Electromagnetic Field-induced Stimulation of Bruton’s Tyrosine Kinase

Daiva Kristupaitis‡, Ilker Dibirdik§, Alexei Vassilev§, Sandeep Mahajan§, Tomohiro Kurosaki†, Alice Chu§, Lisa Tuel-Ahlgren§, Dong Tuong§, David Pond§, Richard Lubén‡, and Fatih M. Uckun‡‡‡

From the ‡Biotherapy Program, University of Minnesota, Minneapolis, Minnesota 55455, the §Division of Biomedical Sciences, University of California, Riverside, California 92521, the †Wayne Hughes Institute, St. Paul, Minnesota 55113, and the ‡Department of Molecular Genetics, Kansai Medical University, Institute for Hepatic Research, Morinomi, Osaka 570, Japan

Here we present evidence that exposure of DT40 lymphoma B-cells to low energy electromagnetic fields (EMF) results in activation of phospholipase C-γ2 (PLC-γ2), leading to increased inositol phospholipid turnover. PLC-γ2 activation in EMF-stimulated cells is mediated by stimulation of the Bruton’s tyrosine kinase (BTK), a member of the Src-related TEC family of protein tyrosine kinases, which acts downstream of LYN kinase and upstream of PLC-γ2. B-cells rendered BTK-deficient by targeted disruption of the btk gene did not show enhanced PLC-γ2 activation in response to EMF exposure. Introduction of the wild-type (but not a kinase domain mutant) human btk gene into BTK-deficient B-cells restored their EMF responsiveness. Thus, BTK exerts a pivotal and mandatory function in initiation of EMF-induced signaling cascades in B-cells.

A number of epidemiologic studies suggested the possibility that EMF1 radiation from residentially proximate power lines, household electrical wiring, and appliance usage may contribute to the risk of childhood acute lymphoblastic leukemia (1–5). A recent study by Linet et al. (6) showed that living in homes characterized by high measured time-weighted average magnetic field levels or by the highest wire code category does not increase the risk of acute lymphoblastic leukemia in children. However, concerns regarding other forms of EMF exposure remain. Since no directly genotoxic effects are exerted by EMF, it is thought that EMF may participate in leukemogenesis of childhood acute lymphoblastic leukemia by influencing their proliferation, survival, and/or differentiation programs (7–9).

In a recent study, we discovered that exposure of B-lineage lymphoid cells to low energy EMF stimulates the Src protooncogene family protein tyrosine kinase, LYN, leading to downstream activation of protein kinase C (10). These results prompted the hypothesis that a delicate growth regulatory balance might be altered in B-lineage lymphoid cells by EMF-induced activation of LYN kinase. In a subsequent study, we examined the molecular mechanism of enhanced inositol phospholipid turnover in lymphoma B-cells exposed to low energy EMF (11). Our findings were consistent with a sequential activation model according to which EMF exposure first leads to activation of Src family protein tyrosine kinases. Src family protein tyrosine kinases domains interact with and phosphorylate as yet unidentified immunoreceptor tyrosine-based activation motifs, leading to recruitment of spleen tyrosine kinase (SYK) as well as PLC-γ2 via their Src homology (SH2) domains to the phosphorylated immunoreceptor tyrosine-based activation motifs. Subsequently, SYK is activated by Src family protein tyrosine kinases and phosphorylates PLC-γ2 leading to its activation. Activation of PLC-γ2 results in increased inositol phospholipid turnover, production of inositol-1,4,5-trisphosphate (Ins-1,4,5-P3) and protein kinase C activation (11).

BTK is a member of the Src-related TEC family of PTKs (12, 13), and its enzymatic activity is regulated by LYN kinase (14, 15). Mutations in the btk gene have been linked to severe developmental blocks in human B-cell ontogeny, resulting in human X-linked agammaglobulinemia (16–18). BTK was also identified as the mediator of apoptosis in B-lineage lymphoid cells exposed to ionizing radiation (19). Recent evidence suggests an important role for BTK in the regulation of PLC-γ2 activity level (20). The concerted actions of both BTK and SYK are required for the B-cell antigen receptor-induced PLC-γ2 activation (20). Therefore, we decided to examine the potential participation of BTK in EMF-induced activation of PLC-γ2. Here, we show that low energy EMF exposure initiates a biochemical signaling cascade intimately linked to BTK. Our study provides unprecedented experimental evidence that BTK is the mediator of EMF-induced enhanced inositol phospholipid turnover in lymphoma B-cells.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The establishment and characterization of wild-type and BTK-deficient, LYN-deficient, and SYK-deficient clones of DT40 chicken lymphoma B-cells were reported previously (19–25). Wild-type and mutant cells were maintained in suspension cultures at 37 °C, 5% CO2 in a humidified incubator. The culture medium was RPMI 1640 (Life Technologies, Inc.), supplemented with 10% fetal calf serum, 2.5% chicken serum, 10 mM l-glutamine, and 50 mM 2-mercaptoethanol as described previously (11, 19).

**EMF Exposure**—A homogeneous vertical magnetic field was set up by using a Merritt’s coil-based in vitro low frequency EMF exposure system (11). Merritt’s four square coil system is known to produce a large volume of uniform magnetic field. Unless otherwise indicated, the applied vertical sinusoidal 60-Hz field was 0.1 millitesla (1 gauss). The current needed to obtain 1 gauss was 0.7 A. The magnetic field was parallel to the coil axis and was uniform near the axis and the center of...
EMF-induced Stimulation of BTK

Activation of BTK in DT40 Lymphoma B-cells Exposed to Low Energy Electromagnetic Fields—LYN kinase plays a pivotal role in ligand-induced signal transduction events in B-lineage lymphoid cells and is thought to mediate its downstream effects (e.g., activation of PLC-γ2 and inositol phospholipid turnover) by activating the tyrosine kinases SYK and BTK (19–20, 22, 24). We previously reported that exposure of B-lineage lymphoid cells to low energy EMF stimulates the protein tyrosine kinase LYN, and activation of LYN kinase was sufficient and mandatory for EMF-induced tyrosine phosphorylation in B-lineage lymphoid cells (10). To further elucidate the EMF-induced signal transduction events in B-lineage lymphoid cells, we decided to examine the enzymatic activity of BTK in DT40 lymphoma B-cells after EMF exposure using immune complex kinase assays. We first exposed DT40 Western blot analyses. Autoradiograms of the kinase assay (KA) and Western blots (WB) using anti-BTK antibody are shown in each panel. A, wild-type (WT) DT40 cells. BTK-deficient DT40 cells were reconstituted with wild-type human btk gene (btk WT) (B) or a mutation in the SH2 domain (btk WT mSH2) (C) or in the PH domain (btk WT mPH) (D). The establishment and characterization of these DT40 clones were described previously in detail (19, 20).

RESULTS AND DISCUSSION

Analysis of Stimulation of Inositol Phospholipid Turnover—Ice-cold perchloric acid (20%) was added after EMF exposure to the cell suspensions to stop further reaction. The test tubes were kept on ice for 20 min and then sedimented at 2,000 x g for 15 min at 4 °C. The supernatant was collected and the pH was neutralized to 7.5 with 5% nonidet P-40 lysis buffer. The lysates were immunoprecipitated with a polyclonal rabbit anti-BTK antibody (3 m g/ml aprotinin, and 1 m M phenylmethylsulfonyl fluoride as protease inhibitors. Lysates were spun twice at 12,000 x g for 10 min at 4 °C prior to immunoprecipitation. 500-μg samples of the cell lysates were immunoprecipitated with a polyclonal rabbit anti-BTK antibody (3 μl/500 μg of lysate) and immunocomplex kinase assays (26–29), as well as anti-BTK Western blot analyses, were performed as described (14, 19). All BTK kinase and Western blot autoradiograms were subjected to densitometric scanning using an automated AMBIS system (Automated Microbiology System, Inc., San Diego, CA), and for each time point a stimulation index was determined by comparing the density ratios of the BTK protein band test sample:density of kinase band/density of BTK protein band baseline control sample. The expression levels of PLC-γ2 and actin in whole cell lysates of wild-type and mutant DT40 B-cells were examined by Western blot analysis using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech) as reported previously (11).

Analysis of Stimulation of Inositol Phospholipid Turnover—Ice-cold perchloric acid (20%) was added after EMF exposure to the cell suspensions to stop further reaction. The test tubes were kept on ice for 20 min and then sedimented at 2,000 x g for 15 min at 4 °C. The supernatant was collected and the pH was neutralized to 7.5 with ice-cold 10 mM KOH and centrifuged again. The supernatant was then collected and stored at −20 °C for subsequent measurement of Ins-1,4,5-P3 levels.

EMF-induced BTK activation in DT40 lymphoma B-cells. DT40 cells were exposed to 60-Hz EMF at field intensities of 1 gauss (G), 3G, 5G, 7G, or 10 G. Controls (CON) were not exposed to EMF. At 10 and 30 min, EMF exposure was stopped with a 1% nonidet P-40 lysis buffer. The lysates were immunoprecipitated with a polyclonal anti-BTK antibody and then subjected to immune complex kinase assays (see "Experimental Procedures"). The radioactivity (counts/min) of the autophosphorylated BTK bands was determined as a measure of the BTK enzymatic activity.

the coil system. Cells were maintained at all times in a low AC (8 milligauss) environment except during a single centrifugation step. This was achieved by using a 2-pole motor tissue incubator (CEDCO model IRE 93) with low AC fields for routine cell culture and by defining the lowest field regions within the incubator. Exponentially growing cells (5 x 10⁶ cells/ml) in serum-free medium in 1.5-ml capacity microcentrifuge tubes were exposed to the 1-gauss, 60-Hz EMF by placing the tubes at the center of the four-coil field generator contained in the incubator with shielding sheets of metal alloy at the bottom of the chambers. Control tubes were simultaneously placed inside a duplicate incubator without the exposure apparatus. To measure the fields in the incubators, the laminar flow hood, centrifuge, and nearby areas, a gaussmeter (MAG model 25, Magnetic Sciences International) was used. EMF strength was constantly monitored with the gaussmeter and adjusted manually if needed. In all experiments, the coils were activated before the cells had been placed into, to avoid fluctuations of the EMF during turning on the apparatus. The coils were turned off only after the cells were taken out of the exposure system.

Immune Complex Kinase Assays of BTK—To evaluate the effects of EMF on the enzymatic activity of BTK, exponentially growing cells were exposed to EMF and, at the indicated time points after EMF exposure, the test tubes were immediately immersed in ice water for 30 s to 1 min. Cells were spun down at 3,000 x g for 5 min at 0 °C and lysed in a 1% nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% nonidet P-40, 1 mM EDTA) containing 1 mM Na3VO4 and 1 mM sodium molybdate as phosphatase inhibitors, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride as protease inhibitors. Lysates were spun twice at 12,000 x g for 10 min at 4 °C prior to immunoprecipitation. 500-μg samples of the cell lysates were immunoprecipitated with a polyclonal rabbit anti-BTK antibody (3 μl/500 μg of lysate) and immunocomplex kinase assays (26–29), as well as anti-BTK Western blot analyses, were performed as described (14, 19). All BTK kinase and Western blot autoradiograms were subjected to densitometric scanning using an automated AMBIS system (Automated Microbiology System, Inc., San Diego, CA), and for each time point a stimulation index was determined by comparing the density ratios of the kinase and protein bands to those of the baseline sample and using the formula: stimulation index (SI) = (density of kinase band/density of BTK protein band) test sample/(density of kinase band/density of BTK protein band) baseline control sample. The expression levels of PLC-γ2 and actin in whole cell lysates of wild-type and mutant DT40 B-cells were examined by Western blot analysis using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech) as reported previously (11).
the enzymatic activity of BTK at various time points after the initiate of the EMF exposure. As shown in Fig. 1, 60-Hz EMF exposure at a field intensity of 1 gauss or 3 gauss induced rapid activation of BTK (p77\textsuperscript{BTK}) kinetic activity, as reflected by increased autophosphorylation. This rapid activation of BTK was observed in four consecutive independent experiments. The magnitude of activation was less at higher field intensities. Therefore, a field intensity of 1 gauss was used in subsequent experiments.

Different domains of BTK are important for its physiologic functions (13, 30). BTK has a pleckstrin homology (PH) domain, a TEC homology domain, a single SH3 domain, a single SH2 domain, and a catalytic kinase domain (12, 13, 17, 30). Mutations in the SH2 domain as well as PH domain of the BTK result in defective B-cell development, leading to human X-linked agammaglobulinemia (16, 31–34). The PH domain is responsible for interactions with various isoforms of protein kinase C,β2 subunits of heterotrimeric GTP-binding proteins and phosphatidylinositol-4,5-bisphosphate, the precursor to Ins-1,4,5-P3 (35–38). The SH3 domain is responsible for interactions with proline-rich sequences such as TEC domains, whereas the SH2 domain facilitates the interactions with tyrosine-phosphorylated proteins (13, 38). It has been shown that the SH2 and PH domains of BTK are required for the activation of PLC-γ2 in B-cell antigen receptor-mediated B-cell activation (20), but the same domains are not essential for the activation of BTK induced by ionizing radiation (19).

Therefore, we decided to determine if the SH2 and PH domains of BTK are required for its activation following EMF exposure. Exposure of control wild-type DT40 cells to EMF resulted in a time-dependent activation of BTK, as measured by enhanced autophosphorylation. While the autophosphorylation showed a 7.9-fold increase at 30 min by densitometric analysis showed a 7.9-fold increase at 30 min as determined by anti-BTK Western blot analysis, showed only the respective genes by homologous recombination knockout. A, BTK-deficient (BTK\textsuperscript{−/−}) DT40 cells. B, LYN-deficient (LYN\textsuperscript{−/−}) mutant DT40 cells. C, SYK-deficient (SYK\textsuperscript{−/−}) mutant DT40 cells.

**Fig. 3.** Time course of EMF-induced BTK activation in mutant DT40 lymphoma B cells. Cells were exposed to 1-gauss low frequency EMF. At the times indicated in the panels, EMF exposure was stopped with a 1% Nonidet P-40 lysis buffer. The lysates were immunoprecipitated with a polyclonal anti-BTK antibody and then subjected to immune complex kinase assay or anti-BTK Western blot analyses. Phosphorylation in kinase assay (KA) was initiated by the addition of radiolabeled ATP and terminated after 10 min. Autoradiograms of the kinase reaction (KA) and Western blots (WB) are shown in each panel. Mutant DT40 clones were rendered deficient for specific tyrosine kinases through target disruption of the respective genes by homologous recombination knockout. A, BTK-deficient (BTK\textsuperscript{−/−}) DT40 cells. B, LYN-deficient (LYN\textsuperscript{−/−}) mutant DT40 cells. C, SYK-deficient (SYK\textsuperscript{−/−}) mutant DT40 cells.

As expected, no BTK bands were detected in immune complex kinase assays or anti-BTK Western blots of DT40 cells, which were rendered BTK-deficient through targeted disruption of the btk gene by homologous recombination knockout, and were used as a negative control (Fig. 3A). Targeted disruption of the lyn gene abolished the activation of BTK after EMF exposure, indicating that LYN kinase acts upstream of BTK in the EMF-induced signaling cascade (Fig. 3B). By comparison, targeted disruption of the syk gene did not abolish the BTK activation. However, the magnitude of the BTK signal seemed markedly attenuated in SYK-deficient DT40 cells (maximum SI: 2.9 at 15 min), consistent with the existence of cross-talk between BTK and SYK in generating an optimal EMF response (Fig. 3C).

**Role of BTK in Activation of PLC-γ2 in DT40 Lymphoma B-cells Exposed to Low Energy EMF**—In accordance with our previous study (11), exposure of DT40 cells to EMF resulted in enhanced inositol phospholipid turnover (Fig. 4A). Because BTK is required for the B-cell antigen receptor-induced PLC-γ2 activation in DT40 cells (20), we then examined the role of BTK in EMF-induced inositol phospholipid turnover. Targeted disruption of the btk gene abolished the EMF-induced Ins-1,4,5-P3 signal (Fig. 4B). Introduction of wild-type (but not a kinase domain mutant) human btk gene into the BTK-deficient DT40 cells restored their ability to respond to EMF with enhanced inositol phospholipid turnover (Fig. 4C and D). The lack of the Ins-1,4,5-P3 signal in BTK\textsuperscript{−/−} and BTK\textsuperscript{−/−} BTK/K\textsuperscript{−/−} cells was not due to lower expression levels of PLC-γ2 enzyme in these cells (Fig. 4E). These results demonstrate that BTK is essential for EMF-induced PLC-γ2 activation in DT40 lymphoma B-cells, and its kinase domain is required for the Ins-1,4,5-P3 response. In summary, we examined the molecular mechanism of enhanced tyrosine phosphorylation and increased inositol phospholipid turnover in DT40 lymphoma B-cells exposed to low energy EMF. Our findings provide unprecedented evidence that EMF exposure initiates a biochemical signaling cascade...
indicated in the figure, the cells were lysed with ice-cold 20% perchloric acid and then assayed for Ins-1,4,5-P$_3$ levels using a radioligand competition assay (see "Experimental Procedures"). A, wild-type DT40 cells (WT). B, BTK-deficient cells (BTK$^-$). C, BTK-deficient DT40 cells reconstituted with wild-type (WT) human btk gene (BTK$^-$rBTK/WT). D, BTK-deficient DT40 cells reconstituted with a human btk gene that contains a mutation in the kinase domain (BTK$^-$rBTK/K$^*$). Results are the mean ($\pm$ S.E.) values obtained in replicate experiments each performed with duplicate measurements at every time point. $n$ = number of independent experiments. E, anti-PLC-$\gamma$2 and anti-actin Western blot analysis of wild-type, BTK-, BTK$^-$rBTK/WT, and BTK$^-$rBTK/K$^*$ DT40 cells.

![Figure 4](image-url)
28. Uckun, F. M., Dibirdik, I., Smith, R., Tuel-Ahlgren, L., Chandan-Langlie, M., Schieven, G. L., Waddick, K. G., Hansen, M., and Ledbetter, J. A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 3589–3593
29. Dibirdik, I., Chandan-Langlie, M., Ledbetter, J. A., Tuel-Ahlgren, L., Obuz, V., Waddick, K. G., Gajil-Perzalska, K., Schieven, G. L., and Uckun, F. M. (1991) *Blood* **78**, 564–570
30. Saouaf, S. J., Mahajan, S., Rowley, R. B., Kut, S. A., Fargnoli, J., Burkhardt, A. L., Tsukada, S., Witte, O. N., and Bolen, J. B. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 9524–9528
31. Vetrie, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flinter, F., Hammarstrom, L., Kinnon, C., Levensky, R., Bebow, M., Smith, C. I. E., and Bentley, D. R. (1993) *Nature* **361**, 226–233
32. Tsukada, S., Saffran, D. C., Rawlings, D. J., Parolini, O., Allen, R. C., Kissak, I., Sparkes, R. S., Kubagawa, M., Mohandas, T., Quan, S., Belmont, J. W., Cooper, M. D., Conley, M. E., and Witte, O. N. (1993) *Cell* **72**, 279–290
33. Vihinen, M., Cooper, M. D, Basile, G. S., Fischer, A., Goud, R. A., Hendriks, R. W., Kinnon, C., Kwan, S.-P., Litman, G. W., Notarangelo, L. D., Ochs, H. D., Rosen, F. S., Vetric, D., Webster, A. D. B., Zegers, B. J. M., and Smith, C. I. E. (1996) *Immunol. Today* **16**, 460–465
34. Bradley, L. A., Sweatman, A. K., Levering, R. C., Jones, A. M., Morgan, G., Levensky, R. J., and Kinnon, C. (1994) *Hum. Mol. Genet.* **3**, 79–83
35. Tsukada, S., Rawlings, D. J., and Witte, O. N. (1994) *Curr. Opin. Immunol.* **6**, 623–630
36. Harlan, J. E., Hajduk, P. J., Yoon, H. S., and Fesik, S. W. (1994) *Nature* **371**, 168–170
37. Hemmings, B. A. (1997) *Science* **275**, 1899
38. Pawson, T. (1995) *Nature* **373**, 573–580
39. Kawakami, Y., Miura, T., Bissonnette R., Hata, D., Khan, W. N., Kitamura, T., Maeda-Yamamoto, M., Hartman, S. E., Yao, L., Alt, F. W., and Kawakami, T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 3938–3942
40. Anderson, Y. S., Teutsch, M., Deng, Z., and Wortis, H. H. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 10966–10971