



EFFECTS OF EXPOSURE TO ELECTROMAGNETIC RADIATION AT 835 MHz ON GROWTH, MORPHOLOGY AND SECRETORY CHARACTERISTICS OF A MAST CELL ANALOGUE, RBL-2H3

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A mast cell line, RBL-2H3, was exposed to 835 MHz for 20 minutes, three times per day for 7 days at a power density of $8.1 \pm 3 \text{ mW/cm}^2$. From day 4 onwards, it was observed that the rate of DNA synthesis and cell replication increased, that actin distribution and cell morphology became altered, and the amount of β -hexosaminidase (a marker of granule secretion) released in response to a calcium ionophore was significantly enhanced, in comparison to unexposed cultures. There were no effects seen on levels of cytoskeletal protein synthesis or of beta-actin mRNA. Morphological changes persisted following subculture for at least 7 days in the absence of further exposure. It is hypothesized that effects of exposure to an electromagnetic field at 835 MHz may be mediated via a signal transduction pathway. © 1997 Academic Press Limited

KEYWORDS: electromagnetic fields; cell growth; secretion; cytoskeleton; mast cells

INTRODUCTION

Electromagnetic fields (EMFs) have been reported to affect a wide range of basic cellular functions. Most of the studies on bioeffects of EMFs have used extremely low frequency (ELF) electromagnetic fields (in the frequency range below 1000 Hz). The cellular functions which have been reported to be affected by exposure to ELFs include proliferation (Kwee and Rasmak, 1995), protein synthesis (Goodman and Henderson, 1988; Blank *et al.*, 1994), gene transcription and expression (Wei *et al.*, 1990; Phillips *et al.*, 1992; Liburdy *et al.*, 1993; Goodman *et al.*, 1994; Broude *et al.*, 1994; Ohtsu *et al.*, 1995), the cell cycle (Rosenthal and Obe, 1989; Antonopoulos *et al.*, 1995), ion transport (Wei *et al.*, 1990; Garcia-Sancho *et al.*, 1994), ligand–receptor binding events (Liburdy, 1995), neurite outgrowth (Blackman *et al.*, 1993), and protein kinase C activation (Monti *et al.*, 1991). Although a common mechanism has not been agreed upon, the cell membrane has been implicated as a target site of interaction (Liburdy, 1995).

It has been suggested that ELF fields affect signal transduction in cells such as PC12 cells (Ohtsu *et al.*, 1995) and T cells (Eichwald and Kaiser, 1995). This seems to be the most 'plausible biological framework currently being investigated for understanding ELF effects on cells' (Liburdy, 1995). It is important to note that some studies have reported no effects of ELF fields on cellular processes such as gene transcription (Desjobert *et al.*, 1995; Lacyhulbert *et al.*, 1995) and proliferation (Paile *et al.*, 1995), and that most of the reports examining DNA damaging effects of ELFs show negative results (Antonopoulos *et al.*, 1995).

In comparison to the extensive literature on ELF effects, there have been relatively few reports of biological effects of radiofrequency fields such as those used by cellular phone systems (800–1800 MHz). Published studies in the radiofrequency range indicate that similar effects to those described above for ELF fields are elicited, including: alterations to lymphocyte proliferation rate at 27 or 2450 MHz (Cleary *et al.*, 1992); cell cycle alterations at 27 MHz in mammalian cells (Cao *et al.*, 1995); reduction of cAMP-independent protein kinase activity in human lymphocytes by a

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450 MHz field (Bawin *et al.*, 1978) and alterations to Ca^{2+} efflux from chick cerebral hemispheres exposed to 450 MHz fields (Byus *et al.*, 1984). In addition, in contrast to most ELF reports, cytogenetic damage following exposure to radio-frequency fields at 954 MHz has been observed (Maes *et al.*, 1995, 1996). This observation is significant, since different mechanisms are likely to operate in the radiofrequency region compared to the ELF-region in view of the much higher efficiency of absorption of energy from the fields. All of these reported effects occurred in the absence of heating of the target cells and tissues.

In the present work we aimed to use a mast cell line, RBL 2H3, with which we were familiar from previous studies (Kawasugi *et al.*, 1995; Ludowyke *et al.*, 1994a,b) to determine effects of exposures to electromagnetic radiation at a frequency used by mobile phone communication on a range of cellular processes. Cells such as mast cells and chromaffin cells which regulate their granule secretion offer a model to investigate effects of microwaves on signal transduction.

In this study, we demonstrate that exposure of RBL-2H3 cells to 835 MHz microwaves elicits alterations in cell proliferation, F-actin distribution, cell morphology and secretion. Some of these alterations were similar to those induced by PMA. After 7 days of exposure the morphological rearrangements persisted through subculture in the absence of exposure to the electromagnetic field. We propose that this frequency may be affecting molecules associated with the signal transduction pathway in these cells.

MATERIALS AND METHODS

Exposure chamber

The experimental exposure chamber consisted of an aluminium box of 1.10 m internal side length. The specimens were placed at a designated location (see below) on a wooden platform located on the mid-plane of the chamber. Power at 835.1 MHz was delivered to a circular loop antenna 9.5 cm in diameter mounted horizontally at the centre of the mid-plane. 1% of the power was reflected back into the source.

Characterization of the field

Since the chamber is essentially a microwave cavity, it has resonant modes labelled with integer

sets (l, m, n). The resonant frequency is given by:

$$f_r = \frac{\omega_r}{2\pi} = \frac{c}{2A} (l^2 + m^2 + n^2)^{1/2}, \quad (1)$$

where c is the velocity of light and A is the side length of the chamber. This gives a resonant frequency of 818.2 MHz for the (442), (424) and (244) modes. The cavity is therefore not strongly resonant at 835.1 MHz, since the resonances in microwave cavities are characteristically sharp with Q values of the order of 10^4 . This gives a half width of the resonance of approximately 1 MHz. The standing wave pattern of the reflected power is however expected to resemble these modal patterns since they are the closest to the driving frequency and will be weakly excited. The field patterns of these modes are therefore calculated in order to interpret the observed field pattern.

The circular loop antenna produces a magnetic field on the midplane of the chamber which is essentially along the vertical or z axis direction of the chamber. The electric field will therefore essentially be transverse, in the xy plane. Thus, it is expected that the transverse electric (or TE) modes will be excited in preference to the transverse magnetic (or TM) modes. A calculation of the modal field patterns in the chamber shows that for the TE_{442} mode, the fields are given by:

$$\begin{aligned} E_x &= E \cos k_1 x \sin k_2 y \sin k_3 z \\ E_y &= -E \sin k_1 x \cos k_2 y \sin k_3 z \\ E_z &= 0 \end{aligned}$$

and

$$\begin{aligned} B_x &= \frac{Ek_3}{i\omega} \sin k_1 x \cos k_2 y \cos k_3 z \\ B_y &= \frac{Ek_3}{i\omega} \cos k_1 x \sin k_2 y \cos k_3 z \\ B_z &= -\frac{E(k_1 + k_2)}{i\omega} \cos k_1 x \cos k_2 y \sin k_3 z \end{aligned} \quad (2)$$

where $k_1 = 4\pi$, $k_2 = 4\pi$, $k_3 = 2\pi$, and x, y and z are the coordinates expressed as a fraction of the sidelength of the chamber.

The electric fields are zero on the mid-plane of the chamber, but at a small distance above the mid-plane, the square of the (transverse) electric field is given by:

$$E_{\text{transverse}}^2 = \sin^2 4\pi x \cos^2 4\pi y + \cos^2 4\pi x \sin^2 4\pi y. \quad (3)$$

The magnetic field is wholly transverse on the mid-plane, with a square given by:

$$B_{transverse}^2 = \frac{4\pi^2 E^2}{\omega_r^2} (\sin^2 4\pi x \cos^2 4\pi y + \cos^2 4\pi x \sin^2 4\pi y) \cos k_3 z. \quad (4)$$

The magnetic field develops a longitudinal (z) component above the mid-plane with a square given by:

$$B_{longitudinal}^2 = \frac{36\pi^2 E^2}{\omega_r^2} (\cos^2 4\pi x \cos^2 4\pi y). \quad (5)$$

The field patterns corresponding to (3) and (5) are shown in Figs 1 and 2.

The corresponding field patterns for the TE₄₂₄ mode are:

$$\begin{aligned} E_x &= E \cos k_1 x \sin k_2 y \sin k_3 z \\ E_y &= -2E \sin k_1 x \cos k_2 y \sin k_3 z \\ E_z &= 0 \end{aligned}$$

and

$$\begin{aligned} B_x &= \frac{2k_3 E}{i\omega} \sin k_1 x \cos k_2 y \cos k_3 z \\ B_y &= \frac{k_3 E}{i\omega} \cos k_1 x \sin k_2 y \cos k_3 z \\ B_z &= -E \frac{(2k_1 + k_2)}{i\omega} \cos k_1 x \cos k_2 y \sin k_3 z \end{aligned} \quad (6)$$

and the squares of the total electric and magnetic fields are shown in Figs 3 and 4. The field patterns of the remaining mode, TE₂₄₄, are simply obtained from the field pattern of TE₄₂₄ by interchanging x and y . It is important to realize that the total electric field intensity and the total magnetic field intensity do not have the same spatial variation, so that it is possible to find places where the electric field is high and the magnetic field is low and vice versa. This exposure chamber therefore allows the possibility of differentiating electric field effects from magnetic field effects.

Measurements of the electric field strength were made using two broad band field strength meters, a Holaday Instruments model HI 3004 fitted with STE probe, and an EMR-20 meter (Wandell & Goltermann, Eningen, Germany). The Holaday probe, which was used to make multiple measurements throughout the exposure chamber consists of three orthogonal dipole antennas and so is equally sensitive to all polarizations of the field. The probe

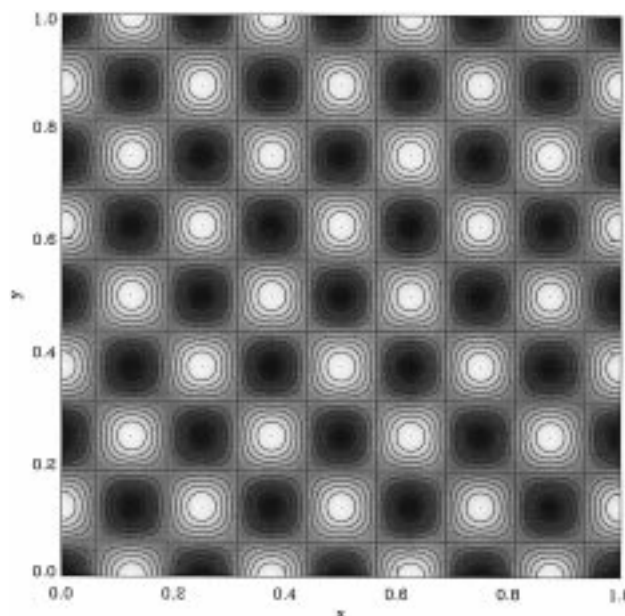


Fig. 1. A plot of the square of the transverse (horizontal) electric field and of the square of the transverse magnetic field for the (442) mode. Light areas represent large values and dark areas represent small values. The electric field vanishes at the midplane but increases with distance above the midplane, while the magnetic field is non-zero at the midplane and decreases with distance above the midplane. x and y are fractional co-ordinates of the midplane of the exposure chamber.

has a diameter of approximately 15 cm and therefore its reading is a weighted average over an approximately spherical volume of space.

Cell culture

RBL-2H3 cells were maintained as an adherent monolayer in a humidified atmosphere at 37°C in 5% CO₂. The normal growth medium contained RPMI-1640 (Gibco Laboratories, NY, U.S.A.) supplemented with 10% (v/v) foetal bovine serum (P.A. Biologicals Co, NSW, Australia), 25 mM Hepes (Commonwealth Serum Laboratories (CSL), Victoria, Australia) and 4 mM glutamine (CSL). The cultures tested negative for Mycoplasma by the Gen-Probe kit (Gen-Probe Inc., CA, U.S.A.). The cells were detached with trypsin/ethylene-diaminetetraacetic acid (trypsin/EDTA—0.05% trypsin, 5.3 mM Na₄EDTA; Multi-Cel, Cytosystems, Australia) and subcultured twice per week. Cells were not passaged more than 20 times.

Exposure of cells

Cells were irradiated three times per day, for 20 minutes at a time, at four-hourly intervals. This

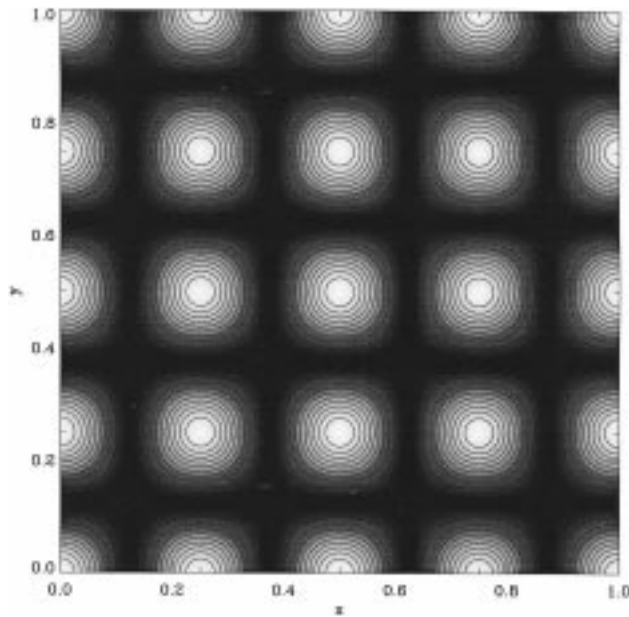


Fig. 2. A plot of the square of the longitudinal (vertical) magnetic field strength for the (442) mode. The z component is zero on the midplane and increases with distance above the midplane.

was done by placing them in the field at a position corresponding to the coordinates in Fig. 1 of $x=5.1$; $y=7.3$. The cultures were placed 0.04 m above the midplane. Control (unexposed) cells were handled in an identical fashion to the treated groups (they were removed from the incubator and placed in the exposure chamber) but without

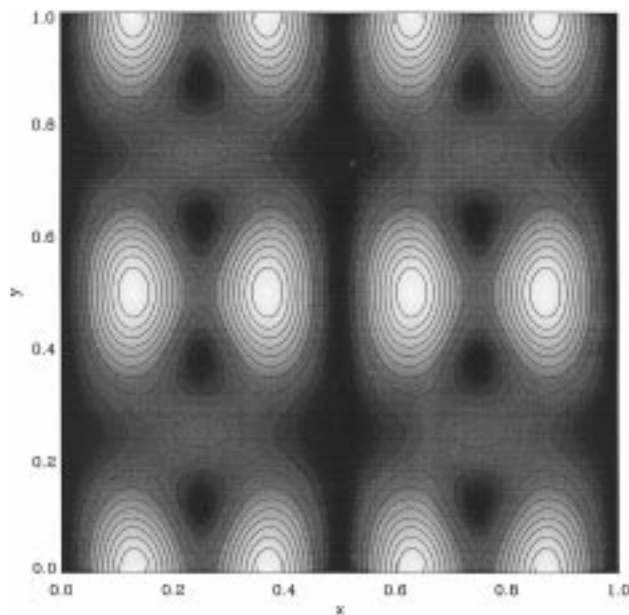


Fig. 3. A plot of the 424 mode total transverse (horizontal) electric field strength and total transverse magnetic field strength. Other conditions as per legend to Fig. 1.

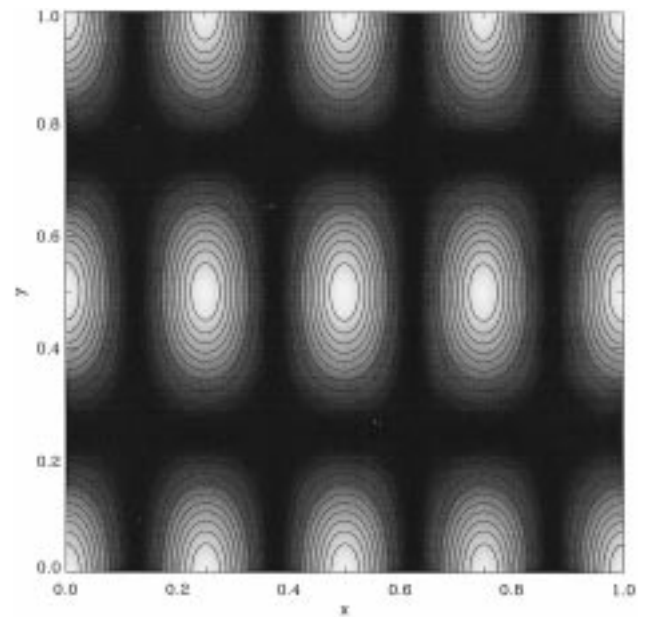


Fig. 4. A plot of the square of the longitudinal magnetic field strength for the (424) mode.

irradiation. Flasks were sealed prior to exposure to eliminate variation in humidity and pH. In order to minimize random variability in field strength, the quantity of culture medium per flask and the position of the flask in the chamber were standardized between experiments as much as possible.

Proliferation assay

The proliferation assay was adapted from that described by Pryor *et al.* (1995). The cells were plated at 1.5×10^3 cells/well in 96-well flat-bottomed tissue-culture plates (Corning, NY, U.S.A.). There were eight replicates at each concentration. The cells were incubated in a humidified atmosphere at 37°C in 5% CO₂ for 48 hours prior to the start of the assay. Medium was aspirated and replaced in all wells on alternate days. Comparison of proliferation was made between control (unexposed), and exposed cells on each day for seven consecutive days. For each day, the wells of one plate from each group were pulsed with 0.5 mCi of (³H)thymidine (specific activity 5.0 Ci/mmol, Dupont, MA, U.S.A.) for 18 hours. The plates were washed twice with phosphate-buffered saline (PBS) and cells detached with trypsin/EDTA as previously described. The cells were harvested with an Inotech automated cell harvester and counted in Ready Safe scintillation fluid (Beckman

Instruments Inc., CA, U.S.A.) on a Tri-Carb 1500 liquid scintillation analyser (Packard, Groningen, The Netherlands).

Cell morphology, actin distribution and measurement of cell height

Immunofluorescence and confocal microscopy were used to determine effects of 835 MHz exposure on F-actin distribution and cell morphology and to measure cell height. Cells were exposed as described above over a 7-day period in either eight-well glass Lab-Tek Chamber Slides (Nunc, Roskilde, Denmark), or in 75-cm² culture flasks (Corning). Cells irradiated in chamber slides were plated at a concentration calculated to give 1.5×10^4 cells per well on each day, in order to avoid variation in degree of confluence between days of treatment. Each day for 7 consecutive days, the cells of one slide from each group were fixed for 10 minutes in 3.5% (v/v) formaldehyde in PBS containing 0.1% (v/v) Triton X-100 at room temperature (RT). The fixed cells were washed twice with PBS. F-actin was visualized by adding a solution of phalloidin-fluorescein isothiocyanate (phalloidin-FITC) (Sigma-Aldrich, Sydney, Australia) diluted 1:100 (v/v) in PBS for 30 minutes at RT. The cells were washed with PBS and mounted for viewing in fluorescent mounting medium containing 90% (v/v) glycerol, 10% (v/v) PBS at pH 8.0 containing 1 mg/ml *p*-phenylenediamine (Sigma-Aldrich). Cells exposed in flasks were initially seeded at 5×10^5 cells per flask and not sub-cultured over the 7-day period. To determine if the effects of exposure persisted in the absence of further treatment, the cells were cultured for a further 1–14 days, then plated onto chamber slides for 24 hours before being fixed and stained as described above. All samples were examined by confocal laser scanning microscopy (CSLM) using a Sarastro 2000 Confocal Microscope (Molecular Dynamics, Sunnyvale, CA, U.S.A.), as described previously (Kawasugi *et al.*, 1995). Measurement of cell height was performed using the z-section scanning function on the confocal microscope. For each day and treatment, 20 cells were measured at random, as previously described (Kawasugi *et al.*, 1995).

Activation of cells

Immediately prior to activation, both 7-day exposed and control cells were washed twice in MEM. The treatments were made up in MEM to

be added to the cells. For activation with phorbol myristate acetate (PMA) a concentration of 25 nM PMA was used. For activation with the calcium ionophore A23187, two concentrations, 1000 nM and 500 nM, were used. The cells were also activated by a combination of PMA and A23187. In these treatments PMA at a concentration of 25 nM was added with A23187 at a concentration of either 1000 nM or 500 nM as previously described (Ludowyke *et al.*, 1994a). Each treatment was added to the wells and incubated for 30 minutes. At the end of the activating period, the plate was placed on ice to halt the reaction. The supernatant was removed from each replicate well and added to a 96-well V-bottomed microtitre plate (Nunc, Roskilde, Denmark) and centrifuged at 1400 rpm for 10 minutes. Samples were taken from these for the measurement of β -hexosaminidase. There were eight replicates for each activation treatment.

β -hexosaminidase assay

The secretion assays were adapted from those described by Ozawa *et al.* (1993). The cells were plated at 1.5×10^4 cells/well in 96-well flat-bottomed tissue-culture plates (Corning, NY, U.S.A.). Twenty-five microliter aliquots of the activation reaction supernatants and cell lysates were added to each well and incubated with 5 mM *p*-nitrophenol- β -D-glucosaminide (P-NAG) in a 0.1 M sodium citrate buffer (pH 4.5) for 2 hours at 37°C. At the end of the incubation, 200 μ l of a stop solution of 1 M Na₂CO₃ was added. The absorbance was measured at 405 nm on a Diagnostics Pasteur LP 400 spectrophotometer. Total β -hexosaminidase was determined by lysing cells with 0.1% (v/v) Triton X-100 after samples had been taken for analysis of released β -hexosaminidase.

Polyacrylamide gel electrophoresis

In order to determine whether there was an effect on protein expression, SDS-polyacrylamide gel electrophoresis was carried out on cytoskeletal and cytoplasmic proteins extracted from control and exposed cells after 7 days using a method previously described (Ludowyke *et al.*, 1994a).

RESULTS

Temperature measurement

The exposed cultures were found to be an average of $0.8 \pm 0.4^\circ\text{C}$ greater (26.2°C for control

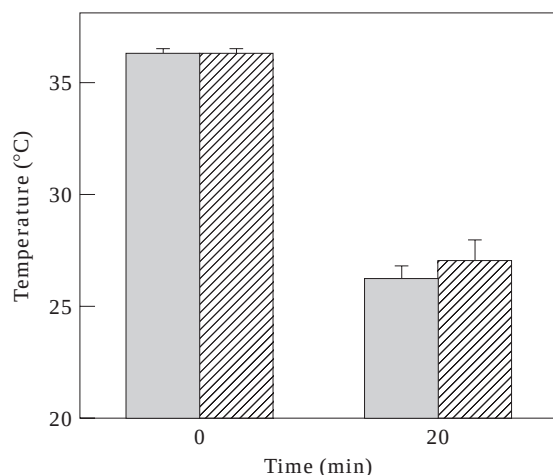


Fig. 5. A plot of the temperature difference of the control and exposed cell culture medium following the exposure period. ▨, Control; ▩, exposed.

cultures, 27.0°C for exposed cultures, Fig. 5), after 20 minutes exposure. This value was used as an independent measure of power density (see later).

Measurement of power density

The results of measurements with the Holaday Instruments probe are given in Table I in units of V/m, together with the expected result obtained

Table 1.
The electric field as measured by the Holaday probe at various positions at the mid-plane of the exposure chamber

Point no.	x-coordinate	y-coordinate	Electric field (V/m)
1	0.075	0.075	60
2	0.150	0.075	45
3	0.300	0.075	175
4	0.500	0.075	50
5	0.925	0.075	90
6	0.850	0.075	30
7	0.700	0.075	150
8	0.845	0.500	120
9	0.710	0.500	20
10	0.500	0.310	110
11	0.430	0.310	120
12	0.570	0.300	85
13	0.540	0.710	70

The x-coordinate and y-coordinate values are the fractions of the dimensions of the chamber. Figure 6 shows the plot of these values vs the expected values as per the calculation of the field characteristics described in Materials and Methods.

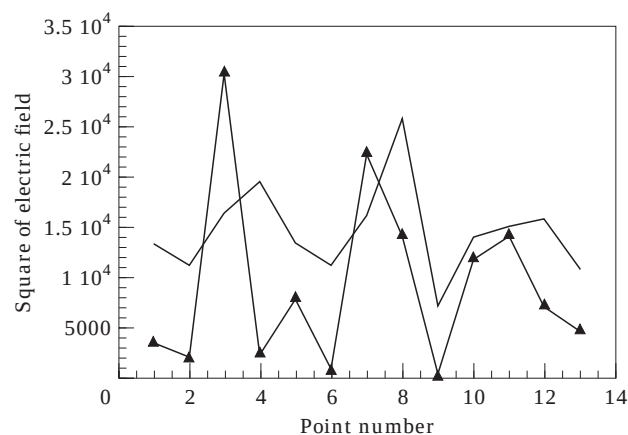


Fig. 6. A comparison of the measured electric field intensity squared (triangles) with the predicted values for the (442) mode (solid line). The point numbers refer to the positions given in Table 1.

from an estimate of the expected modal field strength. Figure 6 shows the squares of the electric field compared to predictions made on the basis that the 442 mode provides the major contribution to the electric field strength. The agreement is reasonable, given that the field due to the exciting antenna is not included in the theoretical estimate and the other two possible TE modes will provide some contribution. The power level in the chamber when the measurements were taken as an average over the measured points was 2.4 mW/cm². This level is just over the maximum occupational exposure level of 2 mW/cm² specified in the ANSI standard. The maximum power level measured using the Holaday probe was 8.1 mW/cm² and with the EMR-20 (Wandell and Goltermann) probe was 9.6 mW/cm².

As objects such as cell culture flasks placed in the chamber will perturb the field, the power density in the flask was also calculated from the temperature difference between the cell culture and the ambient temperature in the chamber under equilibrium conditions. This calculation is valuable as an independent measurement of the power density in the region of the chamber containing the cell culture under the actual experimental conditions. The calculation is not subject to possible errors arising from the perturbation of the fields by the presence of the field meter probe and the different conditions applying in the chamber when the specimens are present. The heat gain by the cell culture from the field can be assumed to be closely equal to the heat loss by convection since it rests on a shelf made from a poor thermal conductor so that conductive and radiative losses are small compared

to convective losses. The power density is then given by

$$S = \frac{6.18At}{\pi r^2 Q_{abs}} \quad (7)$$

The numerator is the convective power loss from a horizontal surface of area A and at a temperature t above ambient (Ozisik, 1985). The denominator is the power absorbed from a field of unit intensity by a specimen of cross section area πr^2 in a situation in which the absorption efficiency is Q_{abs} . The absorption efficiency was calculated from the exact solution obtained by Mie as discussed in Van de Hulst (1957) for a sphere immersed in a planar field. The dielectric properties of the cell culture were estimated from the properties of sea water which has approximately the same conductivity as the cell culture. The properties of sea water at 835 MHz were obtained from the value of the dielectric permittivity of

$$\varepsilon = \varepsilon' + j\varepsilon'' \quad (8)$$

with $\varepsilon' = 69$ and $\varepsilon'' = 60\lambda\sigma$, where λ is the wavelength, and $\sigma = 6.5 \text{ (ohm m)}^{-1}$ (Levanon, 1988). Q_{abs} was found to have the value 0.331 under these conditions for a spherical specimen of radius 3 cm which gives the same volume as the specimen. The power density was then obtained from the average temperature rise of $0.8 \pm 0.4^\circ\text{C}$, giving the value $4.2 \pm 2.1 \text{ mW/cm}^2$. This is in reasonable agreement with the maximum measured field of 8.1 mW/cm^2 , considering the approximations used and that the specimens may be located at a point where the field is somewhat less than the maximum.

Cell proliferation

For the first five days of exposure the rate of ^3H -thymidine uptake was similar between controls and cells exposed to 835 MHz (Fig. 7a). After that, the rate of ^3H -thymidine uptake in the control cells declined due to the cultures becoming confluent. This decline was not seen in exposed cells, whether the cells were plated at an initial concentration of 250, 500 or 1000 cells per well (data for 500 cells/well is shown in Fig. 7). Viable cell counts (Fig. 7b) indicated that the cells in the exposed cultures continued to increase in number, whereas the cells in the control cultures decreased in number after 4–5 days, indicating that the cultures had reached confluence and had started to lose adhesion with the plastic (characteristic of this cell line in our laboratory).

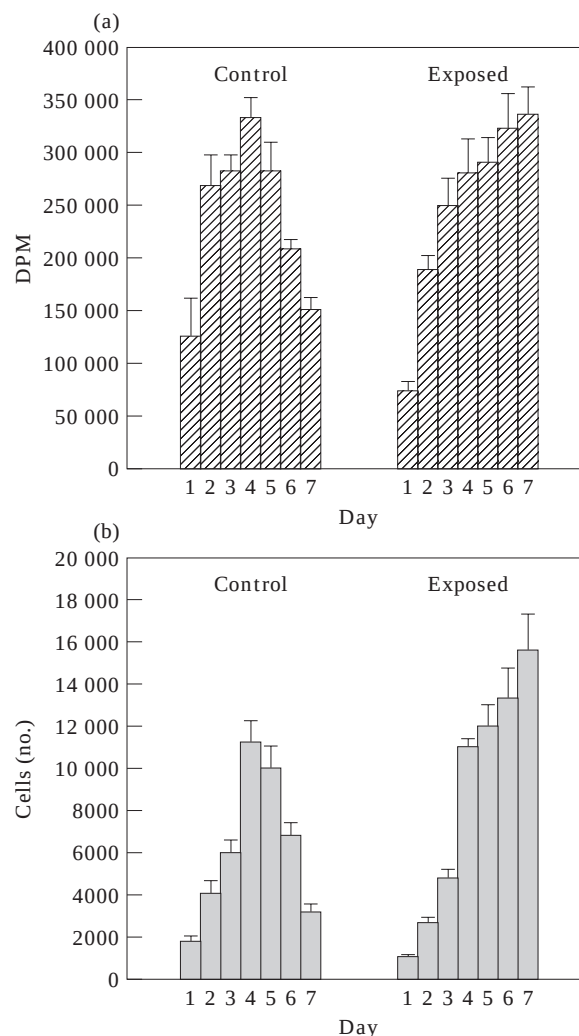


Fig. 7. Effects of 835 MHz exposure on RBL-2H3 cell growth. (a) Comparison of DNA synthesis as measured by uptake of ^3H -thymidine in unexposed (control) cells and cells exposed to 835 MHz. Cells were harvested each day for 7 days. (b) Cell counts. Viable cell counts were made each day for 7 days. The error bars represent standard deviation from the mean. The figures are representative of at least three separate experiments.

Cell morphology, actin distribution and measurement of cell height

After 7 days of exposure, the appearance of actin-containing cell surface ruffles was observed in cells cultured in chamber slides (Fig. 8b). The cells also showed a marked increase in spreading. This arrangement of actin in ruffles was not seen in the control cells, in which actin was observed in a band at the plasma membrane in the predominantly spherical cells (Fig. 8a). When cells were exposed for 7 days in 75-cm² culture flasks, then subcultured and plated onto chamber slides without further exposure, the actin distribution was similar to

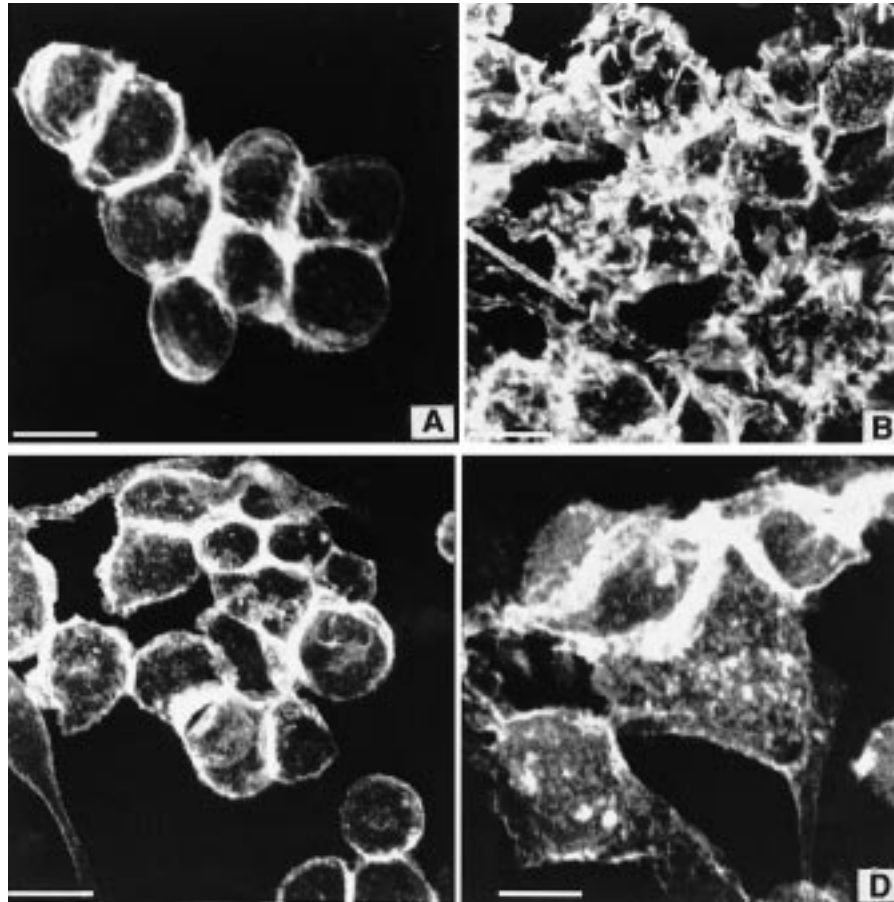


Fig. 8. Changes in cell morphology and actin distribution induced in RBL-2H3 cells by 835 MHz exposure visualized by confocal microscopy of cells stained with phalloidin-FITC. (a) Control cells after 7 days. (b) Cells exposed to 835 MHz three times per day for a 7-day period showing increased cell spreading and prominent cell surface ruffles. (c) Control cells mock exposed for 7 days followed by culture for 14 days. (d) Cells exposed as in (b) cultured for a further 14 days without exposure to 835 MHz. Scale bar = 20 microns.

control cells, but they exhibited increased cell spreading similar to that seen in 7-day exposed cultures (Fig. 8c), indicating that some of the morphological changes induced by microwave exposure persisted through subculture. This was seen up to 14 days after the end of the exposure period.

From 0 to 5 days of exposure, the height of the exposed RBL-2H3 cells was an average of 11.4 microns, compared to 11.3 for the control cells. These measurements are in general agreement with that previously reported for unactivated RBL-2H3 cells (12.77 ± 1.09 microns) (Ludowyke *et al.*, 1994a). After 6 days the exposed RBL-2H3 cells decreased in cell height (Fig. 9). At day 7 the average height measured by confocal microscopy was 8.6 ± 1.2 microns, which was identical to that previously reported by our laboratory for cells activated by ATP γ S for 30 minutes (Ludowyke *et al.*, 1994a).

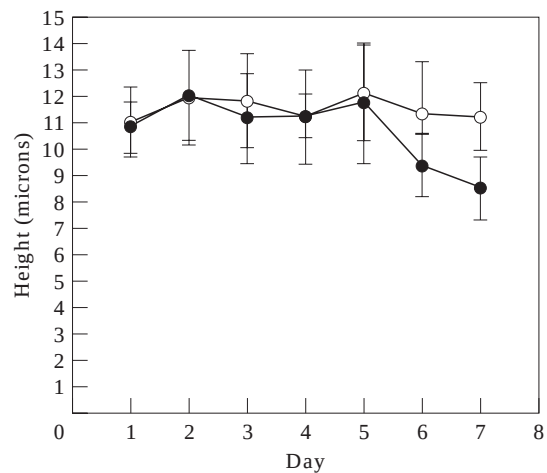


Fig. 9. Changes in RBL-2H3 cell height induced by exposure to 835 MHz over a 7-day period compared to unexposed controls. Cell height measurement was performed by confocal microscopy, on 20 cells per treatment. Means are the average of two separate experiments. —○—, Control; —●—, exposed.

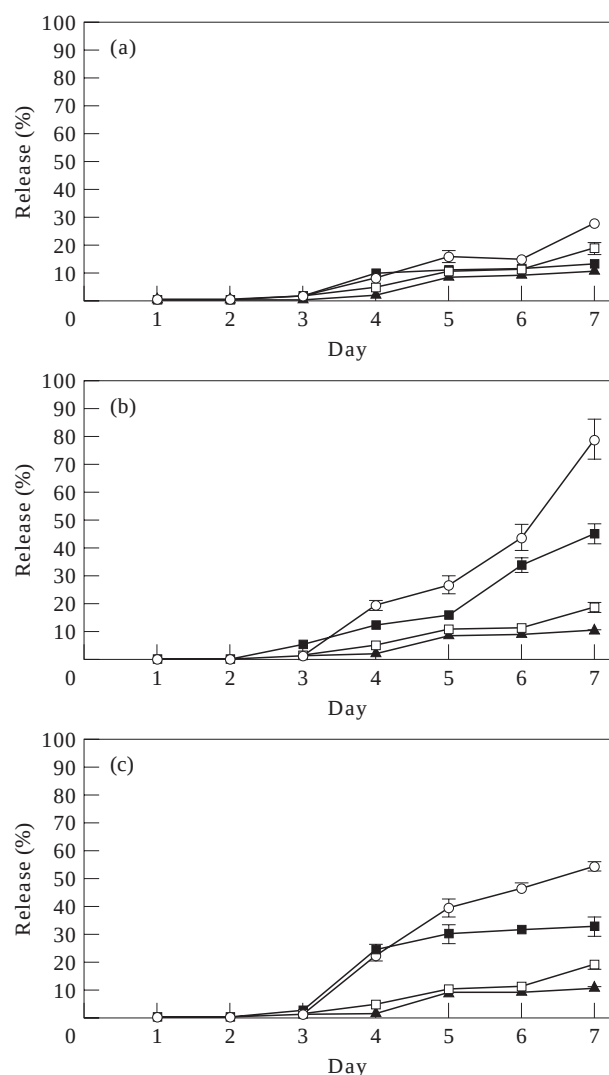


Fig. 10. Effects of 835 MHz on secretion of β -hexosaminidase. (a) Cells treated with 25 nM PMA; —▲—, no treatment; —■—, PMA (25 nM); —□—, 835 MHz; —○—, 835 MHz + PMA (25 nM). (b) Cells treated with 1000 nM A23187; —▲—, no treatment; —■—, A23187 (1000 nM); —□—, 835 MHz; —○—, 835 MHz + A23187 (1000 nM); (c) cells treated with both PMA (25 nM) and A23187 (1000 nM); —▲—, no treatment; —■—, PMA (25 nM) + A23187 (1000 nM); —□—, 835 MHz; —○—, 835 MHz + (PMA [25 nM] + A23187 [1000 nM]).

Secretion

Figure 10 contains data averaged from three separate experiments. Cells exposed to 835 MHz did not spontaneously release β -hexosaminidase in the absence of a secretagogue. Addition of PMA did not cause a significant difference between secretion from control and irradiated cells (Fig. 10a). However A23187 alone, when added to irradiated cells resulted in a significant increase in secretion from

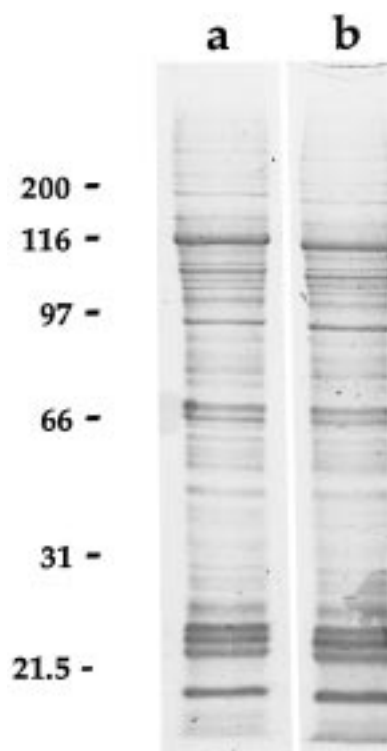


Fig. 11. Gel electrophoresis of the cytoskeletal fraction of control and exposed RBL-2H3 cells. Proteins visualized by Coomassie blue staining. (a) Control, (b) exposed to 835 MHz over a 7-day period.

cells irradiated for 4–7 days, reaching a maximum after 7 days of exposure (Fig. 10b). The increase averaged 1.7 times that seen in the unexposed cells. A combination of PMA and ionophore (at concentrations less than those known to induce maximal secretion) resulted in an increase in secretion from cells which had been exposed for greater than 4 days, compared to unexposed controls (Fig. 10c). The maximum increase was seen in cells which were exposed for 7 days, and this treatment also resulted in a 1.7-fold increase in β -hexosaminidase release than the unexposed cells.

Polyacrylamide gel electrophoresis

From one-dimensional polyacrylamide gels of proteins from cells separated into cytoskeletal and soluble fractions, there was no difference in the level of Coomassie blue staining of any protein band from cells exposed for 7 days compared with control cells (Fig. 11).

DISCUSSION

We and others have demonstrated that activators of secretion from mast cells induce a significant

alteration in the morphology of the cell and a rearrangement of actin and actin-associated cytoskeletal proteins (Pfeiffer *et al.*, 1985; Oliver *et al.*, 1985; Apgar, 1991; Liu *et al.*, 1987; Ludowyke *et al.*, 1994a,b; Kawasugi *et al.*, 1995). Similar changes to those seen in mast cells also occur in other secretory cells such as chromaffin cells and are believed to be necessary for the disassembly of the cytoskeletal barrier to allow the granules to have access to their docking sites on the plasma membrane for the fusion of granule and plasma membrane to occur (Pfeiffer *et al.*, 1985).

We used the rat basophilic leukemia cell line RBL-2H3, a common model for studying mast cell exocytosis, in the present experiments to examine the effects of microwaves at 835 MHz on cellular functions such as cell proliferation, morphology and secretion which are the end results of signal transduction pathways. RBL-2H3 cells have most of the features of mucosal mast cells, including the presence of IgE receptors on the surface, the presence of tryptase, chymotrypsin and histamine within the secretory granules, and the ability to be activatable by the calcium ionophore, A23187, but not by polyamines such as 48/80. The time course of secretion and many of the initial signals which promote secretion in these cells have been well characterized (Ludowyke *et al.*, 1994a). Unactivated RBL-2H3 cells in culture exhibit no focal adhesions or actin stress fibres, despite being adherent. Actin and myosin form a layer at the cell membrane. Resting cells are rounded and smooth-surfaced. Upon activation with several different mediators, both the 'physiological' antigen stimulus (such as DNP-BSA), and others including the PMA and calcium ionophore, the cells undergo dramatic reorganization of actin into surface ruffles, and into stress fibres. The cells flatten and spread and secretion of histamine and other allergic mediators is induced (Ludowyke *et al.*, 1994a,b). The relationship between mast cell morphology and secretion of allergic mediators such as histamine has been an area of some interest. During antigen (Oliver *et al.*, 1985; Spudich, 1994), or PMA plus A23187-induced secretion from intact RBL-2H3 cells (Ludowyke *et al.*, 1994a) and ATP γ S-induced secretion from permeabilized cells (Ludowyke *et al.*, 1994b), major morphological and cytoskeletal changes occur. These include increased cell spreading, actin stress fibre formation and the formation on the cell membrane of ruffles which contain actin and myosin (Pfeiffer *et al.*, 1985; Oliver *et al.*, 1985; Apgar, 1991; Liu *et al.*, 1987; Ludowyke *et al.*, 1994a,b).

In the present study we have demonstrated that some of the same morphological changes occur following six or more days of exposure to 835 MHz radiation at a maximum power density of 8.1 mW/cm², for 20 minutes at a time, three times per day. This treatment induces a marked decrease in cell height, increased cell spreading and the appearance of cell surface ruffles. No actin filaments are observed, however. Furthermore, the secretory characteristics of the exposed cells are altered, so that they secrete significantly more β -hexosaminidase (and by analogy, histamine) in response to the calcium ionophore, A23187, and to PMA + A23187, than do unexposed controls. It is possible that the morphological and cytoskeletal changes may be priming events which enable secretion to occur more rapidly in response to activators. There is other evidence that secretory cells may be sensitive to electromagnetic fields. A study of exposure of chromaffin cells to low frequency magnetic field stimulation showed that this treatment was comparable to nerve growth factor in producing neurite outgrowth and release of noradrenaline (Druckercolin *et al.*, 1994).

Exposure to 835 MHz also resulted in a significant change in cell proliferation, which manifested as a failure to reach a peak of either DNA synthesis or cell replication. Control cultures exhibited an expected bell-shaped curve for both parameters, indicating that as cells reached confluence the rate of cell division reached a peak and then decreased. No inhibition of cell replication occurred in exposed cultures, whether the initial cell concentration was 250, 500 or 1000 per well. It is therefore possible that the normal regulatory mechanism of contact inhibition of cell growth may have been affected by the exposure.

The changes in morphology, secretion and proliferation occurred in the absence of obvious changes in cytoskeleton protein expression, or of alterations to levels of actin mRNA (data not shown), and in the absence of heating of the cell culture medium. Furthermore, an altered morphology persisted through subculture of the exposed cells. The molecular mechanism by which this altered morphology occurs is not known, but the observation may provide evidence for a cumulative effect of microwave exposure at this power density.

The exposure system contains some unique features which provide the ability to examine various parameters of the effects of microwaves on cells.

Firstly, we have used a resonant cavity, which gives rise to the fields illustrated in Figures 1–4. As

we have characterized the scale of the variation of the field both theoretically and experimentally, it can be determined that there would be no 'hot spots' within the volume of the size of the culture vessel used, as this volume is much smaller than the periodicity of the standing wave. It is important that the chamber be not excited too close to its resonant frequency of 818.2 MHz, since in that case the field amplitudes would become very large and also very sensitive to the level of absorption by the specimens or the measurement equipment, preventing a meaningful estimate of the field amplitude being made. At 835.1 MHz, however, the chamber is not strongly resonant. We have taken the maximum measured power density from the electric field measurements (8.1 mW/cm^2) as the appropriate value in order not to underestimate the true power level to which the cultures are exposed. The fact that there are positions in the exposure chamber where the electric field vanishes but where the magnetic field is strong (e.g. at the mid-plane) provides us with a mechanism whereby the contribution of the electric and magnetic fields to the biological effects observed can be determined. Preliminary results indicate that the electric field has a greater contribution to the effects (data not shown). These studies need to be extended before any definite conclusions can be drawn.

Secondly, in the area of biological effects of microwave exposure, the question of heating above 37°C of the cells is raised as a possible explanation of the effects seen. In our system, the cultures are allowed to cool below 37°C during the exposure, and the control cultures are treated identically. Cultured cells are frequently cooled to room temperature when undergoing various manipulations such as sub-culturing, microscopic examination, etc. Our system ensures that the exposure does not cause the cells to be heated above 37°C , and thus the question of heat stress does not arise.

In searching for a mechanism of observed effects of electromagnetic fields on biological systems, Liburdy (1995) and others have hypothesized that EMFs interact with the membrane, triggering an early event in the signal transduction cascade, such as calcium ion influx. This process (calcium ion influx) has been confirmed experimentally (Liburdy, 1992a,b; Eichwald and Kaiser, 1995) in the absence of release of calcium from intracellular stores. These results implicate the cell membrane as being involved in the EMF interaction. Further support for an effect of microwave irradiation on signal transduction is our preliminary finding of the decrease of Ras from the cytosol and a concomitant increase at the membrane (data not shown).

This may reflect an increase in the recruitment of Ras-associated proteins to the cell membrane which leads to Ras activation (Marshall, 1996). The Ras family of GTP-binding proteins are known to be key regulators of eukaryotic cell growth and differentiation, and secretion, and production of actin-containing membrane ruffles is characteristic of activation of one of these proteins, Rac (Qiu *et al.*, 1995). However, it has been previously reported that unmodulated carrier waves do not affect the cell membranes (Byus *et al.*, 1984). The effects we report here induced by unmodulated 835 MHz on RBL-2H3 cells may therefore be acting via an alternative mechanism.

The fact that levels of secretion induced by A23187 were at least as high as those seen with PMA + A23187 in a previous report (Ludowyke *et al.*, 1994b) may indicate that 835 MHz microwave radiation is mimicking the action of PMA in activating protein kinase C in RBL-2H3 cells. Evidence from work in PC12 cells has shown that the effects of ELF (50 Hz) could be inhibited by the PKC inhibitor calphostin C (Ohtsu *et al.*, 1995). Further evidence in support of this proposition is that like PMA, exposure to 835 MHz alone does not induce secretion. It requires a second effector such as A23187 or antigen in order to induce secretion. The activation of protein kinase C and an increase in intracellular Ca^{2+} concentration are thought to be the primary synergistic signals required for secretion (Ludowyke *et al.*, 1994a; Cuhna-Melo *et al.*, 1989).

Another possibility is that the EMF may be acting directly on the medium in which the cells are cultured. Fesenko *et al.* (1995) described such a phenomenon using millimetre microwave irradiation (42.25 GHz) below the levels of thermal effects. Ion-channel activity in Vero cells was altered by the addition of irradiated buffer, but not by control buffer, to the cells.

Which, if any, of the above mechanisms are operating to produce the effects reported above of electromagnetic field-associated cellular changes requires further detailed study.

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