Vol. 212, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 6, 1995 Pages 104-109

Enhancement of β-Galactosidase Gene Expression in Rat Pheochromocytoma Cells by Exposure to Extremely Low Frequency Magnetic Fields

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Received May 31, 1995

Exposure of PC12–VG cells to an extremely low frequency magnetic field (ELFMF) enhanced the β-galactosidase gene expression stimulated by treatment of the cells with forskolin. The enhancing effect of the ELFMF was inhibited by treatment of the cells with a specific inhibitor of PKC, calphostin C, as well as with the Ca^{2+} entry blockers nifedipin and dantrolen. Enhancement appeared within the first hour of a 4h forskolin treatment when the ELFMF was given at different times during culture. We speculate that exposure of PC12-VG cells to an ELFMF during the early response to forskolin treatment affects cell signal transduction, resulting in enhanced gene expression.

Extremely low frequency magnetic fields (ELFMFs), conventionally defined as those produced by alternating current between 30 and 300Hz, are produced by power distribution networks, industrial machinery, and common household electric appliances. Because of the recent increase in the exposure of humans to ELFMFs, there is concern about possible carcinogenic effects. The effects of ELFMFs on certain cellular functions have been reported. Conti *et al.* (1) showed that exposure to 2.3-6.5mT ELFMFs at 3-50Hz inhibits blastogenesis of peripheral blood lymphocytes stimulated by the mitogen concanavalin A. Yost and Liburdy (2) reported that combined exposure to a 42.1 μT ELFMF at 16 Hz and to a 23.4 μT static magnetic field inhibited the calcium influx triggered by concanavalin A in rat thymic lymphocytes. Rodemann *et al.* (3) reported that long-term exposure to an ELFMF (6 mT, 20 Hz) for up to 21 days at 2x6 h per day

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Abbreviations: ELFMF, extremely low frequency magnetic field; NGF, nerve growth factors; PKC, protein kinase C; PKA, protein kinase A; T, tesla; TPA, 12-0-tetradecanoyl phorbol 13-acetate; VIP, vasoactive intestinal peptide.

0006-291X/95 $12.00

induced morphological differentiation in human fibroblasts. Our research on the expression of early response genes showed that c-*fos* expression is induced in HeLaS3 cells exposed to 0.18-0.2 T static magnetic fields (4). Furthermore, Phillips *et al.* (5) showed that in human T-lymphoblastoid cells exposure to 100 μT ELFMF at 60 Hz induced a time-dependent increase in the transcription of the c-*fos*, c-*jun*, and c-*myc* genes, as well as in protein kinase C (PKC) .

Despite the known qualitative effects of ELFMFs on cells, the quantitative estimations of their effects are controversial because of differences in the quality and power of the experimental equipment used and/or the sensitivity of the biological responses. We recently designed new equipment for long-term high-intensity (400mT) exposure of cultured cells to ELFMFs (6). We also established a system for assaying β-galactosidase gene expression stimulated by forskolin, in which the expression plasmid of the β-galactosidase gene is introduced into rat PC12 cells (7). We here report that exposure of rat PC12 cells to ELFMFs modified the intracellular signal transduction induced by stimulation with forskolin.

Materials and Methods

ELFMF exposure unit

Details of the exposure equipment are given elsewhere (6). Briefly the magnetic field is generated by a pair of magnetic cores, 240 x 340mm rectangles 40mm apart, made of stacked, insulated silicon steel plates to reduce the generation of heat. The magnetic field is oriented vertically, the field in the horizontal direction being very weak. The current transformer is AC 200V, 50Hz, triphase, and 35kVA. An 800A peak current (effective AC value 566A*rms*) is supplied to the AC magnets. Two copper coils pass through the steel cores, and the field generated has a magnetic intensity from 0 to 400mT. Culture dishes 35mm in diameter are placed in the exposure chamber, in which the mean current intensity calculated from the equation $J = \frac{1}{2} \times \sigma \times \omega \times r \times Bo$ (8) at 400mT is 140μA/cm^2. An acrylic CO_2 incubator is installed between the cores. Cooling water circulates around the magnetic cores, and warm water circulates around the incubator to keep the temperature of the exposure area at 37 ± 0.5°C. The atmosphere in the incubator is 95% humidified air plus 5% CO_2. The incubator space for ELFMF exposure is 190mm (W) x 220mm (D) x 28mm (H). Ten 6cm culture dishes can be placed in this area that has a uniform ELFMF field (variation < 1%).

Cell and culture conditions

PC12-VG cells, derived from rat pheochromocytoma PC12 cells, were established by stable transfection with the expression plasmid pVIPGAL1 that consists of the *E. coli lac*-Z operon including the β-galactosidase gene connected with the promoter region of the human vasoactive intestinal peptide (VIP) gene (7). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Summit Biotechnology, Greeley, CO), 5% heat-inactivated horse serum (Gibco Laboratories, Grand Island, NY), and 100μg/ml G418 (geneticin disulfate, Gibco Laboratories, Grand Island, NY).

ELFMFs exposures and the β-galactosidase activity assay

Cells were plated at a density of 10^4 cells/plate (35mm diameter) one day before exposure to the ELFMF. They were rinsed with phosphate-buffered saline (PBS), after which 1ml of medium containing 2μM forskolin (Calbiochem, La Jolla, CA) with or without 25ng/ml of TPA (Wako Chemicals, Japan) was added to each dish. To inhibit cell signal transduction we used 1.27μM

calphostin C (Wako Chemicals) as the PKC inhibitor, or 10μM nifedipin (Wako Chemicals) and 10μM dantrolen (Wako Chemicals) as the calcium entry blockers. The treated PC12–VG cells were exposed to a 200 or 400mT ELFMF for 4h. Treated cells were incubated in a conventional CO_2 incubator were used as the unexposed control.

β-galactosidase activity was measured according to Lim and Chae (9). After the dishes had been rinsed with PBS, 1ml of PBS containing 3.5mM o-nitrophenyl-β-D-galactopyranoside (Nacalai Tesque, Japan), 2mM $MgCl_2$, and 0.5% (v/v) Nonidet P-40 (Nacalai Tesque) was added to each one. The optical density of the reaction mixture at 420nm was measured after 30min at room temperature when a faint yellow color had developed. The Student's *t*-test was used for the statistical evaluation of the experimental data.

Results

β-galactosidase activity in the PC12–VG cells without forskolin stimulation was very low whether or not there was ELFMF exposure. Fig. 1 shows that β-galactosidase activity was enhanced by treatment with forskolin and that this enhancement was increased by the combination of forskolin treatment and ELFMF exposure (200mT or 400mT). Results of combined treatment with 2μM forskolin, 25ng/ml of TPA and a 400mT ELFMF exposure on β-galactosidase activity are given in Table 1. Forskolin and TPA stimulation for 4h produced a 2.34-fold increase in enzyme activity. When exposure to ELFMF was combined with forskolin and TPA treatment, enzyme activity was enhanced another 2.52-fold.

β-galactosidase activity enhancement by forskolin was reduced on the addition of nifedipin and dantrolen (Fig.2B, dark bar) or calphostin C (Fig,2C, dark bar). Exposure to an ELFMF under these conditions did not restore β-galactosidase activity (Fig.2B and 2C, gray bars).

PC12-VG cells were treated with 2μM forskolin for 4h, during which time the cells were exposed to an ELFMF for the periods shown in Fig. 3. 2 (C) and 1 (D) h exposures to a 400mT ELFMF followed by 2 (C) and 3 (D) h incubations in a conventional incubator, enhanced β-galactosidase activity to the same degree as a 4h exposure to an ELFMF (B) alone. In contrast,

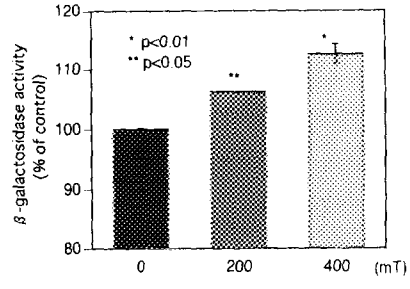


Fig. 1.
ELFMF effects on β-galactosidase activity in PC12-VG cells stimulated with forskolin. PC12-VG cells were treated with forskolin (2μM) then exposed to a 200 or 400mT ELFMF for 4h. Twenty-four plates were used for each trial. Error bars represent the standard errors of the means. Asterisks, *(p<0.01) and **(p<0.05), show the statistic significance as compared to treatment with forskolin alone (0mT).

Table 1

ELFMF effects on β-galactosidase activity in PC12-VG cells stimulated with forskolin and TPA. PC12-VG cells were treated with forskolin and TPA or with forskolin alone, and then exposed to 400mT ELFMF for 4h.[a]

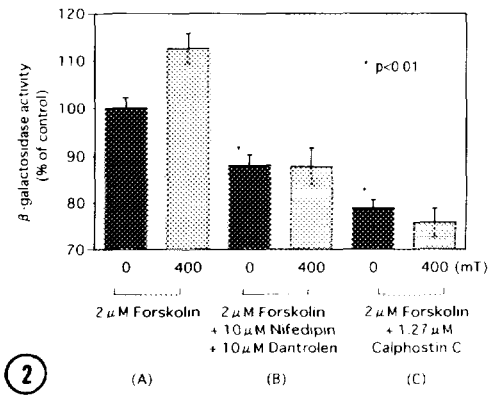
ELFMF	Forskolin (2μM) alone [b]	Forskolin and TPA(25ng/ml)
-	100.0±2.0	234.1±4.1
+	112.6±3.2*	252.4±5.5**

[a] β-galactosidase activity is given as a % of the control.

[b] Six plates were used in each trial, and the experiments were repeated four times.

Asterisks, *($p<0.01$) and **($p<0.05$), show the statistical significance as compared to treatment with forskolin alone (-).

2h of culture in a conventional incubator followed by 2h of exposure to an ELFMF (F) produced no additional enhancement with forskolin alone (A). A 1h exposure to an ELFMF during the 2nd hour of the 4h forskolin treatment (E) also was not effective.



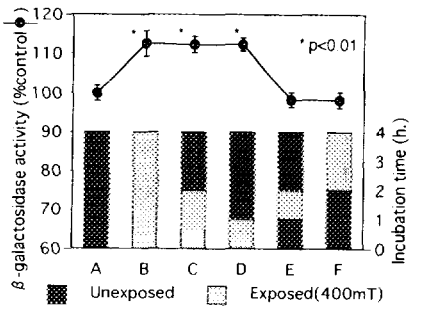


Fig. 2.

ELFMF effects on β-galactosidase activity in PC12-VG cells treated with forskolin and the calcium entry blockers, nifedipin and dantrolen, or with forskolin and calphostin C, a specific inhibitor of PKC. PC12-VG cells treated with forskolin alone (A), forskolin (2μM), nifedipin (10μM) and dantrolen (10μM) (B), and forskolin (2μM) and calphostin C (1.27μM) (black bars) (C), then exposed to a 400mT ELFMF for 4h (gray bars). Six plates were used for each trial, and the experiments were repeated three times. Asterisks (*) show the statistic significance ($p<0.01$) in comparison to treatment with forskolin alone.

Fig. 3.

Effects on β-galactosidase activity in PC12-VG cells due to ELFMF exposure for different periods during treatment with forskolin for 4h. Activity was measured at the end of the 4h treatment with 2μM forskolin. Black bars show the periods of nonexposure to 400mT ELFMF, gray bars the periods of ELFMF exposure. Six plates were used in each trial. The experiments were repeated three times. Error bars show the standard errors of the means. Asterisks (*) indicate significant differences in comparison to the control (column A) ($p<0.01$).

Discussion

PC12 cells release norepinephrine in response to low frequency (500Hz) magnetic fields at 160~850μT (10). The outgrowth of neurite in PC12 and PC12D (a subline of PC12) cells is stimulated by nerve growth factors (NGF) (11). Exposure to an ELFMF of 2.2μT at 50Hz for 20-22h also enhances neurite outgrowth in the absence of NGF (12). These results indicate that PC12 cells are a suitable material for experiments in which the biological effects of ELFMFs are assessed.

Forskolin activates adenylate cyclase and increases the intracellular cyclic AMP level. Pathways by which β-galactosidase is expressed may have two steps: (i) An increase in the cyclic AMP level activates protein kinase A (PKA). The promoter region of the human VIP gene has a cyclic AMP-responsive element (13); therefore, this activation of PKA may enhance the transcription and translation of the stimulation factor(s) that binds to the promoter region of the human VIP gene, thereby enhancing the transcription and translation of β-galactosidase gene. (ii) The promoter region of the human VIP gene has a TPA-responsive element (14), and activation of PKC by TPA may induce the production of stimulation factor(s) that bind to the VIP promoter, resulting in enhanced expression of the β-galactosidase gene.

The enhanced β-galactosidase activity in PC12-VG cells produced by forskolin treatment was further enhanced when exposure to an ELFMF was combined with forskolin treatment (Fig. 1). Although ELFMF exposure alone had no effect on β-galactosidase activity, it could have enhanced the adenylate cyclase activity induced by forskolin and potentiated PKA signal transduction.

Forskolin and TPA together enhanced β-galactosidase activity 2.34-fold as compared with forskolin treatment alone, in combination with ELFMF exposure the activity was enhanced an additional 2.52-fold also was used (Table 1). The enhanced β-galactosidase activity found in PC12-VG cells may be related to the stimulation of PKC signal transduction. Kavaliers *et al.* (15) showed that ELFMF has antagonistic effects on opiate-mediated analgesia in snails, which suggests an alteration in calcium channel function and/or Ca^{2+} flux involving PKC. Walleczek *et al.* (16) showed that exposure of rat thymic lymphocytes to an ELFMF increased the Ca^{2+} influx during mitogen-activated signal transduction. Ca^{2+} is a signal transducer in many signal transduction pathways that involve the activation of PKC. Calphostin C, which interacts with the regulatory domain of PKC, is a specific inhibitor of PKC (17). Calphostin C reduce β-galactosidase activity in PC12–VG cells and inhibited its enhancement when there was ELFMF exposure (Fig.2). In addition to nifedipin and dantrolen, calcium entry blockers reduced the enhancement of β-galactosidase activity when there was ELFMF exposure (Fig. 2). The enhancing effects of ELFMFs on forskolin-stimulated β-galactosidase activity may depend on the calcium ion-PKC pathway.

One hour exposure to an ELFMF during forskolin stimulation had the same effect as a 4h exposure (Fig. 3D and B). A 2h exposure to an ELFMF after 2h of incubation without exposure

was not effective. The ELFMF therefore must affect the early step(s) of the signal transduction related to the activation of the VIP promoter by forskolin. ELFMF produces enhanced changes in the gene transcription of c-*fos*, c-*jun*, and c-*myc* (5); all immediate early response genes. The enhancement of β-galactosidase activity therefore may be due to increases in the transcription of these early response genes.

In explanation of how ELFMF interacts with cells to enhance gene expression, the most popular theories suggest that the plasma membrane is directly or indirectly involved in transducing ELFMF perturbation. The pericellular currents induced by an ELFMF may modify ion flux through the plasma membrane and affect ligand-receptor interactions, including that of adenylate cyclase and the activated form of PKC at the cell surface. In our study, exposure to ELFMF enhanced forskolin-stimulated β-galactosidase activity in PC12-VG cells, which suggests that intracellular signal transduction, involving the PKA- and PKC- pathways, is markedly affected.

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