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An Extremely Low Frequency Magnetic Field Attenuates Insulin Secretion From the Insulinoma Cell Line, RIN-m

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In this study, we investigated the effects of exposure to an extremely low frequency magnetic field (ELFMF) on hormone secretion from an islet derived insulinoma cell line, RIN-m. We stimulated RIN-m cells to secrete insulin under exposure to an ELFMF, using our established system for the exposure of cultured cells to an ELFMF at 5 mT and 60 Hz, or under sham exposure conditions for 1 h and observed the effects. In the presence of a depolarizing concentration of potassium (45 mM KCl), exposure to ELFMF significantly attenuated insulin release from RIN-m cells, compared to sham exposed cells. Treatment with nifedipine reduced the difference in insulin secretion between cells exposed to an ELFMF and sham exposed cells. The expression of mRNA encoding synaptosomal associated protein of 25 kDa (SNAP-25) and synaptotagmin 1, which play a role in exocytosis in hormone secretion and influx of calcium ions, decreased with exposure to an ELFMF in the presence of 45 mM KCl. These results suggest that exposure to ELFMF attenuates insulin secretion from RIN-m cells by affecting calcium influx through calcium channels. Bioelectromagnetics 25:160–166, 2004.

Key words: 5 mT and 60 Hz; insulinoma cell line; KCl stimulation; calcium channels; synaptosomal associated protein; synaptotagmin 1

INTRODUCTION

The possible health effects of exposure to extremely low frequency magnetic fields (ELFMFs) have become a considerable public concern. Several epidemiological studies have shown an association between exposure to ELFMF and elevated risk in children and occupationally exposed adults [Savitz and Loomis, 1995]. Whether exposure to magnetic fields causes significant cellular stress remains a contentious issue in in vitro studies.

Pancreatic islets play a fundamental role in regulating the blood glucose levels of the body through the secretion of hormones such as insulin, glucagon, somatostatin, and pancreatic polypeptide. The release of insufficient amounts of these hormones is the basis of various forms of diabetes. Therefore, it is important to assess the effects of exposure to ELFMF on pancreatic islet function.

Studies evaluating the influence of exposure to ELFMF on pancreatic islet function are scarce, and it is often difficult to compare the existing studies because of the different research methods used. Previously, Joelly et al. [1983] reported that calcium ion content, calcium ion efflux, and insulin secretion during glucose stimulation were reduced when isolated rabbit islets were exposed to low frequency pulsed magnetic fields. Hayek et al. [1984] reported that exposure to low intensity homogeneous magnetic fields inhibited insulin release from isolated newborn rat islets stimulated by high glucose concentration (16.7 mM) and aminophylline (10 mM). Recently, Laitl-Kobierska et al. [2002] reported that long term exposure of rats to ELFMF led to increased synthesis and secretion of

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insulin. Consequently, an association between magnetic field exposure and pancreatic islet function has not been demonstrated unequivocally.

One important approach to overcome the problems of using islets is the use of insulinoma cell lines. RIN-m cells are derived from X-ray radiation induced rat insulinoma [Chick et al., 1977; Gazdar et al., 1980] and have been used to investigate the mechanism of insulin secretion [Yada et al., 1989].

In this study, we have investigated the effects of exposure to ELFMF on insulin release by the insulinoma cell line, RIN-m, using our previously manufactured equipment to expose cultured cells to an ELFMF at 5 mT and 60 Hz.

MATERIALS AND METHODS

ELFMF Exposure Unit

ELFMF exposure, a sinusoidal magnetic field at a frequency of 60 Hz, 5 mT, was performed using a previously described magnetic field exposure apparatus [Miyakoshi et al., 1996; Ding et al., 2000]. The distribution of the magnetic density was measured using a Gauss meter (Model 3251, Yokogawa Electrical Co., Ltd., Tokyo). Briefly, the ELFMF exposure system consists of a magnetic field generator that uses Helmholtz coils built into a CO_2 incubator, a transformer and a thermocontroller. The direction of the field is vertical. The atmosphere in the incubator is maintained with humidified 95% air plus 5% CO_2. The temperature in the exposure space, which is monitored by thermocouple sensor probes, is maintained at 37 ± 0.2 °C. The measured 60 Hz ELFMF exposure during the sham exposure was <0.5 μT. Static magnetic fields other than geomagnetism were undetectable (<0.1 μT).

Cell Culture

RIN-m cells (obtained from the Dainippon Pharmaceutical Co., Osaka, Japan) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C in humidified 95% air plus 5% CO_2. For each experiment, a new vial of frozen cells was thawed, seeded at a density of 1×10^5 cells/cm^2 on 12- or 6-well cell culture plates and cultured. Cells were used from passage numbers 16–23.

Insulin Secretion Tests

Cells were plated on 12-well culture plates at a density of 3.5×10^5 cells/well. Insulin secretion tests were performed 4 days after plating, when the cells were 80–90% confluent. The culture medium was changed to fresh medium 16 h before the insulin secretion tests, and the tests were performed as shown in Figure 1.

On the day of the experiment, the medium was removed, and the cells were washed twice with 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)-buffered Krebs–Ringer solution (119 mM NaCl, 4.74 mM KCl, 2.54 mM $CaCl_2$, 1.19 mM $MgSO_4$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, and 10 mM HEPES at pH 7.4) containing 0.2% bovine serum albumin (BSA), and 3.3 mM D-glucose. The cells were then preincubated in the same buffer at 37 °C for 30 min. The buffer was then removed, and 0.8 ml of basal incubation buffer (HEPES-buffered Krebs–Ringer solution containing 0.2% BSA and 3.3 mM D-glucose) was added and the cells were incubated at 37 °C for 1 h. After the basal incubation period, wells were divided into two groups; one is stimulated under exposure to an ELFMF (Fig. 1, Group A), and the other is stimulated under sham exposure condition (Fig. 1, Group B).

The stimulation was performed as follows: the cells were incubated at 37 °C for 1 h in 0.8 ml of stimulation buffer, which consisted of HEPES buffered Krebs–Ringer solution containing 0.2% BSA, D-glucose, and various reagents, as indicated in the figure legends. At the end of the basal incubation period and stimulation period, an aliquot of the incubation buffer in each well was collected and stored at −20 °C until the insulin measurement was performed. The insulin concentration of the samples was measured by enzyme linked immunosorbent assay (ELISA) using a rat insulin ELISA kit (Sibayagi Co., Gunma, Japan). Insulin secretion from RIN-m cells stimulated various regents were analyzed using the ratio of insulin secretion during the stimulation period/insulin secretion during basal incubation period.

RNA Extraction

After insulin secretion tests were performed using 6-well culture plates, the cells were scraped and the total RNA was prepared using an ISOGEN isolation kit (Nippon Gene Co., Toyama, Japan). Briefly, the cells were homogenized in 1 ml of ISOGEN reagent, and 0.1 ml of chloroform was added to this mixture. The mixture was centrifuged at 15,000*g* for 15 min at 4 °C, and the resulting aqueous phase was transferred to 0.5 ml of isopropyl alcohol. The resulting precipitate was collected by centrifugation at 15,000*g* for 10 min at 4 °C, and the RNA pellet obtained was washed with 75% ethanol, and then dissolved in diethyl pyrocarbonate-treated water. The amount of RNA was measured using an ultraviolet-visible spectrometer at 260 nm, and the purity of the RNA obtained was determined from the absorbance ratio at 260/280 nm. The ratio at >1.8 was used following reverse transcriptase-polymerase chain reaction (RT-PCR).

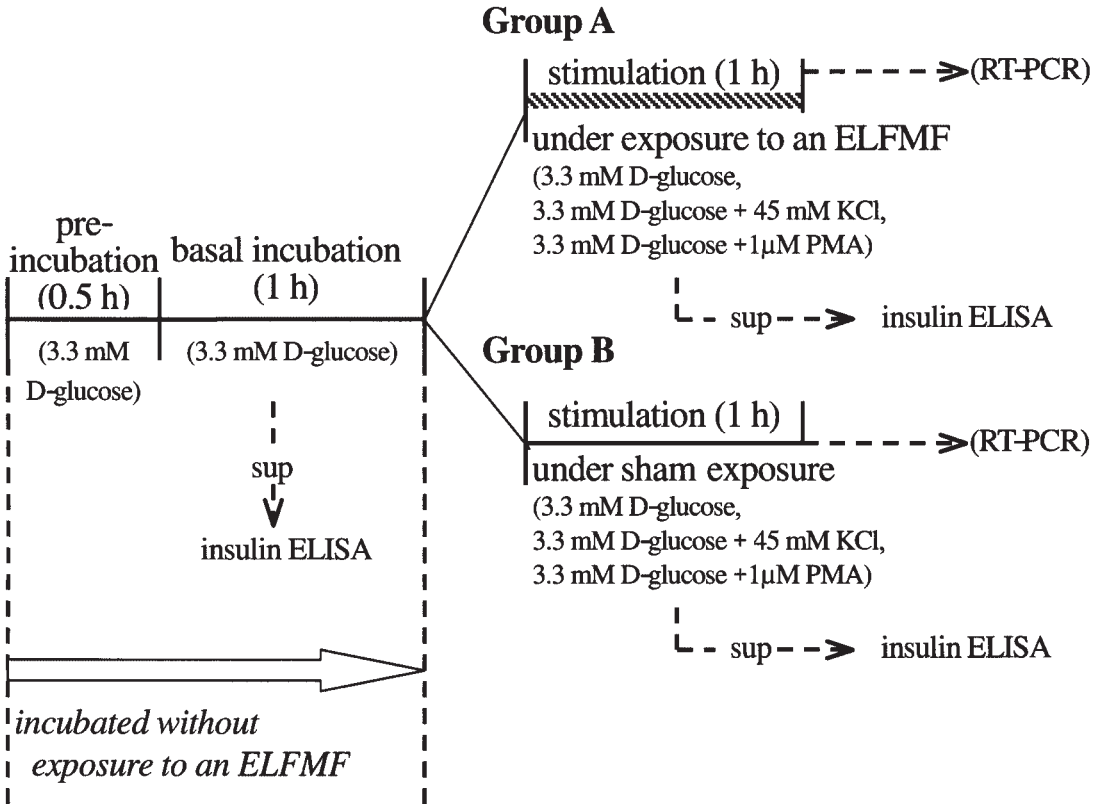

Fig. 1. The outline of insulin secretion tests. Preincubation and basal incubation were performed without exposure to an extremely low frequency magnetic field (ELFMF). After wells were divided into two groups, one group is stimulated under exposure to an ELFMF (**Group A**), and the other group is stimulated under sham exposure (**Group B**). At the end of the basal incubation period and stimulation period, an aliquot of the incubation buffer in each well was collected, and the insulin concentration of the samples was measured by enzyme linked immunosorbent assay (ELISA).

RT-PCR

cDNA synthesis was performed using a $(dT)_{20}$ oligo primer and ThermoScript RT (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. PCR was performed using Taq polymerase (TaKaRa Co, Shiga, Japan). PCR mixtures contained cDNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 400 μM dNTP, 2.5 U/50 μl of Taq polymerase, and 1 μM sense and antisense primers. The primers were as follows, insulin 1: forward 5′-ATGGCCCTGTGGATGCGCTT-3′, reverse 5′-TAGTTGCAGTAGTTCTCCAGCT-3′, insulin 2: forward 5′-ACAGTCGGAAACCATCAGCAA-3′, reverse 5′-GCTGGTGCAGCACTGATCCACG-3′, synaptosomal associated protein of 25 kDa (SNAP-25): forward 5′-GGTTCCTTAACTAAGCACCACTGACTT-3′, reverse 5′-TTTCCCGGGCATCGTTTGTTACC-3′, synaptotagmin 1: forward 5′-ATGGCTGTGTATGACTTTGATCGCT-3′, reverse 5′-GAAGACTTTGTCGATGGCGTCGTT-3′, β-actin: forward 5′-ATGGTGGGTATGGGTCAGAAGG-3′, reverse 5′-ACGCACGATTTCCCTCTCAGTC-3′. The reaction mixture was incubated in a thermal cycler at 95 °C for 45 s, at 59 °C for 45 s, and at 72 °C for 90 s. β-Actin expression was used to normalize the input template cDNA in a semi-quantitative PCR reaction. Serial half dilutions of cDNA were amplified to ensure analysis of products in the linear range of amplification.

Quantification of PCR Products

Each PCR product was analyzed on a 1.8% agarose gel with 0.1 μg/ml ethidium bromide, and the quantification of the PCR products was performed by densitometry of the band intensity using a Kodak Digital Science IS 440 CF System and 1D Image Analysis Software ver. 3.5 (Eastman Kodak Co., Rochester, NY).

Statistical Analysis

Statistical comparisons were performed by analysis of variance and, when appropriate, using the Student's *t*-test. Experimental results were presented as the mean ± SE, and studies were repeated at least three times independently.

RESULTS

Insulin Secretion From RIN-m Cells Under Sham Conditions

Under sham conditions, incubation with stimulation buffer containing a high concentration of potassium (45 mM KCl) caused insulin release to increase approximately 14 fold, compared to the basal incubation conditions. In contrast, both a normal (3.3 mM) and a high (16.7 mM) concentration of D-glucose did not increase insulin release, compared to the basal incubation conditions. Phorbol-12-myristate-13-acetate (PMA) also increased insulin release from RIN-m cells by approximately 15 fold (Table 1). These results are in agreement with previous reports describing RIN-m cells and their subcloned cell line, RINm5F [Praz et al., 1983; Bhathena et al., 1984; Yada et al., 1989].

Effects of ELFMF on Insulin Secretion

Insulin secretion from RIN-m cells in the stimulation buffer containing a normal concentration of D-glucose did not increase under exposure to an ELFMF, compared to the sham exposed cells. On the other hand, in the presence of 45 mM KCl, exposure to ELFMF significantly attenuated insulin release from RIN-m cells by approximately 30%, compared to sham exposed cells. When PMA was included in the stimulation buffer, insulin release was slightly, but not significantly, enhanced by exposure to ELFMF (Fig. 2). These results suggest that exposure to an ELFMF in the presence of chemical reagents affects insulin secretion, but that exposure to ELFMF alone is insufficient to stimulate insulin secretion. These findings are consistent with our previous reports showing that exposure to an ELFMF in combination with chemical reagents enhanced the effects of exposure to ELFMF on cells [Miyakoshi et al., 1998; Yaguchi et al., 2000].

Effects of Nifedipine Treatment on Insulin Secretion Induced by Potassium

Insulin release from RIN-m cells was assessed in the presence of 45 mM KCl after treatment with various concentrations of the calcium channel blocker, nifedipine. Under exposure to an ELFMF and under sham conditions, insulin release decreased in a dose-dependent manner. In the presence of low concentrations of nifedipine (5, 50, or 500 nM), insulin secretion was significantly attenuated under exposure to an ELFMF, compared to sham exposed cells. In contrast, there was no difference in insulin release between cells exposed to an ELFMF and those under sham exposure at high concentrations of nifedipine (5 or 50 μM). The amount of insulin secretion in the presence of 5 nM nifedipine under sham conditions was approximately equal to that in the absence of nifedipine under exposure to ELFMF (Fig. 3). These results suggest that ELFMF attenuated insulin secretion from RIN-m cells is related to an effect on calcium channels.

TABLE 1. Insulin Secretion From RIN-m Cells in Response to Secretagogues Under Sham Conditions

Secretagogue	Fold response, relative to basal incubation[a]
45 mM KC1	14.0 ± 0.15
3.3 mM D-glucose	1.0 ± 0.12
16.7 mM D-glucose	1.1 ± 0.15
1 μM phorbol-12-myristate-13-acetate (PMA)	15.4 ± 0.47

[a]Mean ± SE (n = 3).

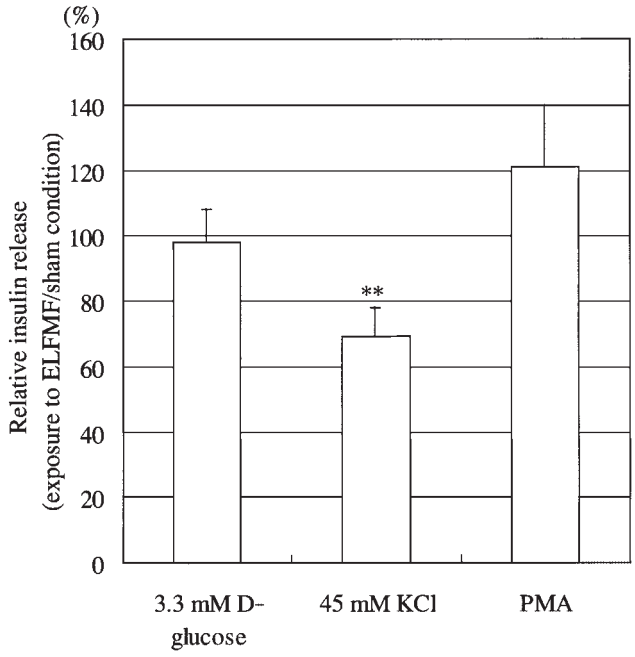

Fig. 2. Relative insulin release from RIN-m cells under exposure to an ELFMF or under sham conditions, measured after 1 h incubation with 3.3 mM D-glucose, or 3.3 mM D-glucose plus 45 mM KCl, or 3.3 mM D-glucose plus 1 μM phorbol-12-myristate-13-acetate (PMA). Data represent the mean ± SE (3.3 mM D-glucose, n = 3; 45 mM KCl and PMA, n = 5). $^{**}P < .01$.

Effects of an ELFMF on mRNA Expression Encoding Insulin 1, Insulin 2, Synaptotagmin 1, and SNAP-25

The effects of an ELFMF on mRNA expression in RIN-m cells were investigated using semi-quantitative RT-PCR. The fragments amplified for insulin 1, insulin 2, SNAP-25, synaptotagmin 1, and β-actin had the expected sizes (331, 333, 493, 456, and 500 base pairs

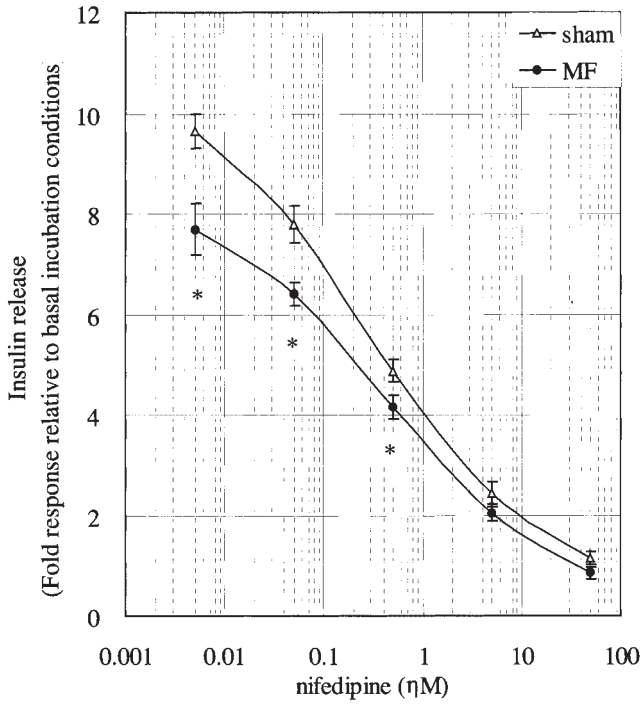

Fig. 3. Nifedipine inhibition of insulin release from RIN-m cells stimulated by 3.3 mM D-glucose and 45 mM KCl under exposure to an ELFMF or under sham conditions. To study the effect of nifedipine, cells were incubated with the indicated concentrations of nifedipine for 30 min, and then incubated for 1 h with stimulation buffer containing 3.3 mM D-glucose and 45 mM KCl. Each point represents the mean ± SE (50 μM nifedipine, n = 3; other concentrations, n = 4). $^{*}P < .05$.

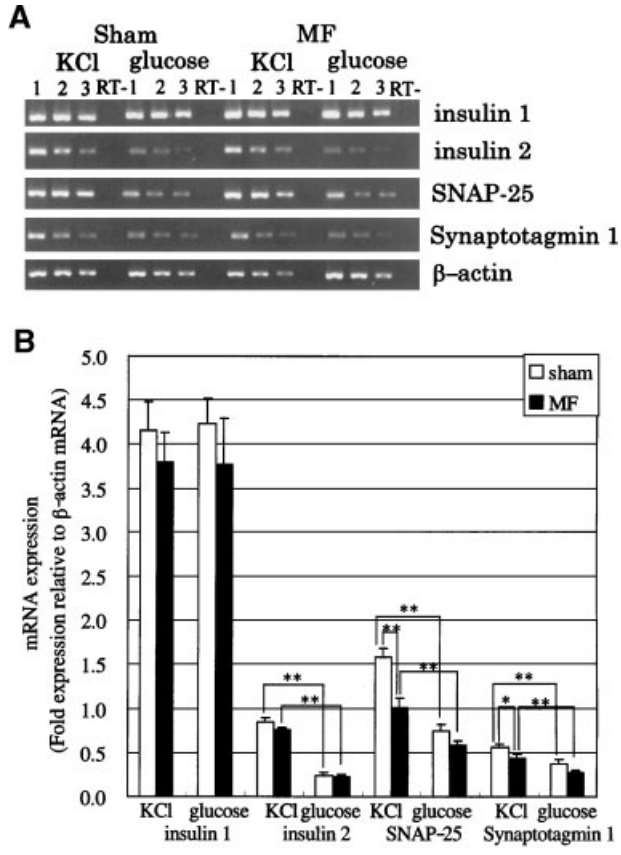

Fig. 4. **A**: Reverse transcription-polymerase chain reaction (RT-PCR) of insulin 1, insulin 2, synaptosomal associated protein of 25 kDa (SNAP-25), and synaptotagmin 1 after an insulin secretion test under exposure to an ELFMF or under sham conditions. Twofold serial dilutions of cDNA were amplified for 25 (insulin 1), 29 (insulin 2 and SNAP-25), 33 (synaptotagmin 1) or 26 (β-actin) cycles. **Lanes 1–3** represent serial dilutions of cDNA sample. Lane 1 was the most concentrated sample in each series. **B**: Semiquantitative RT-PCR analysis of the effects of exposure to an ELFMF on expression of mRNA encoding insulin 1, insulin 2, SNAP-25, and synaptotagmin 1. Insulin secretion tests were performed using stimulation buffer containing 3.3 mM D-glucose or 3.3 mM D-glucose and 45 mM KCl. Data represent the mean ± SE (insulin 1 and insulin 2, n = 3; SNAP-25, n = 5; synaptotagmin 1, n = 6). $^{**}P < .01$, $^{*}P < .05$.

(bp), respectively), and PCR products were undetectable when reverse transcription was performed without reverse transcriptase (ThermoScript RT). Semi-quantitative RT-PCR was performed under conditions where the amplification reaction for the PCR products was within the linear range. For example, the increase in the optical density of the amplified PCR products for β-actin was linear between at least cycles 24 and 29. A reduced intensity of products was observed at each dilution step (Fig. 4A). This result confirmed that the RT-PCR was performed in the exponential portion of the amplification curve.

In the presence of 45 mM KCl, exposure to an ELFMF significantly reduced expression of the mRNA encoding SNAP-25 and synaptotagmin 1 by approximately 36 and 23%, respectively, compared to sham exposed cells. Insulin 2 mRNA expression was slightly, but not significantly, reduced by exposure to ELFMF (approximately 10%), and insulin 1 mRNA expression did not decrease. Insulin 2, SNAP-25, and synaptotagmin 1 mRNA expression increased when RIN-m cells were stimulated by 45 mM KCl, compared to 3.3 mM D-glucose stimulation (Fig. 4B).

DISCUSSION

Recently, human exposure to ELFMFs from various electrical appliances has increased significantly. Thus, the possible health effects of exposure to ELFMF have become a considerable public concern. On the other hand, occurrence of diabetes mellitus has increased progressively in recent years. Insufficient pancreatic islet function is the basis of various forms of diabetes. These two factors motivated us to investigate the effects of exposure to ELFMF on the function of insulin secreting cells. If exposure to ELFMF is deleterious to insulin secreting cells, it is essential that this is

demonstrated and communicated as soon as possible. In contrast, if exposure to ELFMF is beneficial to insulin secreting cells, it might be possible to utilize exposure to ELFMF for medical applications. For example, an ELFMF might be used in diabetes mellitus to decrease blood glucose levels, and to increase insulin levels in blood [Laitl-Kobierska et al., 2002]. Because of these issues, the assessment of the effects of exposure to ELFMF on insulin secretion is very important.

Studies evaluating the influence of exposure to ELFMF on pancreatic islet function are scarce, and it is often difficult to compare the studies that have been performed because of the different research methods used. Hence, an association between magnetic field exposure and pancreatic islet function has not been demonstrated unequivocally. Furthermore, the use of islets results in significant methodological difficulties, due to cellular heterogeneity, limited availability, and rapid deterioration of function. In order to circumvent these problems in the current study, we used insulinoma cells instead of islets. To our knowledge, this is the first investigation in which insulinoma cells have been used to examine the effects of exposure to ELFMF on insulin secreting cells. The advantages of using insulinoma cells were the ease of generation of large quantities of functional cells, and the stability of the resulting cell population.

We investigated the insulinoma cell line, RIN-m. It has been reported that PMA, which acts via the PKC pathway, induces insulin secretion [Yada et al., 1989]. On the basis of the insulin secretory response of RIN-m cells to 45 mM KCl and PMA, we thought that RIN-m cells were suitable for a model to evaluate the effects of exposure to ELFMF on the potassium induced insulin secretion pathway and the PKC cascade. A high concentration of D-glucose induces the closure of ATP dependent potassium channels in insulin secreting cells, which in turn induces membrane depolarization, opening of voltage dependent calcium channels, and insulin secretion. It has been reported that treatment with high concentrations of potassium bypasses the ATP dependent potassium channels of insulin secreting cells and also induces insulin secretion [Hohmeier et al., 2000].

In the current study, insulin secretion induced by 45 mM KCl was attenuated by approximately 30% under exposure to an ELFMF, compared to sham exposure. Treatment with nifedipine reduced the difference in insulin secretion between ELFMF exposed and sham exposed cells. Recent investigations have clarified the molecular mechanisms of exocytosis in neurotransmitter release or hormone secretion [Jones and Persaud, 1998; Gerber and Sudhof, 2002]. In the exocytotic release process, secretory granules are docked at the site of exocytosis and fused with the plasma membrane. SNARE proteins play a role in this tethering/docking process between secretory granules and the plasma membrane. SNAP-25 is a SNARE protein that is expressed in pancreatic islets and is involved in insulin release [Sadoul et al., 1995; Wheeler et al., 1996]. SNAP-25 mRNA expression was significantly increased when chromaffin cells [Garcia-Palomero et al., 2000; Montiel et al., 2003] and rat granulosa cells [Grosse et al., 2000] were stimulated to release neurotransmitters, and these events were closely related to the influx of calcium ions.

Synaptotagmin 1 is a calcium ion sensor protein that is located on the membrane of insulin containing secretory granules [Lang et al., 1997]. It is thought to be play a role in the fusion between secretory granules and the plasma membrane according to the elevation of the cellular calcium concentration [Gerber and Sudhof, 2002].

In this work, the expression of mRNA encoding SNAP-25 and synaptotagmin 1 decreased under exposure to an ELFMF in the presence of 45 mM KCl. From these findings, we conclude that exposure to ELFMF attenuates insulin secretion by reducing the influx of calcium ions through calcium channels. It has been reported that calcium ion content, calcium ion efflux, and insulin secretion during glucose stimulation was reduced when isolated rabbit islets were exposed to low frequency pulsed magnetic fields [Joelly et al., 1983]. It has also been reported that exposure to ELFMF affected neurite growth via voltage gated calcium channels [Morgado-Valle et al., 1998] and affected the differentiation of neuroblastoma cells by antagonizing the shift in cell membrane surface charges and increasing intracellular calcium levels [Tonini et al., 2001]. Our present findings are in agreement with these previous reports.

In the current study, exposure to an ELFMF slightly increased PMA stimulated insulin secretion, but no statistically significant difference was observed at 5 mT and 60 Hz. We have previously reported that exposure to ELFMF at 50 Hz and 400 mT, but not at 5 mT, enhanced the expression of a neuron derived orphan receptor gene induced by treatment with forskolin and PMA [Miyakoshi et al., 1998]. We also reported that the suppression of heat shock protein 70 was observed at a magnetic density of 50 mT, but not at 5 or 0.5 mT [Miyakoshi et al., 2000]. These previous results indicate the dependence of the effects of exposure to ELFMF on the strength of the magnetic field, and they are consistent with the results of the current study.

The results presented here suggest that insulin secretion decreases under exposure to ELFMF. Hence, it might be desirable for diabetic patients who have

insufficient insulin secretion from pancreatic islets to avoid exposure to an ELFMF.

In summary, we investigated the effects of exposure to ELFMF on insulin secretion using the insulinoma cell line, RIN-m. In the presence of 45 mM KCl, exposure to ELFMF significantly attenuated insulin release from RIN-m cells, compared to sham exposed cells. Treatment with nifedipine reduced the difference in insulin secretion between ELFMF exposed and sham exposed cells, and the expression of mRNA encoding SNAP-25 and synaptotagmin 1 decreased under exposure to an ELFMF in the presence of 45 mM KCl. SNAP-25 is important in tethering and docking between secretory granules and plasma membranes and these events are closely related to the influx of calcium ions. Synaptotagmin 1 is a calcium ion sensor protein. These results suggest that exposure to ELFMF attenuates insulin secretion from RIN-m cells by affecting calcium influx through calcium channels.

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