room temperature. The reaction was stopped by adding 1 ml of RIPA buffer (PBS with 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, plus inhibitors), and the immune complex was washed three times with 1 ml of RIPA buffer. The supernatant was removed, and the immune complex pellet was resuspended in 25 \mu l of SDS sample buffer and boiled for 5 min. The BTK immunoprecipitates were resolved on NuPage 4–12% polyacrylamide gels (Novex, San Diego, CA) and electrophoretically transferred (Novex PhastBlot) to 0.45- \mu m Immobilon-polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) followed by PhosphorImager analysis (Storm model 840, Molecular Dynamics; Sunnyvale, CA) for quantitation using the ImageQuant software package (Molecular Dynamics). The gels were stained with Coomassie blue, cut out, and exposed to autoradiography screens. The migration of a 92-kDa protein, which directly linked to horseradish peroxidase (Signal Transduction, Lexington, KY), and visualized by chemiluminescence with Super Signal substrate (Pierce). BTK protein was quantitated by densitometry performed by using a Lynx video densitometer (Applied Imaging Corporation, Santa Clara, CA). BTK kinase activity was normalized as described (2).

The protein-tyrosine phosphatase inhibitor pervanadate (a general term for the variety of complexes formed between hydrogen peroxide and vanadate) was used as a control agent for inducing the phosphorylation of proteins on tyrosine residues and for activation of BTK (8). A stock solution of 50 mM pervanadate was freshly prepared by mixing a 1-gauss field, and chamber A was sham-energized. Each test included a positive control that was done immediately after the EMF or sham exposures to show that the cells increased Ins 1,4,5-P3 production after anti-IgM treatment. Reducing the antibody concentration from 16 \mu g/ml in test 1 to 2 \mu g/ml in test 3. In test 3, chamber B generated the 1-gauss field and chamber A was sham-energized. Each test included a positive control that was done immediately after the EMF or sham exposures to show that the cells increased Ins 1,4,5-P3 production after anti-IgM treatment. For this control, additional samples of the same cells used in the EMF or sham exposures were stimulated with anti-IgM by adding it directly to the 1-ml samples containing 7.5 \times 10^6 cells/ml. In the original investigators’ published work (1), 5 \times 10^6 cells/ml were used. We also used 5 \times 10^6 cells/ml in our initial EMF exposure studies, but a series of studies failed to measure any consistent change in Ins 1,4,5-P3 (data not shown). We increased the cell concentration to 7.5 \times 10^6 cells/ml in an attempt to provide for a measurable amount of Ins 1,4,5-P3 in the unstimulated cells. We also compared two sources of antibodies in test 1, i.e. anti-IgM as research grade (M4) or purchased from a commercial source (M4c). The zero time points for anti-IgM treatment were mock-stimulated with PBS. In response to anti-IgM treatment with either the M4 or M4c antibody, the Ins 1,4,5-P3 production rapidly increased in the 15-s sample and appeared to plateau in the 30-s and 1-min samples. There was a greater response to 4 \mu g/ml M4 antibody at 15 and 30 s than to 16 \mu g/ml commercially available M4c antibody. Thus, the commercially available antibody is ideal as a positive control agent to show the strength of the response between laboratories. We evaluated the effect of reducing the antibody concentration 2-fold in tests 2 and 3. In test 4, we reduced the anti-IgM antibody (M4c) concentration to 2 \mu g/ml and included a PBS mock-stimulated time course that was done immediately after the anti-IgM treatment. Reducing the anti-IgM antibody concentration from 16 \mu g/ml in test 1 to 2

RESULTS AND DISCUSSION

Role of BTK in Ins 1,4,5-P3 Production in DT40 Lymphoma B-cells—Wild-type DT40 and cells deficient in LYN, BTK, SYK, or phospholipase Cγ2 have provided a powerful genetic system to study signals initiated through ligation of the BCR, for a recent review see (9). Wild-type DT40, LYN-deficient cells, restored with a jyn g gene, and BTK-deficient cells reconstituted with the human btk gene have all been shown to be EMF-responsive by measuring an increase in Ins 1,4,5-P3 production (1, 2). Notably, low energy 50-Hz EMFs have been shown to increase Ins 1,4,5-P3 levels in the Jurkat T cell line (10). However, little is known about the biophysical requirements associated with the EMF-responsive state. Therefore, we first defined the response of DT40 and selected transfected lines to BCR-induced signaling before studying EMF-induced signaling events.

BTK-deficient cells reconstituted with the human btk gene have been previously shown to respond to BCR stimulation with a more vigorous Ins 1,4,5-P3 and Ca2+ mobilization than wild-type DT-40 cells (11). In agreement with this evidence, anti-IgM treatment (4 \mu g/ml M4 antibody) of BTK-deficient cells reconstituted with the human btk gene resulted in more Ins 1,4,5-P3 at 15 and 30 s than wild-type cells (data not shown). There was a 3.2-fold increase in the peak amount of Ins 1,4,5-P3 at 30 s in the BTK-deficient cells reconstituted with the human btk gene (17.4 pmol/10^6 cells) when compared with wild-type cells (5.4 pmol/10^6 cells). The increased sensitivity of these cells to BCR-initiated signals affecting Ins 1,4,5-P3 coupled with recent evidence of an enhanced Ins 1,4,5-P3 response when exposed to a 1-gauss 60-Hz EMF (2) indicated that BTK-deficient cells reconstituted with the human btk gene were appropriate for further efforts to define the EMF-responsive state.

No Effect of 60-Hz EMF on BTK or Ins 1,4,5-P3 Production—We next examined the effect of a 1-gauss 60-Hz EMF on Ins 1,4,5-P3 production in BTK-deficient cells reconstituted with the human btk gene. A blinded EMF exposure protocol was developed for these studies (see “Experimental Procedures”) to specifically determine if changes in Ins 1,4,5-P3 were EMF-dependent, protocol-dependent, or both. In the original investigators’ studies (1, 2, 5), a vertical 60-Hz magnetic field was generated by using a Merritt’s four-square coil system. Our exposure system also used a four-coil Merritt arrangement for generating a vertical 60-Hz magnetic field. The original investigators’ exposure system (5) and our coils were wound in a bifilar arrangement to allow the coils to be energized in an opposing arrangement for sham conditions.

The results from a series of four independent blinded experiments, which included anti-IgM stimulation as a positive control, are shown in Fig. 1. In contrast to recently published results (1), there was no causal effect of the 60-Hz field on Ins 1,4,5-P3 production. In tests 1 and 4, chamber A generated the 1-gauss field, and chamber B was sham-energized. In test 2 and 3, chamber B generated the 1-gauss field and chamber A was sham-energized. Each test included a positive control that was done immediately after the EMF or sham exposures to show that the cells increased Ins 1,4,5-P3 production after anti-IgM treatment. For this control, additional samples of the same cells used in the EMF or sham exposures were stimulated with anti-IgM by adding it directly to the 1-ml samples containing 7.5 \times 10^6 cells/ml. In the original investigators’ published work (1), 5 \times 10^6 cells/ml were used. We also used 5 \times 10^6 cells/ml in our initial EMF exposure studies, but a series of studies failed to measure any consistent change in Ins 1,4,5-P3 (data not shown). We increased the cell concentration to 7.5 \times 10^6 cells/ml in an attempt to provide for a measurable amount of Ins 1,4,5-P3 in the unstimulated cells. We also compared two sources of antibodies in test 1, i.e. anti-IgM as research grade (M4) or purchased from a commercial source (M4c). The zero time points for anti-IgM treatment were mock-stimulated with PBS. In response to anti-IgM treatment with either the M4 or M4c antibody, the Ins 1,4,5-P3 production rapidly increased in the 15-s sample and appeared to plateau in the 30-s and 1-min samples. There was a greater response to 4 \mu g/ml M4 antibody at 15 and 30 s than to 16 \mu g/ml commercially available M4c antibody. Thus, the commercially available antibody is ideal as a positive control agent to show the strength of the response between laboratories. We evaluated the effect of reducing the antibody concentration 2-fold in tests 2 and 3. In test 4, we reduced the anti-IgM antibody (M4c) concentration to 2 \mu g/ml and included a PBS mock-stimulated time course that was done immediately after the anti-IgM treatment. Reducing the anti-IgM antibody concentration from 16 \mu g/ml in test 1 to 2
simultaneously exposed under blinded conditions to the sham or EMF to a 1-gauss 60-Hz EMF (see “Experimental Procedures”). Cells were constituted with the human btk gene were evaluated for EMF-responsiveness to a 1-gauss 60-Hz EMF (see “Experimental Procedures”). Cells were simultaneously exposed under blinded conditions to the sham or EMF environment. The assay results are shown as the %B/B₀ to show Ins 1,4,5-P³, levels that were undetectable or equal to B₀, (a value of 100%B/B₀ equals 0 pmol of Ins 1,4,5-P³). The graphs are labeled by the test number; the top figure legend of each graph shows whether chamber A or B was energized in the sham or EMF mode. The anti-IgM (M4 or M4c) antibody treatments were done immediately after the sham or EMF exposures with anti-IgM treatment as a positive control and M4 refers to an anti-IgM antibody from Joe Bolen’s laboratory (DNAX) or 0.66 6 0.02 (mean 0.03; unpaired t test) from the previous zero time point at 45 min.

Fig. 1. Time course of four independent tests of blinded sham or EMF exposures with anti-IgM treatment as a positive control to stimulate Ins 1,4,5-P³ production. BTK-deficient cells reconstituted with the human btk gene were evaluated for EMF-responsiveness to a 1-gauss 60-Hz EMF (see “Experimental Procedures”). Cells were simultaneously exposed under blinded conditions to the sham or EMF environment. The assay results are shown as the %B/B₀ to show Ins 1,4,5-P³ levels that were undetectable or equal to B₀, (a value of 100%B/B₀ equals 0 pmol of Ins 1,4,5-P³). The graphs are labeled by the test number; the top figure legend of each graph shows whether chamber A or B was energized in the sham or EMF mode. The anti-IgM (M4 or M4c) antibody treatments were done immediately after the sham or EMF exposure in the order shown in the figure legend of each graph. M4 refers to an anti-IgM antibody from Joe Bolen’s laboratory (DNAX) and M4c to a commercially available antibody (see “Experimental Procedures”). For tests 1, 2, and 3, the results shown represent the %B/B₀ (mean ± sample deviation) from duplicate measurements. The results shown in test 4 represents the %B/B₀ expressed as the mean ± S.D. from triplicate measurements. In test 4, the last time course consisted of the samples mock-stimulated with PBS (done immediately after the anti-IgM stimulation).

Fig. 2. The amount of Ins 1,4,5-P³ in unstimulated cells is protocol- and time-dependent. Results from test 4 in Fig. 1 are expressed as picomoles of Ins 1,4,5-P³ per 10⁶ cells (mean ± S.D.; n = 3). The amount of Ins 1,4,5-P³ was significantly different (p < 0.02) than the PBS vehicle control 15 s after anti-IgM (2 µg/ml) stimulation. The amount of Ins 1,4,5-P³ measured at the zero time point was unchanged in the samples mock-stimulated with PBS throughout the 5-min time course. No Ins 1,4,5-P³ was detectable in the zero time point sample from unstimulated cells (defined by protocol as 37 min after the cells were resuspended in α-MEM, with 0.01 x LiCl). However, Ins 1,4,5-P³ was detectable in the zero time point samples at 45 and after 52 min. Notably, the zero time point (at 52 min) for the PBS mock-stimulated time course was increased significantly (p < 0.03; unpaired t test) from the previous zero time point at 45 min.

Fig. 1. In contrast to the sham- or EMF-exposed samples, anti-IgM treatment (2 µg/ml) stimulated a rapid increase in Ins 1,4,5-P³ production at 15 s. The amount of Ins 1,4,5-P³ increased from 0.47 ± 0.02 (mean ± S.D.) to 0.66 ± 0.08 pmol/10⁶ cells, a statistically significant increase (p < 0.02). The level of Ins 1,4,5-P³ continued to increase at 30 s (4.2-fold) and 1 min (11.9-fold), reached a maximum after 3 min (21.0-fold), and declined at 5 min (14.9-fold). The results shown in Fig. 2 were generated by rigorously adhering to a timed protocol. The initial zero time point was defined by when the cells were resuspended in α-MEM, supplemented with 0.01 x LiCl. According to protocol, the 5-min time points for the sham and EMF samples were acid-extracted after 43 min, the zero time point PBS control was done at 45 min, and the 15-s time point for the anti-IgM-treated positive control was done approximately at 46 min. Thus, there was less than 4 min between the last sham or EMF time point and the first positive control sample that showed a significant change in Ins 1,4,5-P³ from the mock-stimulated zero time point (mean %B/B₀ of 95.0 or 0.47 ± 0.02 pmol/10⁶ cells). Treatment with anti-IgM for 15 s showed the detection of small changes in Ins 1,4,5-P³ (mean %B/B₀ of 90.3 or 0.66 ± 0.08 pmol/10⁶ cells). There was no significant difference in Ins 1,4,5-P³ between the sham- or EMF-exposed samples. The 5-min time course of samples mock-stimulated by PBS treatment showed that the mean level of Ins 1,4,5-P³ ranged from 1.0 to 1.1 pmol/10⁶ cells. A comparison of the zero time point controls, shown in Fig. 2, indicated that the amount of Ins 1,4,5-P³ increased from an undetectable value at 37 min to 0.47 ± 0.02 pmol/10⁶ cells at 45 min, and to 1.03 ± 0.29 pmol/10⁶ cells after 52 min; a statistically significant increase (p < 0.03). Thus, the amount of Ins 1,4,5-P³ increased during the preincubation period when the cells were maintained in α-MEM, plus 0.01 x LiCl.

It was possible that the conditions used did not allow small changes in Ins 1,4,5-P³ induced by the 1-gauss 60-Hz EMF to be measured. Therefore, in our next studies we increased the preincubation time to provide for more Ins 1,4,5-P³ in the unstimulated cells. The cells were preincubated for 15 min in the sample chamber, as before, but the length of the preincubation time after adding 1 ml of the cell suspension to the
sample tube was increased from the original design of 15 min to 30 and 45 min. Samples were sham- or EMF-exposed according to a timed protocol and the amount of Ins 1,4,5-P₃ determined in triplicate. The effect of these treatments on Ins 1,4,5-P₃ are shown in Fig. 3. There was no significant difference in Ins 1,4,5-P₃ between the sham- or EMF-exposed samples. In contrast, anti-IgM treatment with M4c (2 μg/ml) stimulated an increase in Ins 1,4,5-P₃ production after 1 min (from 0.94 ± 0.01 to 1.91 ± 0.39 pmol/10⁶ cells), which continued to increase at 3 min to 6.36 ± 0.54 (6.8-fold). These data do not support the hypothesis that a 1-gauss 60-Hz EMF stimulated Ins 1,4,5-P₃ production (1, 2).

**No Effect of 1-Gauss 60-Hz EMF Exposure on BTK Activation**—We next evaluated the effect of EMF on the enzymatic activity of BTK, and attempted to duplicate the protocol described in a recent publication (2) where exposure of wild-type DT40 cells resulted in a time-dependent activation of BTK (7.4-fold increase at 30 min; see Fig. 2 of Ref. 2). Exponentially growing cells (resuspended in serum-free α-MEM, at 5 × 10⁶ cells/ml) were preincubated in 1.5-mi capacity microcentrifuge tubes for 30 min then exposed to the sham or 1-gauss 60-Hz EMF (see “Experimental Procedures”). The enzymatic activity of BTK was examined by immune complex kinase assay. BTK activation was determined in a kinase buffer (20 mM Tris-HCl, pH 8.0, and 5 mM MnCl₂) as defined by the original investigators (2). The kinase reaction was in 25 μl of kinase buffer supplemented with 5 μM ATP and 10 μCi of [γ-³²P]ATP. In the original investigators’ work, BTK kinase activity was normalized to BTK protein measured from a separate blot (2) and references cited. In our studies, to rigorously control for differences in BTK protein, BTK activation was normalized against the amount of BTK protein measured on the same blot (see “Experimental Procedures”). The BTK protein adjusted stimulation indices (SI) were 1.3 at 2.5 min, 1.5 at 5 min, 1.3 at 15 min, and 0.9 at 30 min for the EMF-exposed samples and 1.3 at 2.5 min, 1.8 at 5 min, 2.0 at 15 min, and 1.5 at 30 min in the sham-exposed samples (data not shown). The zero time point samples, used for normalizing kinase activity, were taken from samples in the sample chamber (ambient environment). Thus, the 1-gauss 60-Hz EMF had no significant effect on BTK activation.

Although the sample chamber had a low ambient AC field (resultant AC of <3 mG), mu-metal shielding reduces the possibility of stray fields (with shield, the resultant AC was 0.2 mG and the resultant DC was 38 mG). Therefore, in our next studies wild-type DT40 cells were prepared as before, but placed in a mu-metal box in the sample chamber during a 30-min preincubation period. The time course of samples from the mu-metal box or sham or EMF exposure (1-gauss, 60-Hz) are shown in Fig. 4. In an effort to further define the 1-gauss 60-Hz EMF exposure environment, and to evaluate an exposure metric that has been suggested to be important for biological effects, a 78.9-μT DC field was applied parallel with the 1-gauss (100.4 μT) vertical AC field. These conditions provide a test for resonance conditions for Ca²⁺ (12). In this study, after immune complex kinase assay, the samples in SDS sample buffer were divided into two aliquots and resolved on NuPage 4–12% polyacrylamide gels to provide for two separate measurements of the EMF-exposed samples on the same blot with either the sham- or mu-metal box-exposed samples. The zero time point samples were taken from samples in the mu-metal box placed in the sample chamber. As shown in Fig. 4, for the first blot, the BTK protein adjusted SI were 1.6 at 2.5 min, 1.8 at 15 min, and 1.9 at 30 min for the EMF-exposed samples and 1.1 at 2.5 min, 1.1 at 15 min, and 0.9 at 30 min for the sham-exposed samples. For the second blot, the BTK protein adjusted SI were 0.8 at 2.5 min, 1.2 at 15 min, and 1.1 at 30 min for the EMF-exposed samples and 1.4 at 2.5 min, 1.1 at 15 min, and 0.8 at 30 min for the mu-metal box-exposed samples. These data show the reproducibility of the immunoblotting procedure and that the 1-gauss 60-Hz EMF environment had no significant effect on BTK activation.

**Effect of Anti-IgM Stimulation on BTK Activation in Vitro**—The initiation and processing of signals through the BCR (for review, see Ref. 13) provides a well established framework for the assessment of a possible EMF-induced signaling mechanism. Therefore, we evaluated the effect of anti-IgM treatment. Wild-type DT40 and BTK-deficient cells reconstituted with the human btk gene were stimulated with anti-IgM treatment (4 μg/ml M4) and immune complex BTK kinase assays were performed. A portion of the cell lysate was used to evaluate changes in tyrosine phosphorylation by immunoblotting with an anti-phosphotyrosine antibody (see “Experimental Procedures”). The time dependence and magnitude of the tyro-
the amount of BTK protein as described previously (2). Autoradiograms of the kinase assay and immunoblots of the same blot probed with the anti-BTK antibody are shown. The BTK protein adjusted SI were determined by normalizing kinase activity to the amount of BTK protein as described previously (2).

Protein tyrosine phosphorylation was rapidly increased in a large number of proteins in samples after 5 s and continued throughout 15 s, 30 s, and 1, 5, 10, and 30 min of treatment (data not shown). BTK has been shown to be rapidly tyrosine-phosphorylated after 1 min following BCR stimulation in wild-type DT40 cells (14). However, in samples taken at 0, 10, 30, and 60 s after stimulation, there was little change in BTK activity from the unstimulated sample (data not shown). Because BTK is known to be associated with serine/threonine kinases (15), the kinase assay was done as before, but the proteins were eluted by boiling the immune complex in RIPA buffer for a second round of immunoprecipitation. BTK-deficient cells reconstituted with the human btk gene and cell lysates were prepared at 0, 5, 10, and 30 min after treatment. We evaluated the effect of pervanadate on the tyrosine phosphorylation pattern in cell lysates and compared BTK activity in the Hepes kinase buffer system with BTK activity in the Tris buffer control sample. The SI were 1.0 at 5 min, 0.8 at 15 min, and 0.7 after 30 min. In the Tris buffer system directly normalized to the Hepes buffer, the SI were 61.8 for the control, 40.4 at 5, and 43.6 at 30 min after treatment. Therefore, DT40 cells were treated with 25 μM pervanadate, lysed at the indicated time points, and immunoprecipitated with the anti-BTK antibody and immune complex kinase assays were performed in 20 mM Tris, pH 8.0, and 5 mM MnCl2. Reactions were done in 25 μl of kinase buffer supplemented with 5 μM ATP and 10 μCi [γ-32P]ATP (6,000 Ci/mmol) for 10 min at room temperature (see “Experimental Procedures”). After immune complex kinase assay, the samples in SDS sample buffer were divided into two aliquots and resolved on NuPage 4–12% polyacrylamide gels to provide two separate measurements of the EMF-exposed samples on the same blot with either the sham or mu-metal box exposed samples. The zero time point samples were removed directly from the mu-metal box in the sample chamber and placed on ice (see “Experimental Procedures”). Autoradiograms of the kinase assay and immunoblots of the same blot probed with the anti-BTK antibody are shown. The BTK protein adjusted SI were determined by normalizing kinase activity to the amount of BTK protein as described previously (2).

FIG. 4. BTK is not activated in a time course of DT40 cells exposed to a defined exposure metric for Ca2+ resonance. In other studies, we demonstrated that BTK was not activated in DT40 cells exposed to a 1-gauss 60-Hz EMF environment with ambient DC field (see “Results and Discussion”). Therefore, to investigate another exposure metric, the exposure system was set to produce a 789 mG (78.9 μT) vertical DC field in combination with a vertical 1-gauss (measured as rms of 100.4 μT; peak value of 142.0 μT) 60-Hz EMF (1.8 AC/DC intensity ratio) according to the Lednev model for Ca2+ resonance (12). DT40 cells were exposed to the EMF, sham, or mu-metal box environment (see “Results and Discussion”), lysed at the indicated time points, immunoprecipitated with the anti-BTK antibody and immune complex kinase assays were performed in 20 mM Tris, pH 8.0, and 5 mM MnCl2. Reactions were done in 25 μl of kinase buffer supplemented with 5 μM ATP and 10 μCi [γ-32P]ATP (6,000 Ci/mmol) for 10 min at room temperature (see “Experimental Procedures”). After immune complex kinase assay, the samples in SDS sample buffer were divided into two aliquots and resolved on NuPage 4–12% polyacrylamide gels to provide two separate measurements of the EMF-exposed samples on the same blot with either the sham or mu-metal box exposed samples. The zero time point samples were removed directly from the mu-metal box in the sample chamber and placed on ice (see “Experimental Procedures”). Autoradiograms of the kinase assay and immunoblots of the same blot probed with the anti-BTK antibody are shown. The BTK protein adjusted SI were determined by normalizing kinase activity to the amount of BTK protein as described previously (2).

Effect of Kinase Buffer on BTK Kinase Activity Induced by Pervanadate Treatment—Exposure to a 1-gauss 60-Hz field has been shown to result in BTK activation with BTK protein adjusted SI of 1.9 at 2.5 min, 4.6 at 6.4 min, and 7.4 after 30 min of exposure; see Fig. 2 of Ref. 2. Evidence for an EMF-induced effect that increased in a time-dependent manner raised the hypothesis that the effect of EMFs may be similar to pervanadate treatment. Pervanadate, a protein-tyrosine phosphatase (PTP) inhibitor, has been shown to produce effects in B cells similar to BCR-induced signaling, suggesting the existence of a preformed signal transducer complex (13, 16). Other investigators have used pervanadate to activate BTK (8). Therefore, DT40 cells were treated with 25 μM pervanadate, and cell lysates were prepared at 0, 5, 10, and 30 min after treatment. We evaluated the effect of pervanadate on the tyrosine phosphorylation pattern in cell lysates and compared BTK activation in immune complex kinase assays in either a Hepes buffer (20 mM Hepes, pH 8.0, 10 mM MgCl2, and 1 mM Na3VO4 buffer or in the Tris buffer (20 mM Tris, pH 8.0, and 5 mM MnCl2) used in our previous EMF studies. A portion of the cell lysate was used to evaluate changes in tyrosine phosphorylation by immunoblotting with an anti-phosphotyrosine antibody (see “Experimental Procedures”). The anti-phosphotyrosine immunoblot, autoradiograms of the BTK kinase assay, and immunoblots of the same blot probed with the anti-BTK antibody are shown. The BTK protein adjusted SI were determined by normalizing kinase activity to the amount of BTK protein as described previously (2).

FIG. 5. Time course of pervanadate-induced tyrosine phosphorylation and BTK activation in DT40 cells. DT40 cells were stimulated with 25 μM pervanadate, lysed at the indicated time points, and immunoprecipitated with the anti-BTK antibody and immune complex kinase assays were performed in either a 20 mM Hepes, pH 8.0, 10 mM MgCl2, and 1 mM Na3VO4 or in the Tris buffer (20 mM Tris, pH 8.0, and 5 mM MnCl2) used in our previous EMF studies. A portion of the cell lysate was used to evaluate changes in tyrosine phosphorylation by immunoblotting with an anti-phosphotyrosine antibody (see “Experimental Procedures”). The anti-phosphotyrosine immunoblot, autoradiograms of the BTK kinase assay, and immunoblots of the same blot probed with the anti-BTK antibody are shown. The BTK protein adjusted SI were determined by normalizing kinase activity to the amount of BTK protein as described previously (2).

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min, and 1.0 at 30 min. Although the kinase activity was much lower in the Hepes buffer system, only the Hepes buffer showed a time-dependent increase in BTK activation, as reflected by increased autophosphorylation, after pervanadate treatment.

We next prepared a control cell lysate and directly compared BTK activity in immune complex kinase assays prepared from the same lysate but assayed in different kinase buffers. The BTK protein adjusted SI normalized to activity in the Hepes buffer (20 mM Hepes, pH 8.0, 10 mM MgCl₂, and 1 mM Na₃VO₄) were 0.8 for 20 mM Tris, pH 8.0, 10 mM MgCl₂, and 1 mM Na₃VO₄, 2.3 for 20 mM Tris, pH 8.0, and 10 mM MgCl₂, and 27.8 for 20 mM Tris, pH 8.0, and 10 mM MnCl₂ (data not shown). Thus, the normalized BTK activity was essentially the same (SI of 0.8) in the Hepes and Tris buffers with 10 mM MgCl₂ and 1 mM Na₃VO₄. When compared with the same buffer with 1 mM Na₃VO₄, a change from MgCl₂ to MnCl₂ increased the normalized BTK activity by 28-fold (data not shown). These data show the role of the kinase buffer in the apparent BTK autophosphorylation activity, measured in immune complex kinase assays.

As shown in Fig. 6, 50 or 100 μM pervanadate treatment of DT40 cells stimulated BTK 2.5- and 3.4-fold at the 5-min sample, respectively, with the BTK kinase assay in 20 mM Hepes, pH 8.0, 10 mM MgCl₂, and 1 mM Na₃VO₄. Unexpectedly, the amount of BTK protein in the immune complex kinase assay decreased in a dose- and time-dependent manner. To our knowledge, this is the first report showing this effect, which was highly dependent on the pervanadate concentration (data not shown). We speculate that high levels of phosphorylation, induced by pervanadate, may interfere with immunoprecipitation of BTK. Considering that the polyclonal antibodies to BTK were produced with glutathione S-transferase fusion proteins containing the first 150 amino acids of BTK (7), it is noteworthy that the PH domain of BTK has been shown to be phosphorylated on serine residues through an interaction with protein kinase C (15, 17, 18).

To further define the response of DT40 cells to pervanadate, cells were treated with 0, 1, 10, 20, or 50 μM pervanadate and samples were taken at 5 and 15 min. As shown in Fig. 7, there was a marked increase in tyrosine phosphorylation for protein that co-migrated with BTK in the 5-min sample treated with 10 μM pervanadate. The immune complex kinase assay of BTK resulted in BTK protein adjusted SI of 2.1 at 5 min and 1.9 at 15 min. Pervanadate treatment (20 μM) for 5 min resulted in a significant increase in the tyrosine phosphorylation of more substrate proteins and the BTK protein adjusted SI were 1.9 at 5 min and 2.6 at 15 min. The protein tyrosine phosphorylation of a large number of proteins increased with 50 μM pervanadate and the BTK protein adjusted SI were 3.2 at 5 min and 2.2 at 15 min.

No Effect of a 1-Gauss 60-Hz Exposure on Protein Tyrosine Phosphorylation or on BTK Activation—We next treated cells with 0, 10, 20, or 40 μM pervanadate; prepared cell lysates at 0, 5, 15, or 30 min after treatment; and directly compared the effect of pervanadate versus samples exposed to a 1-gauss 60-Hz EMF or sham environment on tyrosine phosphorylation patterns (Fig. 8). Anti-tyrosine phosphotyrosine immunoblotting of cell lysates demonstrated that there was an increase in tyrosine phosphorylation of protein that co-migrated with BTK in the 5-min sample treated with 10 μM pervanadate. There was a significant increase in the tyrosine phosphorylation of more substrate proteins with 20 μM pervanadate at 5 min compared to 5 min with 10 μM pervanadate. With 40 μM pervanadate, the protein tyrosine phosphorylation of a large number of proteins increased in a time-dependent manner. In contrast, there was little difference in the EMF-exposed samples when compared with the sham-exposed samples by phosphotyrosine profiles as determined by immunoblotting (Fig. 8). DT40 cells, stimulated with 25 μM pervanadate for 15 and 30 min in parallel with the sham- or EMF-exposed samples, showed a marked increase in tyrosine phosphorylation of a large number of substrate proteins. Uckun et al. (5) showed that protein tyrosine phosphorylation of substrate proteins was maximally stimulated in DT40 cells exposed for 1 min to a 1-gauss 60-Hz EMF. However, we found no detectable effect on protein tyrosine phos-
phorylation of substrate proteins in samples exposed to a sham or 1-, 2-, 3-, or 5-gauss 60-Hz EMF for 1 min (data not shown). Immune complex kinase assays of BTK performed in 20 mM Hepes, pH 8.0, 10 mM MgCl₂, and 1 mM Na₃VO₄ kinase buffer resulted in BTK protein adjusted SI that were 1.0 at 5 min, 1.1 at 15 min, and 1.2 at 30 min for the EMF-exposed samples and 1.1 at 5 min, 1.0 at 15 min, and 1.0 at 30 min for the sham-exposed samples. In contrast, 25 μM pervanadate treatment gave SI of 1.9 at 15 min and 1.6 at 30 min. These data show that exposure to the sham or EMF environment had no significant effect on protein tyrosine phosphorylation profiles or on BTK activation. Our results do not support the hypothesis that a 1-gauss 60-Hz EMF stimulated protein tyrosine phosphorylation (5) or activated BTK (2) in wild-type DT40 cells.

The magnitude of the reported effect of various intensities of 60-Hz EMF on BTK activation after a 30-min exposure (see Fig. 1 of Ref. 2) was 13-fold at 1 gauss, 29-fold at 3 gauss, and 3-fold at 5 gauss. Such high levels of BTK activation are remarkable, especially when compared with other studies showing no apparent changes in BTK autophosphorylating activity during IL-3 stimulation for 30 min in mouse pro-B cells (19). It is noteworthy that reprobing of the same blot with anti-phosphotyrosine antibody showed that tyrosine phosphorylation was increased (19). As shown here, BTK kinase activity was dependent on the buffer conditions. Although no apparent changes in BTK autophosphorylating activity were observed, we have also shown increased tyrosine phosphorylation by immunoblotting of pervanadate-treated samples (data not shown). The largest time-dependent change we observed in samples treated for 30 min with 25 μM pervanadate (Fig. 5). Notably, we observed that the replacement of MgCl₂ with MnCl₂ in the kinase assay strongly enhanced BTK activity 28-fold. This effect of MnCl₂ has been previously used in the study of BTK activation (7).

In summary, we examined the effect of a 1-gauss 60-Hz field on Ins 1,4,5-P₃ production in BTK-deficient cells reconstituted with the human btk gene. We observed that these cells were more sensitive than wild-type cells to BCR-induced signaling affecting the Ins 1,4,5-P₃ pathway, results in agreement with a previous study (11). As shown here, the robust response of this system to a commercial source of the anti-IgM antibody indicates that this genetic model is appropriate for further replication efforts to extend previous findings suggesting an EMF-dependent signaling pathway (1). However, our findings using cells obtained directly from the original investigators' laboratory, with an experimental design, shared between laboratories, do not support a causal relationship between EMF exposure and biological effects. The reasons for our differences are unknown. However, our studies measuring Ins 1,4,5-P₃ show that the preincubation time is important in this experimental system. It is not known whether the preincubation period was important for the effect of EMF described in the original investigators' work (1).

It is common practice to preincubate cells in serum-free medium before anti-IgM treatment as a mean of lowering the base-line level of PTK activity when measuring changes in phosphotyrosine of substrate proteins. As shown here, our assay for measuring phosphotyrosine profiles by immunoblotting was sufficiently sensitive to show an effect of 10 μM pervanadate after 5 min. However, we could not demonstrate any change in phosphotyrosine profiles in DT40 cells exposed up to 30 min to a 1-gauss 60-Hz EMF. In addition, there was no change in phosphotyrosine profiles in DT40 cells exposed for 1 min to a sham or 1-, 2-, 3-, or 5-gauss 60-Hz EMF.

Pervanadate was used to demonstrate that BTK activation was dependent on the kinase buffer. BTK autophosphorylation activity increased in a dose- and time-dependent manner by pervanadate treatment. In addition, these data show that the immune complex kinase assay was clearly not equally as sensitive as the anti-phosphotyrosine immunoblotting assay for detecting pervanadate-induced signaling. Notably, other investigators have demonstrated that increases in BTK activity, measured by autophosphorylation in immune complex kinase assays, may be difficult to show (19).

Our results do not provide support for the hypothesis that
EMF exposure induced a PTK-dependent signaling pathway acting on Ins 1,4,5-P_3. The results presented here should be of wide interest to other laboratories attempting replication. We have presented our experimental conditions with pervanadate showing BTK activation and the effect of MnCl_2 on the apparent BTK autophosphorylation activity. These data establish the experimental conditions where there is no EMF-dependent effect of a 1-gauss 60-Hz exposure on protein tyrosine phosphorylation or on BTK activation. It may be significant that pervanadate treatment resulted in a time-dependent increase in BTK activation, that is similar in its time dependence, but is of lower magnitude (Fig. 5; 4.2-fold at 30 min) than the effect of 1-gauss 60-Hz EMF (7.4-fold at 30 min) shown by the original investigators (2).

The results presented here raise the hypothesis that some parameter of the EMF exposure environment (4), used by the original investigators, may be responsible for a biological effect that is remarkable in its similarity to the action of pervanadate. This hypothesis is supported by the following evidence. 1) Evidence suggests that the interaction of the magnetic field with the biological system is dependent upon a unique combination of static DC and time-varying AC field vectors. Whether biological effects depend on synergism between a static and time-varying magnetic field is still a controversial issue. However, the literature provides extensive evidence suggesting that both the magnitude and relative field direction are important (12, 20–24). 2) A large number of studies have shown that the tyrosine phosphorylation state of a protein is dependent on the balance between the competing activities of PTKs and PTPs (16, 25–29). PTPs contain an essential cysteine residue at the catalytic site involved in the formation of a thiol-phosphate intermediate during catalysis (30). Pervanadate has been shown to inhibit PTPs by irreversibly oxidizing the catalytic cysteine (28, 31). Whether an undefined EMF exposure metric (4) is responsible for an oxidant-like biological effect remains to be determined. This suggests that additional work is needed in this model system to further evaluate this hypothesis. Most important, replication is needed for the DT40 genetic model to be recognized as appropriate for further efforts to understand the role of PTKs in EMF-induced signaling events mediating the biological effects of power line frequency EMFs.

Acknowledgments—We thank Dr. Fatih Uckun for sending Drs. Ilker Dibirdik and Daiva Kristupaitis to SRI International, and we thank them for their efforts in transferring critical experimental details for measuring Ins 1,4,5-P_3 and BTK in the beginning of this project. In addition, we thank Dr. Joe Bolen and Dr. Mike Tomlinson of DNAX, for antibodies, advice in performing BTK immune complex kinase assays, and sharing their experience working with DT40 cells. Finally, we thank Dr. Mike Galvin and Dr. Gary Boorman of the NIEHS for their support.

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