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Magnetic fields (MF) of 50 Hz at 1.2 μT as well as 100 μT cause uncoupling of inhibitory pathways of adenylyl cyclase mediated by melatonin 1a receptor in MF-sensitive MCF-7 cells

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Magnetic fields (MF) of 60 Hz at 1.2 μT were previously shown to inhibit the antiproliferative effect of melatonin on MCF-7 cells (Liburdy,R.P., 1993, *J. Pimeal Res.* 14, 89–97). In addition, three laboratories (Blackman,C.F. and Benane,S.G., 1998; Luben,R.A. and Morgan,A.P., 1998; Morris,J.E., Chrisler,W.B., Miller,D.L., Sasser,L.B. and Anderson,L.E., 1998; 20th Annual Meeting of the Bioelectromagnetics Society, At. Pete Beach, FL) independently reported results consistent with this finding. In this study, we investigated the molecular basis of the biological effects of MF using MCF-7 cells. Only 1a melatonin receptors were identified by the [^{125}I]melatonin binding assay and RT–PCR analysis. Moreover, preceding exposures to MF of 100 μT for 3, 5 and 7 days blocked the melatonin-induced inhibition of cAMP accumulation in a time-dependent manner, while none of the melatonin receptor functions or GTPase and adenylyl cyclase activities were affected. Estrogen-evoked cell proliferation was not altered by MF either. Exposure to 1.2 μT MF exerted the same effects on the melatonin-signaling pathway as that to 100 μT. Thus, this is the first study to provide evidence that MF may cause uncoupling of signal transduction from melatonin receptors to adenylyl cyclase.

Introduction

Melatonin, a hormone produced primarily by the pineal gland, has been suggested to have antitumor activities as well as a role in the regulation of circadian and seasonal rhythms (5–9). Evidence has accumulated which indicates that melatonin, at least when administrated exogenously, may suppress estrogen synthesis, possibly through reducing luteinizing hormone/follicle stimulating hormone (LH/FSH) secretion (10), interfere with the binding of estrogen to its receptors expressed in breast cancer cells *in vitro* (8,11), ameliorate immune functions (12) and act as a scavenger of free radicals (13). These findings suggest that elevated endogenous melatonin can exert oncostatic action via immunomodulation and/or alterations of reproductive hormones or free radicals.

Wertheimer and Leeper (14) reported a nearly 3-fold increase of breast cancer risk among women under 55 years of age who lived near power lines, suggesting that MF exposure had accelerated development and growth of breast cancer. Furthermore, an increased risk of breast cancer was reported in both women and men (15–19).

It is not known which organs are primarily involved in sensing an MF and changes in an MF. Electrophysiological studies have shown that some of the intrinsic cells of the pineal gland may be affected by an earth-strength MF and that these cells respond with a depression of their electrical activity (20). Concerning the mechanism of 'magnetosensitivity', one can assume that an MF has a direct effect upon the pineal gland because of the electric current induced inside the body (21).

Stevens (22) hypothesized that MF can affect pineal gland melatonin secretion *in vivo*, which, in turn, can influence mammary (breast) carcinogenesis. Since then, a number of experimental studies have been conducted in order to test this hypothesis. Kato *et al*. (23) reported that exposure of albino (Wistar-King) rats for 6 weeks to 50 Hz MF at 1 μT suppressed melatonin concentrations, both during the day and night, in both the plasma and pineal gland. Olcese and Reuss (24) investigated effects of combined 60 Hz vertical electric field and 60 Hz horizontal MF exposure for 6 weeks on non-human primates and found no signs of a reduction in serum melatonin concentrations in a series of three experiments. However, in another small experiment using a different exposure paradigm, they reported nearly complete suppression of the normal nocturnal rise in serum melatonin concentrations, indicating that different animal species respond differentially to different parameters of time-varying magnetic fields (25).

In vitro studies by Hill and Blask (8) and Hill *et al*. (11) demonstrated that melatonin at physiological levels inhibits MCF-7 human breast cancer cell growth. Using MCF-7 cells obtained from Dr D.E.Blask (the Marry Imogene Bassett Hospital Research Institute, NY), Liburdy (1) reported that MF inhibited the antiproliferlative effects of the hormone, allowing the cancer cells to grow in the presence of melatonin. Furthermore, Liburdy (26) revealed the first plausible biological mechanism linking MF exposure to calcium signaling, a fundamental cell process governing many important cellular functions. However, the 'Ca^{2+}' theory is now subject to debate (27,28).

There are many MCF-7 subclones that respond to different degrees of MF as well as to estrogen and melatonin (29). For example, the results of Liburdy (1), Blackman and Benane (2), Lubane and Morgan (3) and Morris *et al*. (4) have not been reproduced with the MCF-7 cells supplied by the American Type Culture Collection (Rockville, MD). Thus, the MCF-7 cells that have 'magnetosensitivity' (designated 'MF-sensitive MCF-7 cells' in this study) are useful in elucidating the molecular basis of the biological effects of MF.

Therefore, we used MF-sensitive MCF-7 cells provided by Dr Liburdy (UCLA, Berkley) in order to reveal the molecular mechanism of the biological effects of MF and found that

Abbreviations: ABTS, 2,2′-azino-di-(3-ethylbenzthiazolinesulfonate); ATCC, American Type Culture Collection; BrdU, bromodeoxyuridine; ELISA, enzyme-linked immunosorbent assay; ERα, estrogen receptor α; IBMX, 3-isobutyl-1-methylxanthine; LH/FSH, luteinizing hormone/follicle stimulating hormone; MF, magnetic fields; NAT, *N*-acetyltransferase; RT–PCR, reverse transcriptase–polymerase chain reaction.

MF exposure causes the uncoupling of the melatonin signal transduction pathway.

Materials and methods

Cell culture and MF exposure

MCF-7 cells were kindly provided by Dr R.P.Liburdy (UCLA, Berkley) and grown in Dulbecco's modified Eagle's medium (Gibco BRL, Rockville, MD) supplemented with 10% FBS (Gibco BRL), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 95% air:5% CO_2 at 37°C. For examination of effects of 17β-estradiol on cell growth, phenol red-free medium (Sigma, St Louis, MO) was used and supplemented with 10% dextran-coated charchoal-stripped FBS and 10 ng/ml epidermal growth factor (Peptide Institute, Osaka, Japan). Cells were subcultured (1:4) one to two times per week. In order to expose the cells to magnetic fields, a 50 Hz sinusoidal MF was generated in a mu-metal chamber with four Merritt-coil devices (30). The mu-metal chamber was a cube that was constructed of nickel (80%) and trace metals. The chamber had four ventilation holes (2.54 cm in diameter) on the top and bottom. A temperature probe was placed inside the chamber to monitor temperature continuously. The anti-parallel mode of operation generated opposing magnetic fields that cancelled each other out and resulted in a true sham exposure (30). When a current was applied to the parallel configuration, a magnetic field was established. Two identical exposure systems were employed in this study. Each coil system was driven by identical signal generators obtained from NF Electronic Instruments (Yokohama, Japan). Cell viability was determined by means of the crystal violet staining method.

Bromodeoxyuridine (BrdU) incorporation

DNA synthesis was determined via BrdU incorporation as previously described (31). MCF-7 cells were grown in a 96-well plate (Coaster, Cambridge, MA). The cells were exposed to no MF (active sham) or MF (1.2 or 100 μT) for 1 week at 37°C. Then 10 μM BrdU was added for a further 3 h at 37°C. The cells were fixed with 70% ethanol in 0.5 M HCl for 30 min at –20°C. Following treatment with nuclease, the cells were incubated with anti-BrdU antibody conjugated to peroxidase (Boehringer-Mannheim, Germany). Bound enzymes were detected with the substrate ABTS® [2,2′-azino-di-(3-ethylbenzthiazolinesulfonate)] (Boehringer) and quantified by measuring absorbance at 405 nm with an enzyme-linked immunosorbent assay (ELISA) plate reader (BioRad, Hercules, CA).

Binding assay

A hormone binding assay was carried out as described previously (32). MCF-7 cells were plated in 24-well plates to a density of 0.75×10^5 cells per well and exposed for 1 week to no MF or 100 μT MF. The cells were then incubated with [^{125}I]melatonin (2200 Ci/mmol, NEN-Dupont, Boston, MA) for 2 h at 37°C in the presence of luzindole (Tocris Cookson, St Louis, MO) or GR135531 (Tocris Cookson). After free radioligands were removed by washing, [^{125}I]melatonin bound to the cells was solubilized with 1 ml 0.5 N NaOH and counted.

Reverse transcriptase–polymerase chain reaction (RT–PCR)

Total cellular RNA was isolated from control and MF-treated cells (1.2–100 μT; 1 week) by single-step guanidinium thiocyanate/phenol/chloroform extraction (33). The First-strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Gibco BRL) with random heximer primers. The PCR reaction mixture contained first-strand DNA, 0.25 mM dNTP, 100 pmol specific primers and *Pfu* polymerase (Stratagene, La Jolla, CA). Thirty-five cycles of PCR were carried out in a Thermal Controller (model PJ 2000; Perkin-Elmer) at 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min, ending with 5 min at 72°C. The primers were as follows: Mel_{1a} receptor, 5′-TCCTGGTCATCCTGTCGGTGTATC-3′ and 5′-CTGCTGTACAGTTTGTCGTACTTG-3′, amplified product of 285 bp; Mel_{1b} receptor, 5′-TCCTGGTGATCCTCTCCGTGCTCA-3′and 5′-AGCCAGATGAGGCAGATGTGCAGA-3′, amplified product of 321 bp; estrogen receptor α, 5′-TGCCAAGGAGACTCGCTA-3′ and 5′-TCAACATTCTCCCTCCTC-3′, amplified product of 263 bp; estrogen receptor β, 5′-TGTTACGAAGTGGGAATGTGA-3′ and 5′-TCTTGTTCTGGACAGGGATG-3′, amplified product of 472 bp. The PCR products were separated by electrophoresis on a 1.2% agarose gel.

Cell-free preparation and enzyme assay

MCF-7 cells were harvested and homogenized in 20 mM Na–HEPES, pH 7.8, 27% (w/v) sucrose, 1 mM EDTA and 20 mM $MgCl_2$. Cell homogenates were centrifuged at 15 000 r.p.m. for 10 min, and the resultant pellets were resuspended in 20 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA and 1 mM dithiothreitol (DTT) and used as the plasma membrane fraction (34).

GTPase activity was measured based on ^{32}Pi liberation from [γ-^{32}P]GTP as previously described (35). Membrane fractions (50 μg protein) were incubated at 37°C for 5 min in a 100 μl reaction mixture containing 50 mM Tris–HCl pH 7.5, 1 μm [γ-^{32}P]GTP (30 Ci/mmol; NEN), 0.5 mM adenyl-5-(β,γ-imino) triphosphate, 0.1 mM ATP, 5 mM $MgCl_2$, 5 mM phosphocreatine, 50 U/ml creatine phosphokinase, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 mM EGTA, 0.2 mM DTT and 1 mg/ml bovine serum albumin. The reaction was terminated by the addition of 650 μl of a 5% suspension of charcoal (Sigma). After centrifugation at 15 000 r.p.m. for 10 min, the supernatants (500 μl) were counted.

Adenylyl cyclase activity was assayed by measuring the production of cAMP from ATP as previously described (35). Membrane fractions (100 μg protein) were incubated at 37°C for 5 min in a 100 μl reaction mixture containing 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 5 mM phosphocreatine, 50 U/ml creatine phosphokinase, 0.5 mM IBMX, 0.2 mM DTT, 1 mg/ml bovine serum albumin and 0.5 mM ATP. The cAMP synthesized was quantified with radioimmunoassay kits (Yamasa Shoyu, Chiba, Japan).

ADP ribosylation

For ADP ribosylation with pertussis toxin, plasma membrane fractions were prepared as described above. ADP ribosylation was carried out in a final volume of 50 μl containing 75 μg membranes, 50 mM Tris–HCl pH 7.5, 1 mM EDTA, 10 mM thymidine, 0.15 mM ATP, 0.1 mM $GDP_\beta S$, [^{32}P]NAD^+ (800 Ci/mmol; NEN), and activated pertussis toxin (Calbiochem, La Jolla, CA) (36). Pertussis toxin activation was at 100 μg/ml in 25 mM Tris–HCl, pH 8.0, 10 mM DTT and 1 mM ATP for 20 min at 30°C. After 30 min of incubation, the samples were centrifuged at 15 000 r.p.m. for 10 min. The resulting precipitates were suspended in 15 μl Laemmli's sample buffer containing 40 mM DTT and analyzed by SDS–PAGE (37) and autoradiography.

Measurement of cAMP accumulation

cAMP accumulation was determined as previously described (34). MCF-7 cells were exposed to 0, 1.2 or 100 μT MF for the indicated number of days. Then the cells were incubated with 100 μM IBMX for 15 min at 37°C. Forskolin (10 μM) was added in the presence of various concentrations of melatonin as indicated for 15 min at 37°C. The incubations were terminated by removal of the medium and addition of 6% trichloro acetic acid. The accumulated cAMP was quantified by radioimmunoassay.

Statistics

Statistical analyses were carried out via Student's *t*-test using the StatView Ver. 5.0 statistical software package (SAS Institute, Cary, NC).

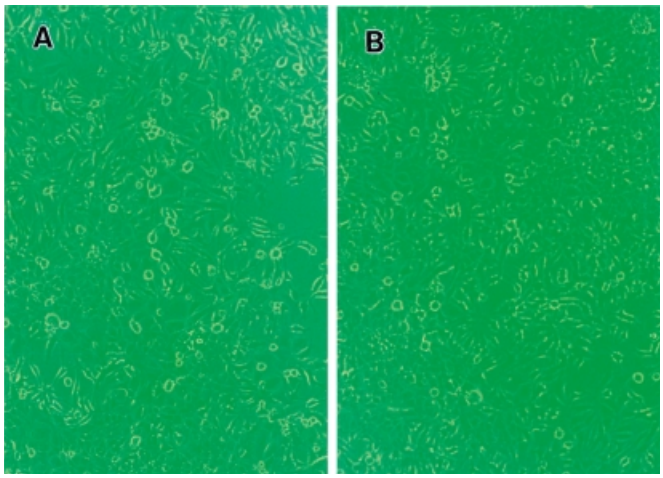

Fig. 1. Phase-contrast micrographs of control MCF-7 cells (**A**) and MCF-7 cells exposed to 100 μT for 1 week (**B**). Original magnification: ×200.

Results

A 50 Hz sinusoidal MF was generated in a mu-metal chamber with four Merritt coils. There was no difference in morphology between control MCF-7 cells (Figure 1A) and cells exposed to 100 μT MF for 1 week in the presence of 10% FBS (Figure 1B). The growth inhibitory effect of melatonin on MCF-7 cells was confirmed by BrdU incorporation (Figure 2A). The cells were exposed to active sham or 100 μT MF for 1 week and then labeled with BrdU. The maximum inhibition caused by 10^{-11}–10^{-9} M melatonin was 20–25% of the control

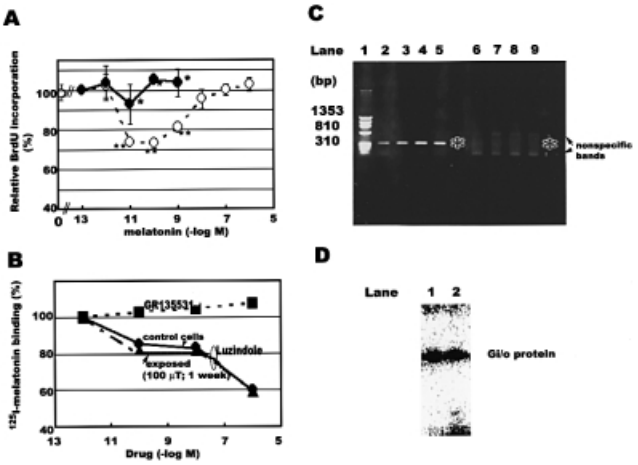

Fig. 2. (**A**) BrdU incorporation into MF-sensitive MCF-7 cells. MCF-7 cells (1×10^4) were exposed to active sham as a control (○) or to 100 μT MF (●) for 1 week in the presence of various concentrations of melatonin as indicated. Following incubation with 10 μM BrdU for another 3 h at 37°C, the incorporated BrdU was quantified by ELISA. The results are presented as a percentage of the value obtained with zero melatonin. The absorbance reflecting BrdU incorporation at zero melatonin and 10^{-11} M melatonin was 0.414 ± 0.018 and 0.311 ± 0.007 (mean ± SE), respectively. Asterisks denote significant differences between control cells and MF-exposed cells in each test (mean ± SE) ($P < 0.05$). Double asterisks denote a significant reduction by melatonin of BrdU incorporation in control cells ($P < 0.05$). (**B**) Binding of [^{125}I]melatonin to MF-sensitive MCF-7 cells. MCF-7 cells, plated in 24-well plates to a density of 0.75×10^5 cells per well, were exposed to 0 (● and ■) or 100 μT MF (▲) for 1 week . The cells were then incubated with [^{125}I]melatonin (2200 Ci/mmol) for 2 h at 37°C in the presence of luzindole (● and ▲) or GR135531 (■). After removing free radioligands by washing, [^{125}I]melatonin bound to the cells was solubilized and counted. (**C**) RT–PCR analysis of the subtype of melatonin receptor expressed in MF-sensitive MCF-7 cells. Total cellular RNA was isolated from control and MF-treated cells (1.2–100 μT; 1 week). First-strand DNA was synthesized using Moloney murine leukemia virus reverse transcriptase with random heximer primers. Thirty-five cycles of PCR were carried out in a Thermal Controller at 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min, ending with 5 min at 72°C. The Mel_{1a} receptor primers were 5′-TCCTGGTCATCCTGTCGGTGTATC-3′ and 5′-CTGCTGTACAGTTT-GTCGTACTTG-3′, and they amplified a band of 285 bp (lanes 2–5). The Mel_{1b} receptor primers were 5′-TCCTGGTGATCCTCTCCGTGCTCA-3′and 5′-AGCCAGATGAGGCAGATGTGCAGA-3′ and they amplified a band of 321 bp (lanes 6–9). The PCR products were separated by electrophoresis on a 1.2% agarose gel: control (lanes 2 and 6), active sham (lanes 3 and 7), 1.2 μT (lanes 4 and 8) and 100 μT (lanes 5 and 9). The expected positions of PCR products are marked by asterisks. (**D**) Autoradiogram of homogenates from control or MF-exposed (100 μT; 1 week) MCF-7 cells labeled with pertussis toxin and [^{32}P]NAD^+. Homogenates (30 μg protein) prepared from control or MF-treated (100 μT; 1 week) MCF-7 cells were labeled with [^{32}P]NAD^+ in the presence of activated pertussis toxin, electrophoresed and autoradiographed. The band migrating at an M_r of ~40 000 represents the [^{32}P]ADP-ribosylated α-subunits of pertussis toxin-sensitive G proteins.

(zero melatonin) ($P < 0.05$). This inhibition was blocked by 100 μT MF at about 0–15% of the control ($P < 0.05$), suggesting that MF has a biological effect on the signal transduction pathway of the hormone. These results were consistent with previous reports (1–4).

To investigate the signal transduction pathway of melatonin in MF-sensitive MCF-7 cells, we carried out [^{125}I]melatonin binding (Figure 2B) and reverse transcriptase–polymerase chain reaction (RT–PCR) analysis (Figure 2C). As shown in Figure 2B, [^{125}I]melatonin binding competed with melatonin type 1 receptor antagonist, luzindole, but not with the type 2 receptor analogue, GR135531. [^{125}I]Melatonin binding was not changed by exposure to MF (Figure 2B). The melatonin 1a receptor was successfully amplified from cDNA (Figure 2C, lanes 2–5) whereas the melatonin 1b receptor was not (Figure 2C, lanes 6–9). Amplification levels of melatonin 1a receptor, as determined by PCR, were not significantly changed by 1.2 (lane 4) or 100 μT (lane 5) MF. Thus, these data indicate that the major melatonin receptor expressed in an MF-sensitive MCF-7 cell is the melatonin 1a receptor and that cells exposed to MF (100 μT; 1 week) did not significantly change in terms of [^{125}I]melatonin binding or expression of the melatonin 1a receptor.

Table I. Effects of MF (100 μT; 1 week) on GTPase and adenylyl cyclase activities in MF-sensitive MCF-7 cells

Cells	Enzyme activity	
	GTPase (Pi pmol/min/mg protein)	Adenylyl cyclase (cAMP pmol/ min/mg protein)
Control cells ($n = 4$)	49.03 ± 1.71	39.25 ± 6.69
Exposed cells ($n = 4$)[a]	56.63 ± 2.88	46.25 ± 8.68

[a]Exposed cells: 100 μT for 1 week.
Values are mean ± SE of four determinants.

Since the cloned melatonin 1a receptor was shown to be coupled to the inhibitory G protein (38), we performed ADP ribosylation with pertussis toxin to identify the sensitive G protein in MF-sensitive MCF-7 cells. Figure 2D shows that both control cells and cells exposed to MF (100 μT; 1 week) were equally labeled with activated pertussis toxin and [^{32}P]NAD^+, indicating that the cysteine residue of the CAAX box of G protein was not modified by MF exposure. We also determined the activities of GTPase and adenylyl cyclase to examine whether or not exposure to MF influences their activities. Homogenates were prepared from control or MF-treated (100 μT; 1 week) MCF-7 cells. Membrane fractions of the homogenates were assayed for GTPase and adenylyl cyclase activities. Table I shows that GTPase activity was not significantly decreased by MF exposure (control cells, 49.03 Pi mol/min/mg protein versus MF-exposed cells, 56.63 Pi pmol/ min/mg protein) ($P < 0.01$). Adenylyl cyclase activity was also not significantly decreased by MF exposure (control cells, 39.25 pmol cAMP/min/mg protein versus MF-exposed cells, 46.25 pmol cAMP/min/mg protein) ($P < 0.05$).

We investigated whether or not MF exerts an effect on the coupling of the inhibitory pathway of adenylyl cyclase mediated by the melatonin 1a receptor. As shown in Figure 3A, forskolin-stimulated cAMP accumulation was inhibited by melatonin in a dose-dependent manner. The percentage inhibition by 1 μM melatonin was 41.9%. ID_{50} was about 1 nM. Surprisingly, for cells exposed to 100 μT MF for 3 days, the percentage inhibition by melatonin of forskolin-stimulated cAMP accumulation was significantly decreased to 27% ($P < 0.005$); a longer period of exposure (7 days) of cells to MF (100 μT) completely disrupted the inhibitory activity of melatonin ($P < 0.005$). Figure 3B shows the rate of uncoupling by 1 μM melatonin as a function of MF exposure time. There was a linear correlation ($r^2 = 0.94$).

We further examined the effects of low exposure to MF on cell growth and the melatonin-signaling pathway. Cells were incubated in the presence or absence of 10^{-11} M melatonin under 0 or 1.2 μT MF for 1 week. Then the cells were labeled with BrdU for an additional 3 h at 37°C, followed by quantification of incorporated BrdU with ELISA

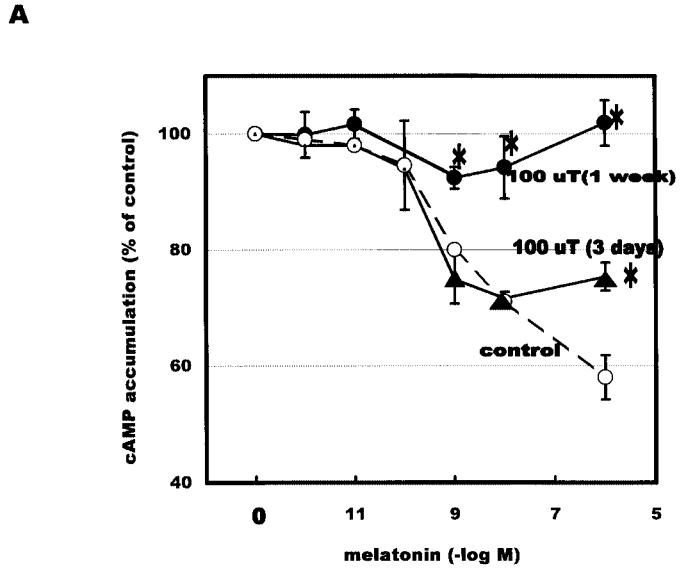

Fig. 3. Uncoupling by MF-exposure of melatonin-mediated inhibitory pathway of adenylyl cyclase. (**A**) MCF-7 cells exposed to 0 (control; ○) or 100 μT MF for 3 (▲) or 7 (●) days. Followed by incubation with 100 μM IBMX for 15 min at 37°C, 10 μM forskolin was added in the presence of various concentrations of melatonin as indicated and incubated for 15 min at 37°C. Accumulated cAMP was extracted with 6% trichloroacetic acid and quantified by radioimmunoassay. The results are expressed as the percentage of values obtained for control cells. Asterisks denote significant differences between control cells and MF-exposed cells in each test (mean ± SE; $P < 0.005$). (**B**) The rate of uncoupling by 1 μM melatonin of forskolin-stimulated cAMP accumulation was calculated using the following formula: Uncoupling (%) = $(A - B)/A \times 100$ where A = percentage inhibition by 1 μM melatonin of forskolin-stimulated cAMP accumulation in control cells, whose value was 41.9%; and B = percentage inhibition by 1 μM melatonin of forskolin-stimulated cAMP accumulation in MF-exposed (100 μT) cells.

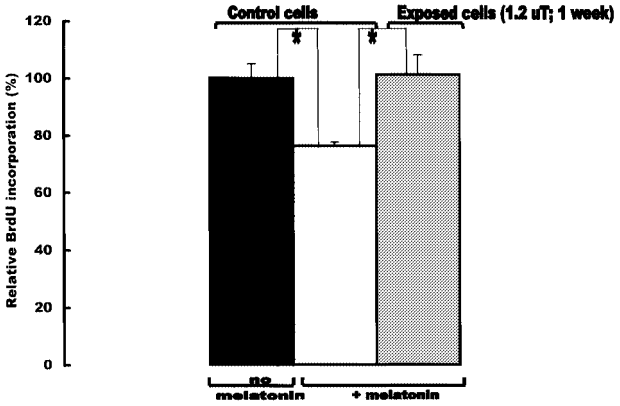

Fig. 4. Inhibition by MF (1.2 μT; 1 week) of antiproliferative effects of melatonin in MF-sensitive MCF-7 cells. MCF-7 cells (1×10^4) were exposed to active sham as a control (□) or to 1.2 μT MF (grey shading) for 1 week in the presence of 10^{-11} M melatonin. Following incubation with 10 μM BrdU for another 3 h at 37°C, the incorporated BrdU was quantified by ELISA. ■, Cells exposed to active sham in the absence of melatonin. Asterisks denote significant differences in BrdU incorporation (mean ± SE; $P < 0.05$).

Table II. Uncoupling of melatonin-mediated inhibitory pathway of adenylyl cyclase by exposure to MF (1, 2 μT; 1 week)

Cells	Inhibition of cAMP accumulation by 1 μM melatonin (%)
Control cells	46.73 ± 0.60
Exposed cells[a]	31.31 ± 1.52*

[a]Exposed cells: 1.2 μT; 1 week.
Value are mean ± SE of four determinants.
*Significantly different from control cells ($P < 0.05$).

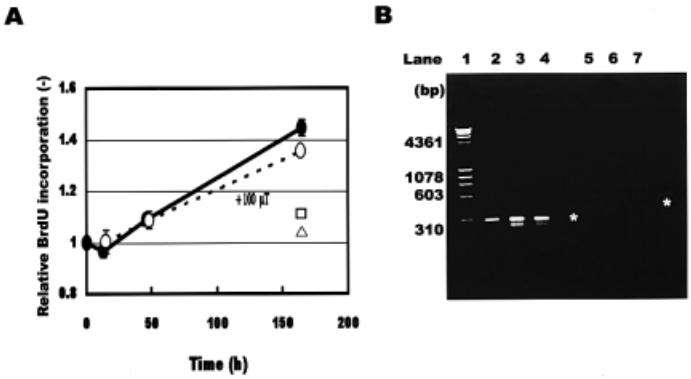

Fig. 5. (**A**) BrdU incorporation into MF-sensitive MCF-7 cells. MCF-7 cells (1×10^4) were exposed to active sham as a control (●) or 100 μT MF (○) in the presence of 10^{-7} M 17β-estradiol for the indicated periods. Following labeling with BrdU, the incorporated BrdU was quantified by ELISA. □, Cells grown in the absence of 17β-estradiol under active sham; △, cells exposed to 100 μT MF in the absence of 17β-estradiol. There was no significant difference between the two kinetics of estrogen-treated cells ($P < 0.05$). (**B**) RT–PCR analysis of estrogen receptor subtypes expressed in MF-sensitive MCF-7 cells. Total cellular RNA was isolated from control and MF-treated cells (1.2–100 μT; 1 week). Thirty-five cycles of RT–PCR were carried out in a Thermal Controller at 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min, ending with 5 min at 72°C. The primers used for amplification are as follows: ERα, 5′-TGCCAAGGAGACTCGCTA-3′ and 5′-TCAACATTCTCCCTCCTC-3′, giving an amplified product of 263 bp (lanes 2–4); ERβ, 5′-TGTTACGAAGTGGGAATGTGA-3′ and 5′-TCTTGTTCTGGACAGGGATG-3′, giving an amplified product of 472 bp (lanes 5–7). The PCR products were separated by electrophoresis on a 1.2% agarose gel: 0 (lanes 2 and 5), 1.2 (lanes 3 and 6) and 100 μT (lanes 4 and 7). The expected positions of PCR products are marked with asterisks.

(Figure 4). Melatonin inhibited BrdU incorporation into the cells (20–25%) under no MF. However, it was blocked by 1.2 μT MF, as previously reported (1).

We also examined if a lower exposure to MF causes the uncoupling of the inhibitory pathway of adenylyl cyclase mediated through melatonin receptors, as seen in the case of exposure to 100 μT MF (Table II). Control cells and cells exposed to 1.2 μT MF for 1 week were incubated with forskolin in the presence of 1 μM melatonin for 15 min at 37°C. Then accumulated cAMP was determined. The percentage inhibition by 1 μM melatonin of forskolin-stimulated cAMP accumulation was significantly decreased to 31% ($P < 0.05$).

Figure 5A shows the time course of 17β-estradiol-treated BrdU incorporation in MF-sensitive MCF-7 cells. Cells were

cultured in phenol red-free medium in the presence of 10^{-7} M 17β-estradiol for the indicated periods under 0 or 100 μT MF. Then, BrdU was incubated for an additional 3 h at 37°C and quantified by ELISA. Up to at least a 1 week-exposure of MF, there was no significant difference between the two kinetics in the presence of 17β-estradiol ($P < 0.05$). In the absence of 17β-estradiol, relative BrdU incorporation in control cells and cells exposed to MF (100 μT; 1 week) was 1.10 and 1.03, respectively.

PCR analysis showed the presence of estrogen receptor α (ERα) but not estrogen receptor β (ERβ) in MF-sensitive MCF-7 cells, indicating that the proliferative effects of 17β-estradiol seen in Figure 5A were mediated through ERα (Figure 5B). The expression of the ERα was not altered by MF (Figure 5B, lanes 2–4).

Discussion

There has been considerable public concern and controversy about the potential effects of MF on humans. Availability of MF-sensitive cell lines may allow us to reveal the molecular basis of the biological effects of MF. Using MF-sensitive human breast cancer MCF-7 cells, we have shown in this study that the disruption of the inhibitory activity of melatonin on forskolin-stimulated cAMP accumulation is one possible biological effect of MF. This disruption was observed after exposure to 1.2 and 100 μT MF. The rate of disruption by MF linearly increased with exposure time. In contrast, enzyme activities, such as GTPase and adenylyl cyclase, involved in melatonin signaling and melatonin binding were not altered by MF exposure. Furthermore, estrogen-evoked cell proliferation was not changed by MF. Thus, the uncoupling of melatonin-mediated inhibitory pathways by MF might be specific.

The MCF-7 cell line was first reported to be responsive to the mitogenic effects of 17β-estradiol by Osborne *et al*. (39); however, many investigators working with these cells have reported differences in their estrogen responsiveness (40,41). Different levels of estrogen receptors, estrogen-binding activity or expression of variant forms of estrogen receptor mRNA with different deleted exons have been reported in MCF-7 cells (42,43). In addition, variations in the expression of the melatonin 1a receptor have been shown in MCF-7 cells (29); there is also a general correlation between expression of the melatonin 1a receptor and responsiveness to melatonin-induced growth inhibition. American Type Culture Collection supplied MCF-7 cells are only minimally responsive to melatonin (44). Thus, investigators who use such an MCF-7 subclone might fail to reproduce the results that Liburdy (1), Blackman and Benane (2), Lubane and Morgan (3) and Morris *et al*. (4) obtained.

The MCF-7 cells used in this study had estrogen receptors (Figure 5B) and 17β-estradiol stimulated cell growth (Figure 5A). In the absence of melatonin, MF (100 μT; 1 week) did not significantly affect the estradiol pathway in the cells (Figure 5A). It was shown that melatonin was able to specifically block estrogen-induced proliferation in MCF-7 cells (45). Therefore, it appeared that the antiproliferative effects of melatonin were mediated through the estrogen-response pathway. Thus, MF would be expected to interfere with the inhibition of the estrogen-response pathway by melatonin.

Liburdy (26) reported that in lymphocytes an initial rise in calcium level was not altered by MF over the first 100 seconds, but the steady-state level of calcium influx sustained at the plateau phase was enhanced. Lindstrom *et al.* (46,47) demonstrated, in real time, MF-induced changes in intracellular free calcium $[Ca^{2+}]_i$ in Jurkat cells using the intracellular calcium probe fura-2. However, several investigators have failed to detect an effect of MF on calcium metabolism or flux in a variety of cell types (48–52). Possible explanations for the conflicting results may invoke physical complexities related to MF exposure, biological complexities related to heterogeneity of biological response, or the need to establish a responsive biologic state in cells. Since cloned melatonin 1a receptors were shown to cause both attenuation of adenylyl cyclase activity and stimulation of phospholipase C activity (53,54), both signaling systems might be affected by MF in MF-sensitive MCF-7 cells.

The results of several *in vivo* studies show that exposure to MF can alter melatonin secretion by the pineal gland (23,25). Kato *et al*. (23) reported that melatonin concentration in plasma as well as in the pineal gland in rats was suppressed after exposure to a circularly polarized 50 Hz magnetic field at 1, 5, 50 or 250 μT for 6 weeks. Olcese and Reuss (24) showed that 30 min magnetic field exposure inhibited the *N*-acetyltransferase (NAT) activity and the melatonin content of the pineal gland in both albino Sprage–Dawley and pigmented Long–Evans (black-hooded) rat strains. Since melatonin has been shown to suppress chemically induced mammary tumorigenesis in the rat (55,56), a decrease in pineal melatonin production by MF has been implicated in the carcinogenesis of mammary tumors (22). Intramuscular implantation of MF-sensitive MCF-7 cells into *nu/nu* mice would be a reproducible means of elucidating the physiological effects of MF in animals.

A coupling defect of the adenylyl cyclase system was observed in the pathogenesis of diseases such as hypertension (57). Since melatonin regulates a variety of physiological and pathophysiological processes, such as hypothalamic control of circadian rhythm, regulation of reproductive function in seasonally breeding species and regulation of temperature, sexual development and the immune system (5–7), a disorder of melatonin responses caused by MF may consequentially lead to dysfunction of these processes. Therefore, epidemiological studies on the biological effects of MF should be conducted with a focus on such aspects.

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