Calcium Protects Differentiating Neuroblastoma cells During 50 Hz Electromagnetic Radiation

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Running title: Magnetic field modulates NG 108-15 cells differentiation

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Abstract

In spite of growing concern about electromagnetic radiation, the interaction between 50/60 Hz fields and biological structures remains obscure. Epidemiological studies have failed to prove a significantly correlation between exposure to radiation fields and particular pathologies. We demonstrate that a 50/60 Hz magnetic field interacts with cell differentiation through two opposing mechanisms: it antagonizes the shift in cell membrane surface-charges that occur during the early phases of differentiation and it modulates hyperpolarizing K-channels by increasing intracellular Ca. The simultaneous onset of both mechanisms prevents alterations in cell differentiation. We propose that cells are normally protected against electromagnetic insult. Pathologies may arise, however, if intracellular Ca regulation or K-channel activation malfunctions.

Introduction

There is growing concern about the increase in environmental pollution due to the emission of electromagnetic waves. The mechanism of interaction between extremely low frequency electromagnetic fields (ELF-EMF) and biological structures, if it exists, is still obscure. Epidemiological studies have failed to find a correlation in live subjects between the continuous presence of ELF-EMFs at different intensities and the appearance of any particular pathology (Reipert et al., 1997;Lacy-Hulbert et al., 1998) (Hatch et al., 1998;Day, 1999). However, several epidemiological studies have demonstrated increases in childhood leukemia and other related diseases in children from populations exposed to extremely low (50/60 Hz) frequency electromagnetic fields (Thomson et al., 1988)such as those produced by major power lines in the proximity of residential areas. However, none of these studies has found a significant correlation between the presence of ELF-EMFs and increases in pathological conditions. Likewise, not only do macroscopic analyses of cell survival using homogeneous primary cultures obtained from humans and animals show conflicting results, but investigations at subcellular level have also failed to explain the sporadic alterations observed after treatment with an ELF-EMF source (Reipert et al., 1997;Feychting et al., 1998). In general, if a detectable modification does exist it is not constant and always occurs following activation of a complex cell mechanism (Hojevik et al., 1995;Grynkiewicz et al., 1985;Eichwald and Walleczek, 1996). The most evident effects induced by magnetic waves are the mobilization of intracellular calcium and, occasionally, morphological changes, although cell signals, when present, are extremely variable (Liburdy et al., 1993;Karabakhtsian et al., 1994;Goodman et al., 1995;Barbier et al., 1996;Loscher and Liburdy, 1998).

Our study is based on the possibility that ELF-EMFs could interfere with dynamic cell conditions such as division, differentiation and membrane voltage fluctuation as well as changes in intracellular calcium concentrations. During our experiments we used field intensities of the same order of magnitude as those measured in the proximity of household appliances or electric power lines (source http://www.hsph.harvard.edu/Organizations Canprevent/emf.html).

All our experiments, from cell culture to patch-clamp, were performed in the constant presence of a magnetic field. We were not expecting to find that exposure to magnetic fields produces drastic effects on cell physiology. Our aim was to reveal sudden alterations that, in most cases, could well be buffered by alternative cytosolic pathways without triggering degenerative damage to cells.

In the present study we show that ELF-EMFs interfere with the differentiation of NG108-15 neuroblastomaXglioma cells *in-vitro*. The differentiating agent, by acting on the surface charges, modulates the normal "depolarization-repolarization" response to growth factors. The resulting change in cell membrane potential represents the cell's commitment to differentiation (Arcangeli et al., 1987;Olivotto et al., 1996). The mechanism of interaction that we propose exists between electromagnetic fields and chemically-induced differentiation is based on two antagonistic cellular events. An ELF-EMF is able to prevent the shift in the surface charge potential and, thereby, hyperpolarization. However, it simultaneously stimulates an increase in intracellular calcium in a dose-dependent manner. By opening calcium-dependent potassium channels, the increase in cytoplasmic divalent ions, acts as a rescue agent in cell membrane hyperpolarization, re-establishing the cell's commitment to differentiation. In NG108-15 cells exposed to low intensity ELF-EMFs the two mechanisms appear separate, where the magnetic field continues to counteract hyperpolarization but intracellular calcium is not sufficiently raised. The simultaneous onset of the two mechanisms probably prevents major damage during cell differentiation. Problems with exposure to ELF-EMFs may be linked with pathological situations in the event of a malfunctioning of intracellular calcium control mechanisms.

Methods

Cell cultures and FACS analysis

NeuroblastomaXglioma culture cells (NG108-15) were grown in DMEM-high glucose with 10% heat-inactivated fetal calf serum in a 5% CO_2 humidified atmosphere at 37°C (Seidman et al., 1996).

Flow cytometric analyses were performed on cell samples taken after 72 hours in the presence of BT2cAMP (96 hours of growth). Frequency histograms of PI-emitted fluorescence intensity (FL2-Height; proportional to the cell DNA content) were obtained from parallel and independent cultures at the indicated field intensities (6 cultures at 0 and 120 µT; 5 cultures at 0 and 360 µT). Signals due to dead cells, cell aggregation and/or polyploidization contributed to about 10% of the recorded distributions and were discarded before calculating the cell frequencies in G1, S and G2/M.

Electromagnetic field exposure

A 50 Hz electromagnetic field was continuously provided inside the cell incubator using a Helmoltz device connected to a custom-made variable magnetic field generator. The field was measured at the beginning and end of each experiment. The maximum value of the induced electric field was negligible, about 0.45 mV/m. The cell incubation temperature was 37 ± 0.6°C throughout each experiment and was directly monitored. During patch-clamp whole-cell experiments the magnetic field was delivered by a single copper coil connected to a custom-made variable magnetic field generator placed around the petri dish containing the cells. The ELF-EMF was measured directly before and after each recording using an accurately calibrated custom-made detection device.

Electrophysiology and calcium measurement

Standard whole-cell and perforated-patch voltage- and current-clamp techniques were used. The bath solution (pH 7.32) contained (in mM): 133 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 5 Glucose. The electrode solution (pH 7.32) for resting potential monitoring contained (in mM): 10 NaCl, 120 KAsp, 2 $MgCl_2$, 4 $CaCl_2$, 10 HEPES, 10 EGTA, Mg-ATP 3, Na_2-GTP 0.2, and amphotericine for experiments in perforated-patch measurements. To record Ca current we used (in mM): 72 CholineCl, 3 KCl, 1 $MgCl_2$, 10 $CaCl_2$, 10 HEPES, 50 TEA-Cl, 10 4AP, 24 Glucose in the bath (pH 7.32) and 20 TEA-Cl, 120 CsAsp, 2 $MgCl_2$, 10 HEPES, 10 EGTA, 4 Mg-ATP, 5 CP-Tris, 0.1 GTP-Tris and CPK 20 u/ml (pH 7.32) to fill the patch-pipette. BT2cAMP (*Sigma*, Milano, Italy), and other chemicals were added using a fast perfusion system. We used an Axon 200B patch-clamp amplifier (Axon Instruments, Novato, CA, USA) to record membrane voltage and current. Experimental traces were digitized (0.2 msec sampling rate) on a VCR (Panasonic, Milano, Italy). Data were analyzed on a PC computer after filtering at 1000 Hz using pClamp 7 programs (Axon Instruments, Novato, CA, USA). For electrophysiological measurements in the presence of an electromagnetic field, the experimental chamber was encircled with an isolated multispiral copper wire to calibrate each field intensity.

Ca was monitored with Indo-1 fluorescence as previously described (Grynkiewicz et al., 1985).

Results

The effects of different field intensities on the growth and differentiation of NG108-15 neuroblastomaXglioma cells are shown in Fig. 1. The cell proliferation rate is altered by the magnetic field only if the cells are chemically induced to differentiate using BT2cAMP. Cell growth inhibition by the differentiating agent was relieved by field strength of between 120 and 240 microTesla (μT). In contrast, the behavior of cells is not statistically different from that of untreated controls at lower (60 μT) or higher (360 μT) field intensities (Fig. 1A). In particular, after 96 hours of growth, cell cultures subjected to a 120 or 240 μT field reached higher densities, of 20-30% more than the non-stimulated NG108-15 cells, whereas at 360 μT there was no statistically significant variation in cell numbers. Undifferentiated cells showed the same growth rate and final cell density as control cells at every field intensity tested. Thus in our differentiating cell line some of the effects on proliferation of the interaction between the electromagnetic field and differentiation reached their maximum in a specific window of field intensities. To monitor the field effects on the cell cycle of NG108-15 cells we performed flow cytometric analysis of cell DNA content during the differentiation process (Fig. 1B). Three days after the addition of BT2cAMP (96 hrs of growth) most control cells (0 μT) were found in the G1 phase (71 $\pm$ 5 %; n = 6), whereas the S compartment was nearly empty. 29 $\pm$ 0.7 % (n = 6) percent of cells showed a 2C DNA content, as part of the population was still dividing (see Fig.1A, left). In comparison, under a 120 μT 50Hz magnetic field a lower percentage of neuroblastoma cells was reproducibly found in the G1 phase (57 $\pm$ 4 %; n = 6). The population showed a remarkable accumulation of cells in both the S and G2/M phases (13 $\pm$ 1 and 30 $\pm$ 3 % respectively; n = 6) (Fig 1 B). Notably, after 3 days BT2cAMP stimulated the growth of long, branched neurites, while these morphological changes were almost completely inhibited by exposure to a 120 μT field (Fig. 1 C). Furthermore, in these conditions the average size of the cell bodies in populations subjected to the 120 μT field was larger than in controls, as can be directly appreciated in Fig.1C and as indicated by a 15-30% increase in average FSC values (Fig. 1 B). Taken together with the increase in cell numbers, DNA content and cell morphology this observation further indicates that proliferation is not inhibited by the differentiating molecule. Significantly, cells exposed to 360 μT showed the same DNA/cell size profile as well as the same morphology as control cells (Fig.1B, right, and Fig.1C), fully confirming that the effects of the magnetic field are not proportional to the intensity but are confined to an intensity window. In conclusion, the differentiating effects elicited by BT2cAMP on NG108-15 cells can be inhibited by an electromagnetic field of between 120 and 240 μT. We obtained similar results using Retinoic Acid (RA) and DMSO as inducers of differentiation (Wanke et al., 1979) (Olivotto et al., 1996). Figure 2 shows cell growth curves obtained in the presence of 10 μM RA (Fig. 2 A) or 50 mM DMSO (Fig. 2 B) with or without the presence of a 120 μT EMF. In both cases the field has a similar effect on cell growth as that already described for BT2-cAMP and reported in Figure 1 A. To strengthen the hypothesis that magnetic fields interfere with the mechanism of differentiation by maintaining cells in a cycling state we repeated the experiment of cell growth in the presence of an EMF using a synchronized cell population. By following the growth rate of synchronized undifferentiated cells we were able to establish the time lapse between release from the synchronized procedure (arrow in Fig. 2 C) and the first cell division (Fig. 2C solid triangle). BT2-cAMP-stimulated cells remained almost unchanged during this interval (Fig. 2C solid circle). Cells in the presence of the differentiating agent and subjected to a 50 Hz 120 μT EMF showed similar behavior to undifferentiated cells (Fig. 2C solid square).

Most differentiating agents, whether polar or apolar (Wanke et al., 1979), are thought to interfere with growth factor-dependent pathways by acting on surface charges present on the cell membrane (Arcangeli et al., 1987;Olivotto et al., 1996). In undifferentiated NG108-15 cells membrane potential values (V_{rest}) (Ferroni et al., 1996) show a multiple Gaussian

distribution that identifies three main subgroups (Fig. 3 A), possibly related to different cell-cycle phases (Binggeli and Weinstein, 1985;Dubois and Rouzaire-Dubois, 1993). On average (n = 183) the lowest potential recorded in cells cultured for 24 hours was about -50 mV. Acute application of the differentiating agent BT2cAMP induced membrane potential hyperpolarization in the cells with a more depolarized V_{rest}. RA and DMSO act in the same way (data not shown), according with the similarity between the results presented in Figure 1 A and Figure 2 A and B. These cells were driven to a membrane potential of –50 mV within 10-15 minutes (Fig. 3 B). Fig 3 C compares the averages of 16 experiments in which 8 cells (solid circles), in addition to stimulation with BT2cAMP (open circle), were exposed to 1 mM TEA and 100 mM Apamin to block Ca-activated K-channels (K_{Ca}). The plot shows the rate of V_{rest} over the initial membrane potential value (V_{rest-I}) during 13 minutes of continuous recording. On average hyperpolarization was fully abolished by the application of K_{Ca} blockers, suggesting a mobilization of intracellular calcium due to the action of the differentiating agent. Voltage-gated Ca channels did not appear to be directly related to this phenomenon, since the application of 1 mM cadmium and 1 mM nickel was not able to prevent BT2-cAMP-induced hyperpolarization (data not shown).

The change in the membrane resting potential due to BT2-cAMP can be correlated with the initial events that occur during commitment to differentiation (Binggeli and Weinstein, 1985;Arcangeli et al., 1993). Since a 120 µT field is able to prevent BT2-cAMP-induced differentiation, we investigated whether an ELF-EMF field might act primarily by affecting V_{rest} hyperpolarization. Figure 4 A shows how, following BT2cAMP application (open square), cell hyperpolarization is completely abolished by the constant presence of an ELF-EMF at 120 µT intensity (Fig.4 A, filled square). In Fig. 4 B the average results obtained in 14 cells (7 control, solid circles, versus 7 ELF-EMF-treated cells, open circles) are shown. In all cases the 120µT field suppressed the hyperpolarizing effects of BT2cAMP.

The question arises about the possibility that the prevention of BT2-cAMP hyperpolarization by an EMF is mediated by an opposite action on the surface charges. We therefore performed experiments to test the influence of adding to the external milieu two ions that are known to act, *inter alia*, on the surface charges of the cell membrane (Kajimoto and Kirpekar, 1972;Zipes and Mendez, 1973;Dorrscheidt-Kafer, 1981;Sanguinetti and Jurkiewicz, 1990). Figure 5 shows the changes induced by 10 µM La^{+++} (Fig. 5 A) and Mn^{++} (Fig. 5 B) in the membrane resting potential of NG108-15 cells and the consequent action of 120 and 240 µT 50 Hz EMFs. Both La^{+++} and Mn^{++} -induced depolarization was enhanced by increasing the strength of the field. However, the present results were inconsistent and repeatable only in 50% of the experiments (7 out of 14 trials). In the others, the action of the di- and trivalent ions either produced no effect (n = 4), or induced slight hyperpolarization (n = 2) or 70 mV depolarization (n = 1). For our experiments we then used the 50% of cells in which BT2-cAMP and La^{+++} independently and reproducibly provoked a change in V_{rest}. In the sequence of Fig. 5 C we show the hyperpolarizing effect of BT2-cAMP, the null combined action of BT2-cAMP and a 120 µT ELF-EMF, the depolarizing effect of 10 µM La^{+++}, followed by the enhanced action of an EMF. The simultaneous presence of the differentiating agent and the trivalent cation did not change the cell membrane potential. In the latter conditions the application of a 120 µT magnetic field caused slight depolarization. The time interval between different stimulation procedures was 10 minutes in each experiment presented in Fig. 5.

In contrast to a 120 µT ELF-EMF, the application of a 360 µT field did not alter the hyperpolarization induced by the differentiating molecule. This result is in agreement with the data obtained on growth curve experiments (Fig. 1). In the example of Fig. 6 A we show that the hyperpolarization induced by the contemporary presence of BT2-cAMP and a 360 µT field (open squares) can be eliminated by 1 mM TEA and 100 mM Apamin (filled squares), confirming the involvement of K_{Ca} in the modification of the membrane potential.

Significantly , a 360 µT ELF-EMF by itself (Fig. 6 B, open circles) was able to reproduce the hyperpolarization due to the differentiating agent (solid circle). However, although the final target is the same, namely the K_{ca} current, the mechanism of intracellular calcium mobilization promoted by a 360 µT 50 Hz field appears to be different. In particular, 10 mM Nifedipine (Fig. 6 C, solid squares) prevents the hyperpolarization due to a 360 µT ELF-EMF (Fig. 6 C, open circles), contrary to what we observed in the case of BT2-cAMP-induced hyperpolarization, as previously stated. 1 mM nickel and 1 mM cadmium, or total removal of extracellular calcium also impaired hyperpolarization at a field intensity of 360 µT (data not shown). In Fig. 6 D we report experimental averages in the different conditions (BT2-cAMP stimulation n = 7: open circles; 360 µT ELF-EMF n = 8: open squares; 360 µT ELF-EMF, Nifedipine block n = 12: solid squares). To provide further support for the hypothesis of a calcium ion-mediated effect during electromagnetic field exposure, we constructed growth curves of NG108-15 cells in the presence of an EMF and low external calcium (Fig. 7). In this case the difference in cell growth observed in the experiments of Fig 1 B is present not only at intensities of 120 (Fig. 7 A) and 240 µT (Fig. 7 B) ELF-EMFs, but also at 360 µT (Fig. 7 C). Figure 7 D depicts the comparison between cells cultured in the constant presence of a magnetic field with 1.8 (gray bars) and 0.1 (black bars) mM external calcium (n = 6).

The results reported in Figures 6 and 7 together with previous studies (Goodman et al., 1995) argue in favor of an interaction between magnetic fields and the mechanisms that regulate intracellular calcium mobilization. Measurement of the intracellular calcium signal by Indo-1 fluorescence during ELF-EMF irradiation (Grynkiewicz et al., 1985) could furnish direct evidence of the dynamics of the divalent ions under the influence of magnetic fields. Intracellular Ca concentration rose modestly if a 120 µT magnetic field was applied (Fig. 8 A, n = 18). The increase was much greater under a 360 µT field (n = 4). When we changed the intensity of the field applied to a single cell (Fig. 8 B) the increase in intracellular calcium was 3 times greater at 360 µT than at 120 µT.

We investigated the possibility that BT2cAMP and a 120 µT 50 Hz magnetic field could interact antagonistically during the differentiating process by affecting the membrane surface charges. To this end we monitored the activation properties of the low-threshold Ca current (Becchetti et al., 1992) in undifferentiated NG108-15 cells challenged by BT2cAMP and/or an electromagnetic field. In the example in Fig. 9 A, currents were recorded with or without a voltage step (from −50 to +10 mV) during continuous cell exposure to a 120 µT field. Ca current was obtained by subtracting the trace in the absence of the voltage challenge (bottom). This procedure was used to construct the activation curve of a low-threshold Ca current in the presence of a 120 µT field (Fig. 9 B). The comparison with control cells (n = 7) and cells treated with BT2cAMP (n = 5) showed that the magnetic field and the differentiating agent had opposing effects, shifting the activation curves respectively to more positive or negative potentials (top). This was confirmed in cells simultaneously exposed to BT2cAMP and a 120 µT field in which the shifts were not observed (n = 4) (bottom). The effect of an ELF-EMF on surface charges is proportional to the field intensity. Fig 9 C shows that the shift of an L-type Ca-current activation curve towards a more negative potential averages 4 ± at 120, 5.6 ± at 240 and 7.4 ± at 360 µT.

Discussion

In spite of growing concern about electromagnetic radiation, the physical mechanisms of interactions between 50/60 Hz fields and biological structures remain obscure. So far biophysical models can provide only indications of the mechanisms underlying the reported field effects (e.g local field effect at the cell membrane, including interference with the surface charges of the plasmalemma or with ligand-receptor binding) (Walleczek, 1992). Most investigations have been carried out on immune system cells since epidemiological studies have shown a correlation between exposure to ELF-EMFs and childhood leukemias (Lacy-Hulbert et al., 1995). These studies indicated the Ca signal as a plausible candidate for the mediation of ELF-EMF effects on cell processes. However the action of magnetic fields on Ca regulation at the molecular level is still poorly understood. Many reports have demonstrated that ELF-EMFs do not affect the extent of Ca release from intracellular stores, but increase Ca influx through the plasma membrane (Karabakhtsian et al., 1994;Liburdy, 1992;Fanelli et al., 1999).

In this paper we have tried to accumulate data to support the hypothesis that extremely low electromagnetic fields act primarily on the surface charges of the cell membrane. Our results show that ELF-EMFs: (i) disturb the action of several differentiating agents (Fig. 2); (ii) enhance the interaction of La^{+++} and Mn^{++} with the cell membrane (Fig. 5); (iii) modify intracellular Ca and Ca-related mechanisms in a dose-dependent way (Fig. 9). A magnetic field of 50 Hz, which approximates the magnitudes measured in the proximity of household appliances or electric power lines (source http://www. hsph.harvard.edu /Organizations /Canprevent/emf.html), is probably not sufficient to trigger primary cell functions directly. We think it more probable that, by acting on cell membrane surface charges the magnetic field acts as a destabilizing agent of complex protein-to-protein interactions that occur, for example, during the cell differentiation process, although at present we have no reason to believe that this is the only mechanism through which ELF-EMFs may interact with biological material. A direct action on single proteins and/or the activation of ion transporters could reasonably take place. Alternatively, magnetic fields could have an as yet unidentified effect on water molecules. Regardless of the mechanisms involved, the final conclusion of our work is that a repeatable and consistent interaction between ELF-EMFs and some biological functions does exist.

The results we obtained concerning the opposite effects on Ca current activation-curves promoted by an ELF-EMF and by the differentiating agent (Fig. 9), together with the data obtained using La^{+++} and Mn^{++} (Fig. 5), could be a sufficient indication that the field interacts with the surface charges on the plasma membrane. Our conclusion is supported by the study of Arcangeli and colleagues (Arcangeli et al., 1993) showing the modulation of surface charges by different classes of differentiating agents. In order to demonstrate the hypothesis of an interaction between surface charges and ELF-EMFs, we directed our attention to the effects that changes in the surface potential may have on Ca-dependent processes. We therefore attempted to understand which Ca permeation pathway was directly involved. Specifically, we considered the role of voltage-gated calcium channels in the calcium protection of cell differentiation during exposure to an ELF-EMF. The model we suggest to explain the effect of a low magnetic field on induced differentiation of the NG108-15 cell line is the following: (i) growth factors contained in fetal calf serum are able to promote depolarization-repolarization of membrane potentials fundamental for progression of the cell-cycle (Arcangeli et al., 1987); (ii) a coupling between growth factor receptors and various effector proteins allow regulation of the Ca influx through an undetermined molecular pathway. As a consequence of Ca influx, the K_{Ca} opens, leading to cell hyperpolarization. When cytoplasmic buffers lower intracellular calcium, the K_{Ca} closes and the cell again depolarizes (Conley E.C., 96 A.D.); (iii) differentiating agents slow down the depolarization-

repolarization process of membrane potentials adsorbing at the charged surface. The inducer first affects the Ca influx, which then activates the K_{Ca} (Arcangeli et al., 1987;Conley E.C., 96 A.D.).

Membrane potential measurements, obtained in the constant presence of complete culture medium, showed hyperpolarization during acute application of 5 mM BT2-cAMP (Fig. 3 B). The effect was detectable in a cell subpopulation with membrane resting potential more depolarized than –50 mV. In particular the cell population with a membrane resting potential of –30 mV is likely to be in a specific cell-cycle phase and can be identified using the "inducible" cells reported in the paper by Arcangeli et al. 1987. It has been previously suggested that chronic hyperpolarization of the membrane potential might actually inhibit progression through cell cycle (Binggeli and Weinstein, 1985). In our experiments we show that the acute application of Bt-cAMP results in a sustained hyperpolarization, as large as cells are depolarized. In the presence of Bt2-cAMP the resting membrane potential is held at value close to –50 mV, possibly impairing all the cell cycle process that would respond only to a change in membrane potential (Binggeli and Weinstein, 1985). Base on this view, the ultimate effects of Bt2-cAMP on cell replication would therefore take at last a period of time close to the Td (duplication time) to be manifested.

This idea is supported by our experiment on synchronized cells (Fig. 2). Growth curves split between EMF stimulated cells and controls occurred in the time lapse of the first cell division. Apamine / TEA, prevented the hyperpolarization (Fig. 3 C), in agreement with the role of the K_{Ca} in a depolarization- repolarization process modulated by the differentiating agent (Conley E.C., 96 A.D.). Experiments reported in our study show that neither nickel /cadmium or Nifedipine prevented the hyperpolarization induced by BT2-cAMP. In Fig.9 we show that BT2-cAMP shifts the activation curve of low-threshold Ca current to more positive potentials (+10 mV) in agreement with its effect on surface charges (Arcangeli et al., 1993). This result indicates that at –30 mV, the mean resting potential of a BT2-cAMP-responsive cell population, the current carried by the voltage-gated calcium channel is reduced in the presence of BT2-cAMP, and voltage-dependent calcium currents can therefore not account for activation of the K_{Ca} .

This last consideration suggests that in our system BT2-cAMP interferes with the surface potential, and indirectly increases K_{Ca} activity, modulating the Ca influx not through voltage-gated calcium channels. It was previously stated that extracellular calcium mediated the depolarization-repolarization process rather than the release of calcium from the stores (Arcangeli et al., 1987). In this case, the shift in the activation curve of L-type calcium channels promoted by BT2-cAMP is only an indirect effect of changes in the surface charges with no relevance for the mechanisms underlying BT2-cAMP-induced hyperpolarization.

120 µT ELF-EMF completely prevents BT2-cAMP-induced hyperpolarization (Fig.4). The magnetic field appears to cause a shift in the surface potential in the opposite direction to that caused by BT2-cAMP. In the experiment in Fig. 5 C we produce direct evidence supporting this behavior. The contemporary presence of La^{+++} and BT2-cAMP completely abolish the change in membrane potential. Furthermore, the effect of ELF-EMF, applied in these experimental conditions, promoted a slight depolarization (Fig. 5 C_e), increasing the effect of La^{+++} . Further experimental evidence, probably of greater physiological significance, was obtained using L-type calcium currents as voltage sensors of surface potential. Since the shift in the activation curves promoted by BT2-cAMP is not directly related to hyperpolarization, the primary effect of a 120 µT ELF-EMF may counteract the adsorption of BT2-cAMP to the surface charges. A 360 µT ELF-EMF, in contrast, was able to hyperpolarize the cell both in the presence and in the absence of the differentiating agent (Fig. 6 B). Hyperpolarization was prevented by Apamine/TEA (Fig. 6 A) as well as by Nifedipine (Fig 6 C) or by Nickel /Cadmium (data not shown). This is the major factor indicating that, in the presence of a 360 µT ELF-EMF, the magnitude of the shift in the surface potential is able to increase the amount of the Ca current through L-type channels. The increment is likely to

be sufficient to activate K_{Ca}, independently of the mechanisms that couple growth factor receptors and Ca influx. We were able to separate the calcium influx dependent on growth factor stimulation and the calcium permeating through L-type channels because in undifferentiated NG108-15 cells the density voltage-dependent calcium current is very low (Lukyanetz, 1998). Although 120 µT ELF-EMF shifts the activation of the calcium current, the current density is too low to activate K_{Ca} independently of BT2-cAMP. At 360 µT, this shift is grater and an additional mechanism is therefore turned on, antagonizing the primary effect on differentiation due to ELF-EMF interaction with surface charges.

In conclusion, we can say that the L-type voltage gated calcium channel primarily mediates the protection by Ca of differentiating neuroblastoma cells from EMF insults.

Our initial hypothesis was that the magnetic field could interfere with cell division, differentiation, and membrane voltage fluctuations, possibly by altering intracellular Ca concentration.

In our study we demonstrate that a 50/60 Hz magnetic field interacts with cell differentiation through two opposing mechanisms. ELF-EMF is able to prevent the shift in surface charges potential promoted by differentiating agents. Simultaneously, it stimulates the increase in intracellular calcium in a dose-dependent manner. The increase in cytoplasmic divalent ions, by opening the K_{Ca}, acts as a rescue agent reestablishing cell's commitment to differentiation. The peculiarity of NG108-15 cells (Lukyanetz, 1998) offered the possibility to dissect these two opposing mechanisms. The simultaneous onset of both mechanisms prevents alterations in differentiation. We propose that cells are normally protect against electromagnetic insult. The scenario just described might be very different in cells with an efficient Ca permeability system (Brown and Higashida, 1988a;Brown and Higashida, 1988b) In these cases, the onset of Ca-dependent hyperpolarization induced by the magnetic field would be expected to be more efficient and the above mechanisms difficult to dissociate. The inhibition of differentiation exerted at particular field intensities could be compensated by the ability to mobilize Ca ions. On this basis, Ca signaling may be seen as an agent protecting against damages produced by exposure to electromagnetic fields. In the presence of even silent alterations in mechanisms acting on intracellular Ca or K_{Ca} channels, chronic exposure to magnetic fields might induce pathological conditions. These considerations may help to explain the contrasting results obtained in epidemiological studies, in which no significant correlation between electromagnetic exposure and pathological insurgency could be found (Reipert et al., 1997;Lacy-Hulbert et al., 1998;Hatch et al., 1998;Day, 1999).

Figure legends

Figure 1. Effects of a 50 Hz electromagnetic field on a differentiating neuroblastoma cell line. A. Growth curves of NG108-15 cells in the absence (dotted line) and presence (continuous line) of a 120 µT field (cell plated at *time = 0*). Cells were undifferentiated (left) or supplied with 1 mM BT2cAMP (right) after 24 hours (arrow). Right part: relative increase in cell numbers after 96 hours in BT2cAMP-treated populations. The ratio between the cell numbers reached by cultures at the indicated field intensities and that observed at 0 µT is reported (n = 25). B. Distribution of Cell DNA content in differentiating cells exposed to different intensity fields. Flow cytometric analyses were performed on cell samples taken after 72 hours in the presence of BT2cAMP (96 hours of growth). Frequency histograms of PI-emitted fluorescence intensity (FL2-Height; proportional to the cell DNA content) were obtained from parallel and independent cultures at the indicated field intensities (6 cultures at 0 and 120 µT; 5 cultures at 0 and 360 µT). Signals due to dead cells, cell aggregation and/or polyploidization contributed to about 10% of the recorded distributions and were discarded before calculating the cell frequencies in G1, S and G2/M (see text). Two representative examples are also shown as density plots between FL2-Height and the intensity of Forward Light Scatter (FSC-Height; proportional to cell size). A warmer color indicates more events and and a cooler color fewer events. C. Phase contrast pictures of differentiating NG108-15 cells exposed to different intensities (0, 120 and 360 µT) of electromagnetic field and supplied with BT2cAMP for 72 hours.

Figure 2. A and B: growth curve of NG108-15 cells obtained in the presence of 10 µM RA (Fig. 2 A) or 50 mM DMSO (Fig. 2 B) with or without the presence of a 120 µT EMF. C: cell growth in the presence of an EMF using a synchronized cell population. Undifferentiated cells were used to establish the time of the first duplicating/duplication? cycle after release from the synchronized procedure (▲solid line). BT2-cAMP-stimulated cells did not change in number during this interval (■broken line). Cells to which the differentiating agent was added and a 50 Hz 120 µT EMF delivered show similar behavior to undifferentiated cells (●dotted line).

Figure 3. Effects of BT2cAMP and a magnetic field on the resting potential of undifferentiated NG108-15 cells. A. Distribution of cell membrane potential values after 24 hours in culture (n = 183). Gaussian fitting identifies three main subgroups. B. Membrane potential changes induced by 5 mM BT2cAMP (supplied at time zero) in the three subgroups. Potentials were monitored during whole-cell current-clamp experiments for 20 minutes (n = 28). The serial resistance value was systematically controlled. C. Averages from experiments in the presence of 5 mM BT2cAMP (O) and during the simultaneous application of 5 mM BT2cAMP and 5 mM Apamine and 1 mM TEA (●) plotted as the ratio between the resting potential at any recorded time (V_{rest}) and the initial resting potential (V_{rest-i}).

Figure 4. Effects of a 120 µT ELF-EMF on BT2-cAMP-induced hyperpolarization. A. Membrane voltage recording performed on a single cell initially showing a −30 mV resting potential in the presence (O) or not (●) of a 120 µT electromagnetic field. B. Average results obtained with more cells exposed (O) or not (●) to the field as the ratio between the resting potential at any recorded time (V_{rest}) and the initial resting potential (V_{rest-i}). Recording was made by whole-cell (n = 3) or perforated-patch experiments (n = 2).

Figure 5. Effects of La^{+++} and Mn^{++} on cell membrane potential. The figure depicts the changes induced by 10 µM La^{+++} (Fig. 4 A) and Mn^{++} (Fig. 4 B) in the membrane resting potential of NG108-15 cells and the increment in depolarization caused by 120 and 240 µT 50 Hz EMFs. Fig. 4 C shows a sequence of: hyperpolarizing effect of BT2-cAMP, null action of

BT2-cAMP when a 120 µT ELF-EMF is simultaneously present, depolarizing effect of 10 µM La^{+++}, enhanced action of EMF, no changes in the membrane potential during the simultaneous presence of BT2-cAMP and La^{+++} and slight depolarization caused by a 120 µT ELF-EMF applied in the latter conditions.

Figure 6. Effect of a 360 µT ELF-EMF on undifferentiated NG108-15 cells. A. Single-cell measurements in the simultaneous presence of 5 mM BT2-cAMP and a 360 µT magnetic field with (■) or without (□) the addition of 5 mM Apamine and 1 mM TEA in the bath solution. B. Comparison between the cell membrane hyperpolarization induced by 5 mM BT2-cAMP (●) and by a 360 µT ELF-EMF (○). C. 10 µM Nifedipine (■) completely prevents the hypepolarization produced by exposure of the cell to a 360 µT ELF-EMF (○). D. Relative membrane potential changes. 0 µT (○, n = 7) , 360 µT (□, n=8) or 360 µT field + Apamine + TEA (■, n = 12).

Figure 7. Growth curves of NG108-15 cells in the presence of an EMF and low external calcium. The difference in cell growth between cells exposed to a magnetic field and controls is always present : at 120 µT (Fig. 7 A), at 240 µT (Fig. 7 B) and at 360 µT (Fig. 7 C). Figure 7 D depicts the comparison between cells cultured in the constant presence of a magnetic field with 1.8 (gray bars) and 0.1 (black bars) mM external calcium (n = 6).

Figure 8. Effect of an electromagnetic field on intracellular calcium. A. Intracellular calcium in a single cell subjected to a 120 µT field intensity. B. Intracellular calcium increased in a single cell sequentially exposed to 0, 120, 360 and again 0 µT intensity fields; data were fitted by linear regression for each interval at the specific field intensity.

Figure 9. Low-threshold Ca current in cells challenged by an electromagnetic field and BT2cAMP. A. Example of whole-cell Ca current recordings during continuous exposure of cells to a 120 µT field (bottom). Current traces were obtained by digital subtraction after (top, thin line) and before (top, thick line) the application of a voltage step (from holding potential of −50 to + 10 mV). B. The top panel depicts activation curves of low-threshold Ca current obtained in control cells (n = 7) (●), after perfusion with 5 mM BT2cAMP (n = 5) (▲) and in the presence of a 120 µT field (n = 5) (■) (in this case using the method described in *a*). The lower panel shows low-threshold Ca current activation curves in control conditions (●) and during the simultaneous presence of a 120µT electromagnetic field and 5 mM BT2cAMP (n = 4) (■). C. Results of 5 experiments in which the activation-curve negative shift was calculated and plotted as a function of the increasing electromagnetic field delivered.

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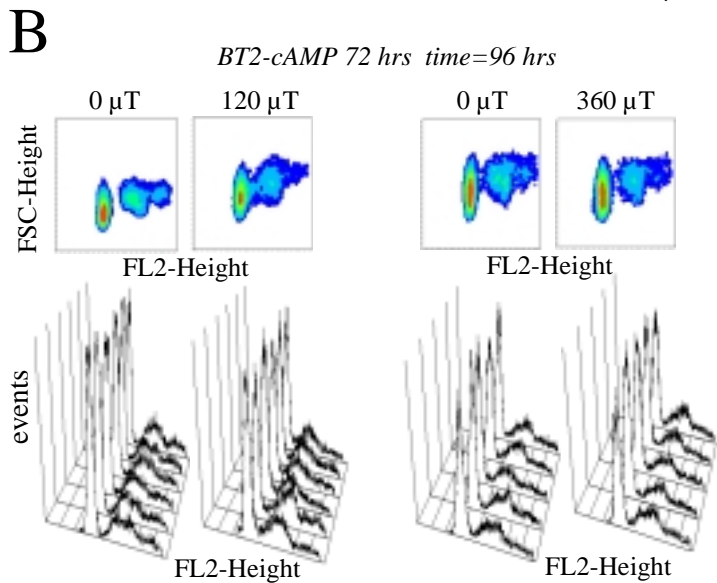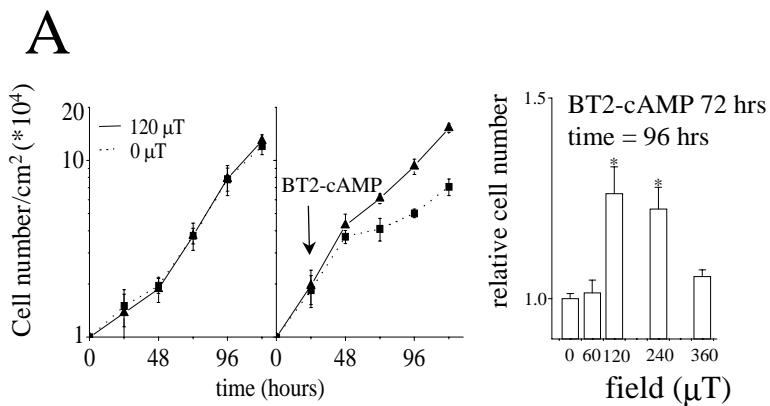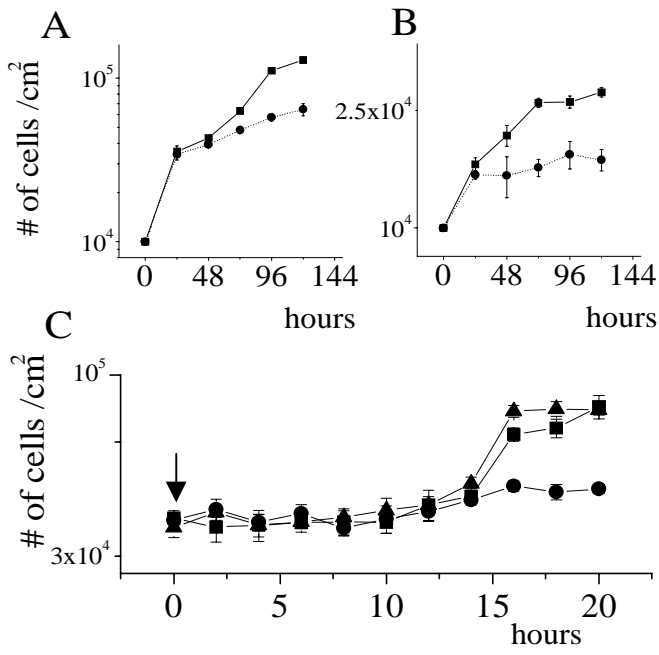
A

Cell number/cm² (*10⁴)

— 120 µT
···· 0 µT

BT2-cAMP

time (hours)

relative cell number

BT2-cAMP 72 hrs
time = 96 hrs

field (µT)

B

BT2-cAMP 72 hrs time=96 hrs

0 µT   120 µT   0 µT   360 µT

FSC-Height

FL2-Height   FL2-Height

events

FL2-Height   FL2-Height

C

BT2-cAMP 72 hrs time=96 hrs

0 µT   120 µT   360 µT

Figure 1

Figure 2

A

20

frequency count

0

-50   -30   mV

B

V$_{rest}$

-30

-40

-50

0   300 600 900

sec

C

V$_{rest}$ / V$_{rest-i}$

1.5

1.0

400 sec

Figure 3

A

V_{rest}

-30

-40

-50

400 sec

B

V_{rest}/V_{rest-i}

1,5

1,0

400 sec

Figure 4

A

La⁺⁺⁺   La⁺⁺⁺ 120 μT   La⁺⁺⁺ 240 μT   V_rest

B

Mn⁺⁺   Mn⁺⁺ 120 μT   Mn⁺⁺ 240 μT   V_rest

200 sec

C

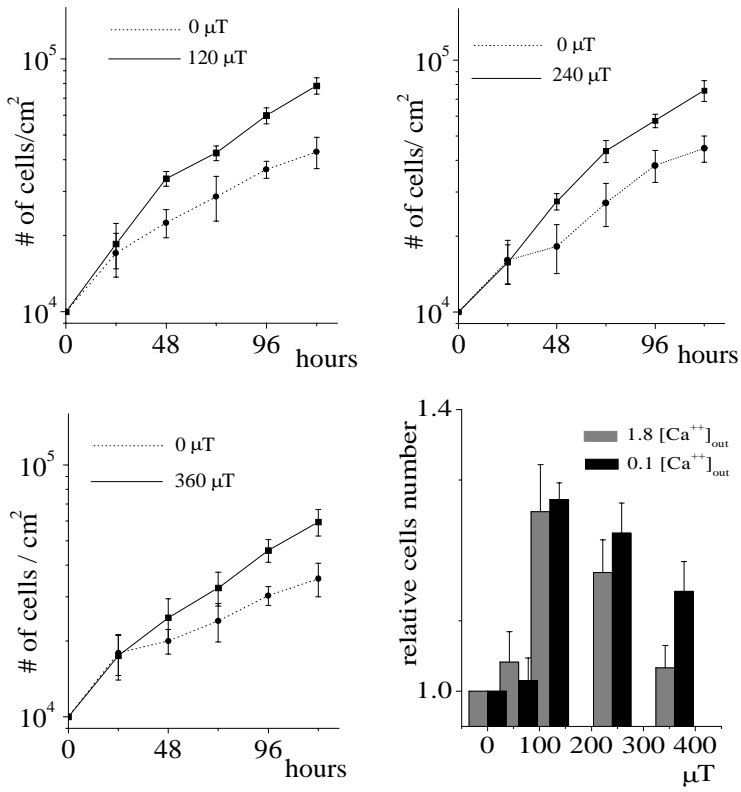BT2-cAMP   BT2-cAMP 120 μT   La⁺⁺⁺   La⁺⁺⁺ 120 μT   BT2-cAMP La⁺⁺⁺   BT2-cAMP La⁺⁺⁺ 120 μT   V_rest

500 sec

Figure 5

Figure 5

Figure 6

Figure 7

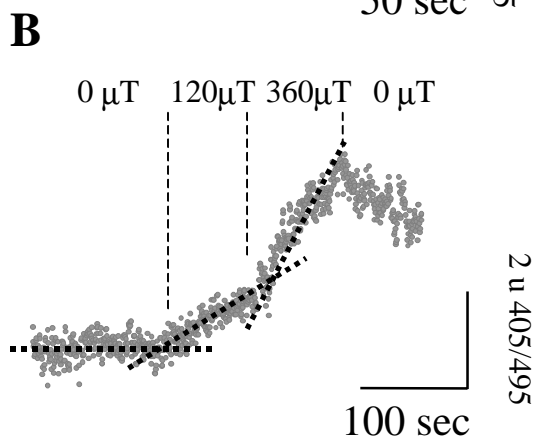**A**

120 µT

5 u 405/495

50 sec

B

0 µT   120µT 360µT 0 µT

2 u 405/495

100 sec

Figure 8

A

B

500 pA

100 pA

50 msec

1,2

I/Imax

120 µT

control

BT2cAMP

0,4

0,0

-60 -30 0 30

1,2

I/Imax

control

120 µT +
BT2cAMP

0,4

0,0

-60 -30 0

V_rest (mV)

C

magnetic field (µT)

100 200 300 400

-4

-6

-8

shift (mV)

Figure 9