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In vitro increase of the fluid-phase endocytosis induced by pulsed radiofrequency electromagnetic fields: importance of the electric field component

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Abstract

Nowadays, due to the wide use of mobile phones, the possible biological effects of electromagnetic fields (EMF) become a public health general concern. Despite intensive research, there are no widely accepted theories about the interactions between EMFs and living cells, and the experimental data are often controversial. We examined the effects of mobile phones EMF (envelope frequency of 217 Hz, carrier frequency of 900 MHz and pulse duration of 580 μs) or its pure, low-frequency pulsed electric field component on fluid-phase endocytosis. In both cases, with exposures exceeding 10 min, an increase of the fluid-phase endocytosis rate was observed ($\approx$ 1.5-fold), on three different cell types. This increase is an all-or-nothing type of response that is occurring for threshold values comprised between 1.3 and 2.6 W/kg for the delivered EMF powers and between 1.1 and 1.5 V/cm for the electric fields intensities depending upon the cell type. The electric component of these EMFs is shown to be responsible for the observed increase. Variations of frequency or pulse duration of the electric pulses are shown to be without effect. Thus, EMF, via their electrical component, can perturb one of the most fundamental physiological functions of the cells—endocytosis.

Keywords: Fluid-phase endocytosis; Electromagnetic field; Electric field

1. Introduction

Since epidemiological studies linked electromagnetic fields (EMF) to increased cancer risks, particularly leukemia [1] and brain cancer [2], the scientific interest in how EMFs generated by mobile phones, high-voltage power lines and other electrical devices might affect biological functions [3,4] and health [5] in humans is longstanding but conclusions are often controversial. The interpretation of the biological effects resulting from cells or animals exposure to EMFs remains difficult due to the dispersion of the biological models, the nonstandardized EMFs exposure, the uncertainty of biologically meaningful exposure metrics and the difficulties in rigorously performing the required controls [6,7].

Among the variety of biological responses induced by EMFs and electric fields, from DC to the GHz range, that have been reported are alterations in cell proliferation [8] and cell cycle [9], perturbations in cell migration [10] and adhesion [11], chromosome damage [12] and interactions

Abbreviations: EMF, electromagnetic field; LY, Lucifer Yellow; FD, FITC-Dextran; GSM, Global System for Mobile communications; CW, continuous wave; SAR, specific absorption rate

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[1] Deceased during the revision of the manuscript, which the authors dedicate to her memory.

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with the immune system [7]. The EMFs can also influence DNA synthesis [13], DNA damage [14] or RNA synthesis [15]. Under EMFs exposure, the expression of protooncogenes is altered, especially the "early response genes" such as c-fos [16], c-jun and c-myc [15] and the expression of heat shock proteins such as Hsp 70 [17] and Hsp 16 [18] is induced. Moreover, EMFs or electric fields are capable to reorganize the cytoskeleton [19], to increase intracellular calcium ion concentration [6], and to induce metabolic oscillations [20] and modifications in free radicals production [21].

As for effects at the level of the cell membrane, Bawin et al. [22] already showed in 1978 that radiofrequency fields with amplitude modulated by sinusoidal signals induced an increase of Ca^{2+} efflux from isolated chicken cerebral tissue. Low-energy EMFs were also reported to provoke membrane-related biological effects like the inhibition of bone cells response to parathyroid hormone [23], the enhancement of Ca^{2+} uptake in activated thymocytes [24] and the induction of cell-surface receptors redistribution [25] or transmembrane potential changes [26].

In the particular case of the brief and intense electric pulses leading to cell electroporation, it has been shown that, after cell electroporation, the endocytosis was stimulated for 5–120 min [27] and a long-lived macropinocytosis observed for up to 60 min [28]. Moreover, lower, non-electroporating electric fields (60–100 V/cm), applied for longer durations, induced an endocytotic-like process into different cell types and into photosynthetic membrane vesicles (thylakoids) [29].

The present study analyzes the effects of EMFs typically generated by mobile phones on the fluid-phase endocytosis of three different cultured cell lines, and the involvement of the low-frequency pulsed electric component corresponding to these EMFs, including the influence of the various electrical parameters. For the three cell lines, an increased fluid-phase endocytosis was observed, essentially characterized by a threshold value for electric field intensity, an all-or-nothing type response and the absence of delayed effects after the end of exposure.

2. Methods

2.1. Chemicals and cells

Fluid-phase endocytosis was quantified using 15 mM fluorescent Lucifer Yellow dipotassium salt (LY) in PBS without Ca^{2+} and Mg^{2+} (Life Technologies, Cergy-Pontoise, France) and fluorescent FITC-Dextran MW 71 000 (FD) (Sigma Chemical Co., St Quentin Fallavier, France) 1 mM in Eagle MEM (Life Technologies). Trypan Blue solution contained 0.08% trypan blue and 0.005% *p*-hydroxybenzoic methyl acid sodium salt in PBS (Sigma). Final concentrations of antioxidant mixture [30] in the medium were 1 mM sodium pyruvate (Life Technologies), 50 μM α-thioglycerol (Sigma) and 20 nM bathocuproindisulfonic acid disodium salt (Sigma).

The B16-F1 murine melanoma cells and the Chinese hamster DC-3F lung fibroblasts [31] were cultured at 37 °C, 5% CO_2, in Eagle MEM supplemented with 10% fetal calf serum (Life Technologies), 100 units/ml of penicillin and 125 μg/ml of streptomycin (Panpharma, Fougères, France). The B16-F1 and the DC-3F cell lines were kindly provided by Dr. J. Belehradek Jr., Villejuif, France. Human head-and-neck A253 carcinoma cells [31] were cultured in McCoy 5A medium (Sigma) supplemented with 10% fetal calf serum and antibiotics. The A253 cell line was kindly provided by Professor John S. Lazo, Pittsburg, PA.

2.2. Determination of LY or FD uptake

One day before the experiment, the cells were seeded into 35-mm Petri dishes or on cover slips (24×32 mm) in order to have a cell monolayer in exponential growth at the time of exposure (approximately 0.7×10^6 cells per dish or cover slip, except otherwise stated, for example for experiments dealing with the effects on confluent cells in stationary phase). Cells were incubated for various time durations (ranging from 10 to 90 min) with 2 ml of culture medium containing 2 mM LY or 1 mM FD, in the presence or the absence of the applied EMF. For exposure to pulsed electric fields (PEF), the cover slips with cells were placed into glass dishes and incubated with 4 ml of culture medium containing 2 mM LY or 1 mM FD, in the presence or absence of PEF. Immediately after the incubation, cell monolayers were rapidly washed three times with PBS, trypsinized, centrifuged (1000×*g*, 10 min, at 4 °C) and the cellular pellets resuspended in 1 ml PBS. An aliquot of 50 μl was diluted 1:1 with Trypan Blue solution in order to count the cells and to determine the cell viability. Then, the cell suspension was sonicated three times 15 s, at 100 W on ice (250/450 Sonifier, Branson Ultrasonics Corporation, Danbury, USA). The incorporated fluorescence representing the total fluorescence released by the disrupted cells was measured using a spectrofluorometer (SFM 25, Kontron Instruments, Herts, England). The excitation and, respectively, emission wavelengths used for fluorescence measurements were 423 and 525 nm for LY, and 485 and 515 nm for FD. The baseline was obtained for cells not exposed to the fluorescent dye. The fluorescence of cells for less than 5 s with LY or FD matched the baseline proving the good washing efficiency. Using a fluorescence standard curve and the number of cells per sample, the fluorescence values were converted into the number of molecules incorporated per cell. All experiments were performed at 31±1 °C, similar dye uptake being observed for temperatures ranging between 30 and 37 °C.

In Figs. 2–8, each data point represents the mean±S.E. of two to seven independent experiments, each experiment being performed in triplicate. For each figure, the individual values of the controls of all the corresponding

independent experiments were used to determine the control average value and the standard deviation. The uptake of the fluorescent dye of EMF- or PEF-exposed cells was calculated with respect to the average uptake of control cells considered to be 100%. The results are reported using histograms except for the figures dealing with time dependence of fluid-phase endocytosis. However, the results in Figs. 2A and 8A are represented also by histograms because the reported mean did not include the same number of samples (the corresponding experiments did not include all the incubation times). The statistical significance of the data (exposed vs. control cells) was determined using the unpaired Student's *t*-test (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

2.3. Exposure to radiofrequency EMFs

Two kinds of EMFs waves were used: (i) pulsed waves (GSM-EMF because they correspond to the EMFs delivered by the GSM mobile phones) consisting in 580-μs-long square pulses delivered with a low frequency envelope at 217 Hz (thus, with a cyclic ratio of 1/8) and with a high-frequency carrier sine wave at 900 MHz (Fig. 1A); (ii) continuous wave (CW-EMF) consisting in a high-frequency sine wave at 900 MHz (Fig 1B).

The signal generator RF S 900-60 (RFPA, Bordeaux, France) delivers these two kinds of signals at adjustable powers ranging from 1 to 7 W (mean incident powers) for GSM-EMF and from 1 to 56 W for CW (mean or peak incident powers being identical for the CW-EMF). EMFs were applied using a new in vitro exposure device specifically designed for studying the effects of the GSM signal on cultured cells [32]. This exposure system, a wire patch antenna measuring 15×15×2.9 cm, was designed in order to expose simultaneously up to eight samples (four columns of two superposed samples placed symmetrically on the floor of the wire patch antenna). The 35-mm Petri dish with the cultured cells was placed inside a 60-mm Petri dish, used as a sample holder. The same depth of 2.5 mm of the volume of medium was respected in both the 35- and the 60-mm dishes in order to preserve the continuity of the dielectric properties and to reduce the edge effects at the periphery of the 35-mm dish. Therefore, a fair homogeneity of the EMF was achieved for the whole of the exposed cell populations. The direction of the magnetic component of the GSM-EMF was parallel to the plates of the wire patch antenna and had an orthoradial symmetry around the wire, while the electric field component of the GSM-EMF was parallel to the wire, and thus normal to plate's surface. Due to the geometry of all these components, this wire patch antenna allowed an optimized utilization of the EMFs power produced by the generator since it presents a very good matching at 900 MHz [32].

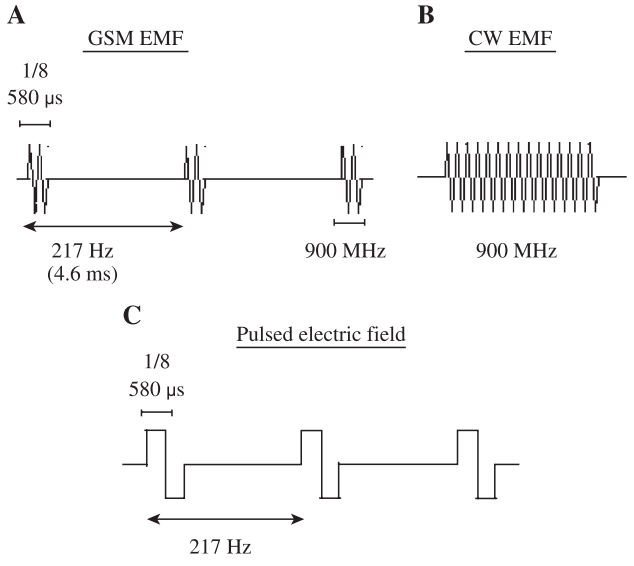

Fig. 1. Shapes and characteristics of the different signals used. (A) Electromagnetic fields generated by mobile phones (GSM-EMFs). (B) Continuous electromagnetic field (CW). (C) Pulsed electric fields (PEF).

The parameter chosen to quantify the amount of power absorbed per unit mass of culture medium is the specific absorption rate (SAR) defined as follows: $SAR=\sigma E^2/\rho$ (W/kg), where E is the root-mean-square local electric field strength, σ is the electric conductivity and ρ is the sample density. The theoretical calculations of SAR distribution were validated experimentally using local probes for electric field and thermal elevation [33]. The effectiveness of the exposure device was also determined by experimental measurements: in the case of GSM-EMF, the mean SAR measured was 0.65 W/kg for 1 W of incident power [33]. Therefore, the maximum power that it was possible to deliver under our experimental conditions was 4.55 W/kg absorbed power for 7-W incident power. It is important to note that the maximal intensity of the pure electric field component of the GSM-EMF could not exceed 2 V/cm (Table 1). The electric component intensity of the GSM-EMF was calculated using the equation of SAR and E for medium conductivity σ of 1.88 S/m (experimentally measured) [33] and medium density ρ of 1 kg/l.

In order to avoid generator disturbance due to the power reflected by the antenna into the wires, the system included a circulator (C3-900, Sodhy, France), which selectively directed the incident EMF power towards the wire patch antenna and the reflected power towards a wattmeter (RW GSM, ORITEL, France) and a 50-Ω resistor (CA 50 NH, Sodhy) that finally absorbed the reflected power. The wattmeter allowed the measurement of the reflected power; therefore, reflected power could be subtracted from the incident power. However, due to the good matching of the antenna, reflected power remained negligible compared to the incident power when the Petri dishes were correctly placed in the antenna.

For all experiments, cells were subjected to either EMF exposure or sham exposure, under identical experimental conditions, in particular regarding to the location of the dishes in the wire patch antenna and the location of the

Table 1
Correspondences between GSM- or CW-EMF incident power, mean SAR, peak SAR and associated local electric field strength

A	B	C	D	E
GSM signal: incident power (W)	GSM signal: mean SAR (W/kg)	GSM signal: peak SAR (W/kg)	CW signal: incident power (W)	Electric field strength (V/cm)
0.12	0.08	0.6	1	0.26
0.5	0.32	2.6	4	0.53
1	0.65	5.2	8	0.74
2	1.30	10.4	16	1.05
3	1.95	15.6	24	1.29
4	2.60	20.8	32	1.49
4.5	2.92	23.4	36	1.58
5	3.25	26.0	40	1.66
6	3.90	31.2	48	1.82
6.5	4.22	33.8	52	1.90
7	4.55	36.4	56	1.97

Column D—CW signal: incident power to deliver the peak SAR in column C. Column E—Peak (GSM) or mean (CW) electric field strength component of the EMF signal.

antenna in the incubator. Importantly, media temperature was measured immediately after each experiment, using a digital thermometer: no significant rise in temperature was found at the powers used in the experiments, in agreement with the theoretical calculations.

2.4. Exposure to pulsed electric fields

Cells were exposed to low-intensity electric fields consisting of bipolar symmetric square pulses. A bipolar signal was chosen to avoid electrophoresis and to minimize electrochemical reactions at the electrodes. Comparison with GSM signal was performed using runs of bipolar symmetrical square pulses of 580 μs for half pulse duration, delivered at a frequency of 217 Hz (Fig. 1C) (which corresponds to a cyclic ratio of 1/8 for the positive part of the pulse). The electric field strength of the positive part of the pulses was varied from 1.2 to 8 V/cm, pulse duration from 75 to 580 μs, frequency from 50 to 400 Hz and total exposure duration from 5 to 90 min. Electric field strengths were chosen on purpose in the same range of values as the strengths of the electric fields associated to the GSM-EMFs.

The electric generator (Micro I, CELTEM, Antony, France) was placed outside the incubator and a digital storage oscilloscope (VC-6025, Hitachi Denshi Ltd., Japan) was used to check the characteristics of the effectively delivered signal. Measurements of the electric current intensity were performed using a voltage probe (AT-10 AP, Hitachi Denshi) plugged to the oscilloscope.

To expose the cells to PEF, the cover slips with cultured cell on were placed into hand-made glass dishes (surface 5×4 cm) containing the electrodes (i.e., two parallel Pt/Ir 90%/10% rods inserted into the dish glass wall at 4-cm distance) [34]. Because of the geometry of this exposure chamber, the field lines perpendicular to the electrodes and parallel to the cover slip surface were quite uniformly distributed over the whole cover slip, placed equally distant between the electrodes. Therefore, all cells on the cover slip were exposed to the same strength electric field, the value of which could be approximated by the ratio of the applied voltage to the inter-electrodes distance.

The controls were incubated under identical experimental conditions less the electric field exposure. Immediately after the end of the electric field or of the sham exposure, the temperature was measured using a digital thermometer, and no significant increase (i.e., more than 1 °C) of temperature was detected for electric field intensity up to 3 V/cm. For higher voltages, we measured an increase of about 2 or 3 °C for 5.3 or 7–8 V/cm, respectively, for a 10-min exposure. For longer exposure durations, temperature increase was the same, probably due to heat dissipation.

2.5. Fluorescence microscopy

To visualize the intracellular location of the fluorescent dye after cell exposure, the cells were washed three times with PBS, trypsinized and centrifuged (1000×*g*, 10 min, at 4 °C). The cellular pellets were diluted in 1 ml PBS. The cells were observed with the appropriate filter for each dye (BV, BP400–440, Olympus for LY and B, BP450–480, Olympus for FD) on a fluorescence microscope (Olympus AX70, Olympus Optical Co., Ltd, Tokyo, Japan) at a 400× magnification.

3. Results

3.1. Increase of dye uptake in B16-F1 and A253 cells exposed to radiofrequency EMFs

Cells were incubated for various durations with either 2 mM LY or 1 mM FD and concomitantly exposed or not to GSM-EMFs of 3.2 W/kg (mean value of the SAR, corresponding to a peak electric field intensity of 1.7 V/cm, Table 1). The exposed B16-F1 melanoma cells displayed an increased dye uptake compared to the control cells (Fig. 2A), of about 1.5 times after a 10-min incubation. This increase was neither cell nor fluorescent dye specific. Indeed, the same outcome was also obtained using the A253 carcinoma cells exposed to 4.5 W/kg in the presence of FD instead of LY (Fig. 2B). After 10 min, FD uptake of exposed cells was about 1.8 times higher in respect to the controls. Longer exposures also induced an increase in LY and FD uptake: 1.2-fold for B16-F1 cells exposed for 60 min (Fig. 2A), and 1.7-fold for A253 cells exposed for 30 min (Fig. 2B).

Fluorescence microscopy of cells exposed or not to the EMFs revealed the presence of the dye in small spots, and not homogeneously distributed in the whole cytosol (data not shown), proving an effective location of the fluorescent dyes into cytoplasmic vesicles. The viability of these two cell lines after their exposure to 3.2 W/kg EMF, tested by Trypan Blue exclusion and cell counting, was similar to the viability in the controls (data not shown).

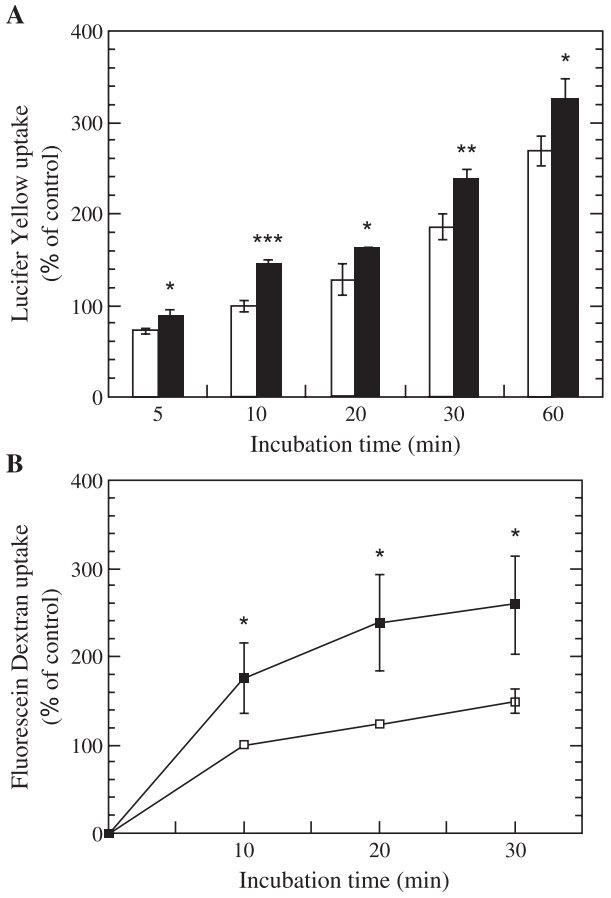

Fig. 2. Increase of fluorescent dye uptake as a function of the duration of cell exposures to GSM-EMF. (□) Control cells exposed only to LY or to FD. (■) Cells exposed to LY and to GSM-EMFs (carrier frequency 900 MHz, envelope frequency 217 Hz, pulse duration 580 μs). The experiments were performed at a temperature of 31±1 °C. (A) B16-F1 cells exposed to 3.2 W/kg. One hundred percent corresponds to control cells uptake in 10 min, i.e., to (6.2±1.8)×10^6 LY molecules per cell. (B) A253 cells exposed to 4.5 W/kg. One hundred percent corresponds to control cells uptake in 10 min, i.e., to (3.4±0.6)×10^6 FD molecules per cell. Statistical significance: unpaired Student's *t*-test, **P*<0.05, ***P*<0.01, ****P*<0.001.

For a 10 min of exposure, a SAR range between 0.6 and 4.5 W/kg (the maximum SAR that could be delivered by the EMFs generator in the above presented experimental conditions) was tested on these two cell lines. While the exposure to SARs between 0.6 and 1.3 W/kg had no significant effect on LY uptake either on B16-F1 or A253 cells (Fig. 3A,B), exposure to SARs between 2.6 and 4.5 W/kg significantly increased the LY uptake. For 3.2 W/kg, there is a 1.5-fold increase of the B16-F1 cells dye uptake and a 1.7-fold for the A253 cells. This increase of the fluorescent dye uptake is thus dependent on the delivered GSM-EMF power, and there is an apparent threshold under which no effect is detected. Threshold values are between 1.3 and 2.6 W/kg and they correspond to peak electric field strengths between 1.1 and 1.5 V/cm. A similar increase of approximately 1.5-fold is obtained for SARs ranging between 3.2 and 4.5 W/kg (corresponding to peak electric field strengths between 1.7 and 2.0 V/cm), SARs which are above the mentioned threshold. Therefore, the fluorescent dye uptake increase looks like an all-or-nothing response.

Exposure of B16-F1 cells to 2.6 W/kg CW-EMF (a continuous high-frequency sine wave at 900 MHz) during 10 min had no detectable effect on LY uptake (Fig. 3A) comparatively to the exposure to GSM-EMF with the same mean SAR of 2.6 W/kg but, of course, not the same peak

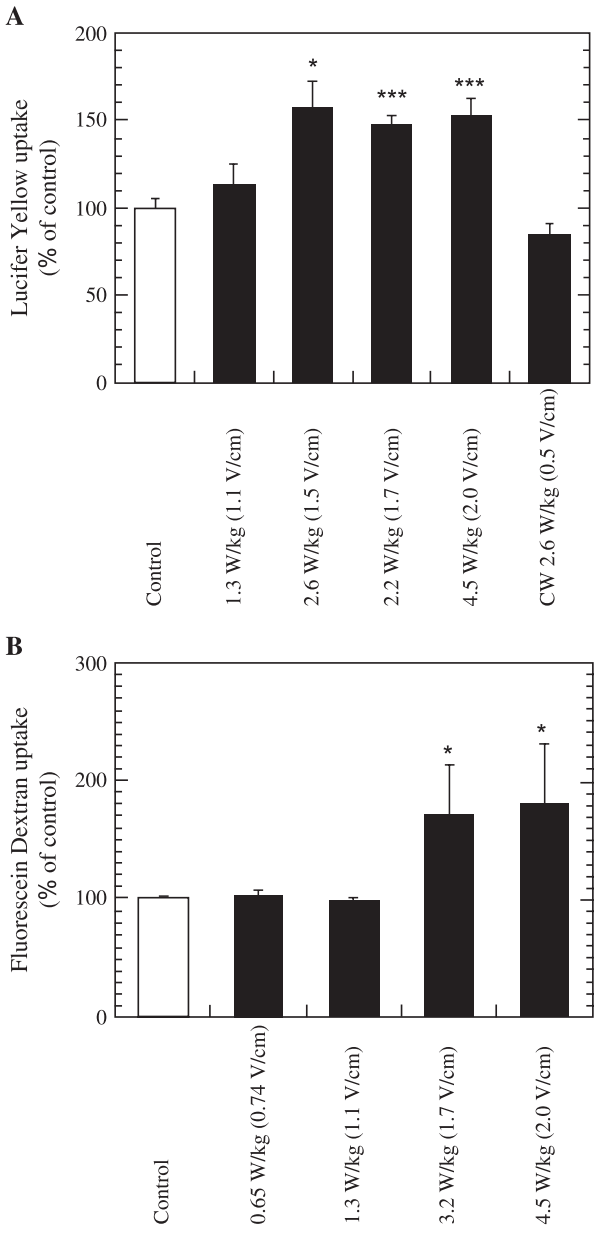

Fig. 3. Increase of fluorescent dye uptake as a function of the power of the delivered GSM-EMFs or CW-EMFs. (□) Control cells exposed for 10 min to LY only. (■) Cells exposed to LY and to GSM-EMFs (carrier frequency 900 MHz, envelope frequency 217 Hz, pulse duration 580 μs) or CW-EMFs (frequency 900 MHz) for 10 min. Values in brackets represent the peak electric field intensities of the corresponding electric component of the EMFs. (A) B16-F1 cells. One hundred percent corresponds to control cells uptake in 10 min, i.e., to (9.0±2.2)×10^6 LY molecules per cell. (B) A253 cells. One hundred percent corresponds to control cells uptake in 10 min, i.e., to (11.4±2.2)×10^6 LY molecules per cell. Statistical significance: unpaired Student's *t*-test, **P*<0.05, ***P*<0.01, ****P*<0.001.

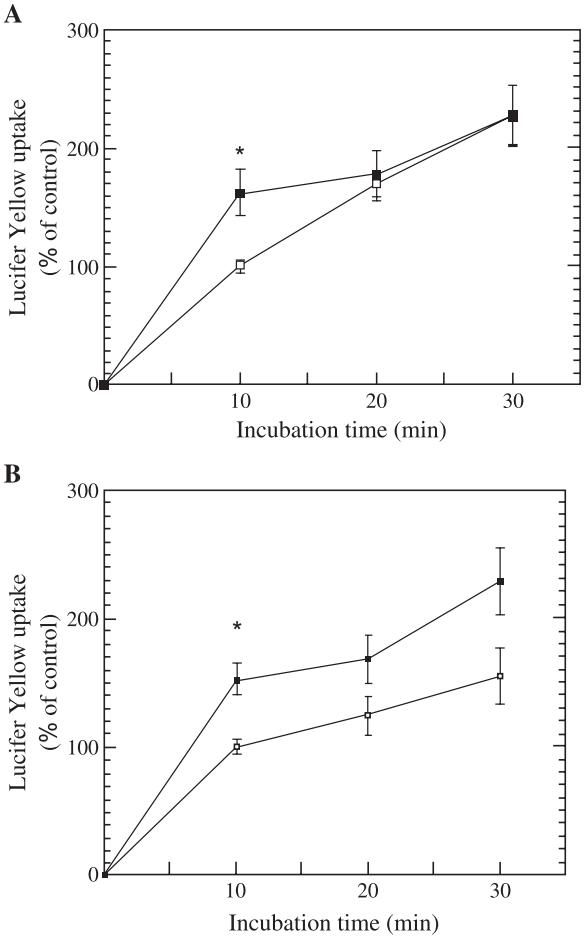

Fig. 4. Increase of LY uptake as a function of the duration of cell exposures to low strength and extremely low frequency PEF. (□) Control cells exposed to LY only. (■) Cells exposed to LY and to the PEF (2.6 V/cm, 217 Hz, 580 μs). (A) B16-F1 cells. One hundred percent corresponds to control cells uptake in 10 min, i.e., to $(5.6\pm1.0)\times10^6$ LY molecules per cell. (B) A253 cells. One hundred percent corresponds to control cells uptake in 10 min, i.e., to $(10.0\pm1.4)\times10^6$ LY molecules per cell. Statistical significance: unpaired Student's *t*-test, $^*P<0.05$.

SAR (Table 1; a CW-EMF of 20.8 W/kg, the peak value of the GSM-EMF, could not be tested because of the generator limitations). The peak SAR and not the mean SAR seems to be the important parameter for the induction of dye uptake increase.

The B16-F1 cells were also exposed to GSM-EMFs at 3.2 W/kg for 10 min in the presence or the absence of an antioxidant mixture [30]: LY uptake was identical, and again about 1.5 times higher than the nonexposed controls (data not shown). Therefore, the dye uptake increase is not linked to a free radical production.

3.2. Increase of dye uptake in B16-F1 and A253 cells exposed to pulsed electric fields

To investigate the effects of the electric field component of the GSM-EMF alone on the fluorescent dye uptake, cells were exposed to PEF with characteristics similar to those of this component. When B16-F1 cells and A253 cells were exposed to 2.6 V/cm PEF, LY uptake was increased about 1.5-fold in both cell types after 10 min of exposure (Fig. 4A,B). This increase became less evident for longer exposures, and even disappeared at 30 min in the case of the B16-F1 cells.

Using fluorescence microscopy, after PEF exposure, a LY distribution in cytoplasmic dots (data not shown), similar to GSM-EMF exposure distribution, located the LY into cytoplasmic vesicles. The viability of both B16-F1 and A253 cells after exposure to PEF (217 Hz, 580 μs and 2.6 V/cm), was identical to the nonexposed cells' viability (data not shown).

For a 10-min exposure, a voltage range between 1.3 and 5.3 V/cm was tested on this two cell types. For the B16-F1 cells, PEF above 1.6 V/cm increased 1.5-fold the LY uptake

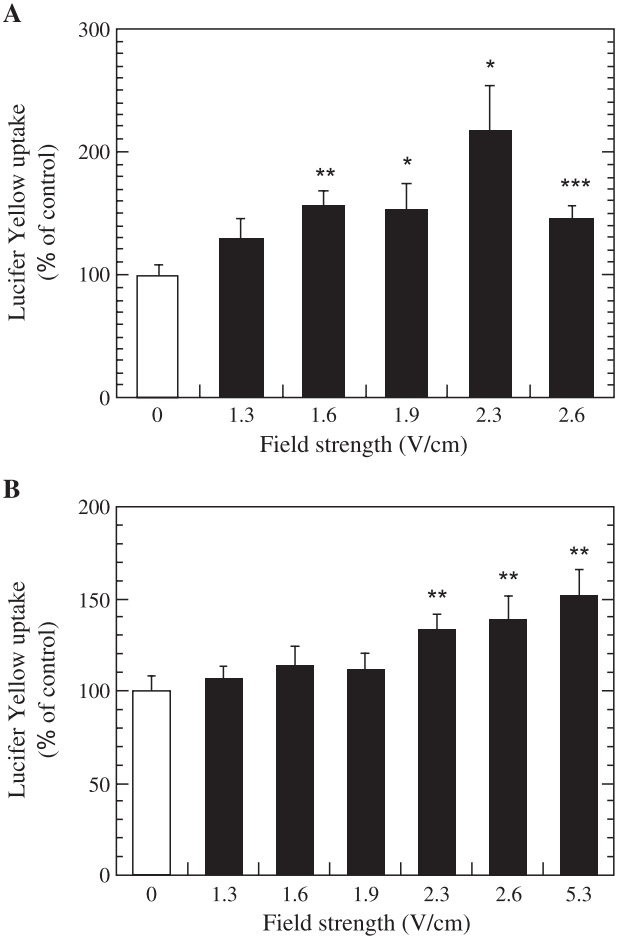

Fig. 5. Increase of LY uptake as a function of the strength of the delivered electric field. (□) Control cells exposed for 10 min to LY only. (■) Cells exposed to LY and to PEF for 10 min (217 Hz, 580 μs). (A) B16-F1 cells exposed to field intensities ranging between 1.3 and 2.6 V/cm. One hundred percent corresponds to control cells uptake in 10 min, i.e., to $(6.0\pm1.0)\times10^6$ LY molecules per cell. (B) A253 cells exposed to field intensities ranging between 1.3 and 5.3 V/cm. One hundred percent corresponds to control cells uptake in 10 min, i.e., to $(8.7\pm1.3)\times10^6$ LY molecules per cell. Statistical significance: unpaired Student's *t*-test, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

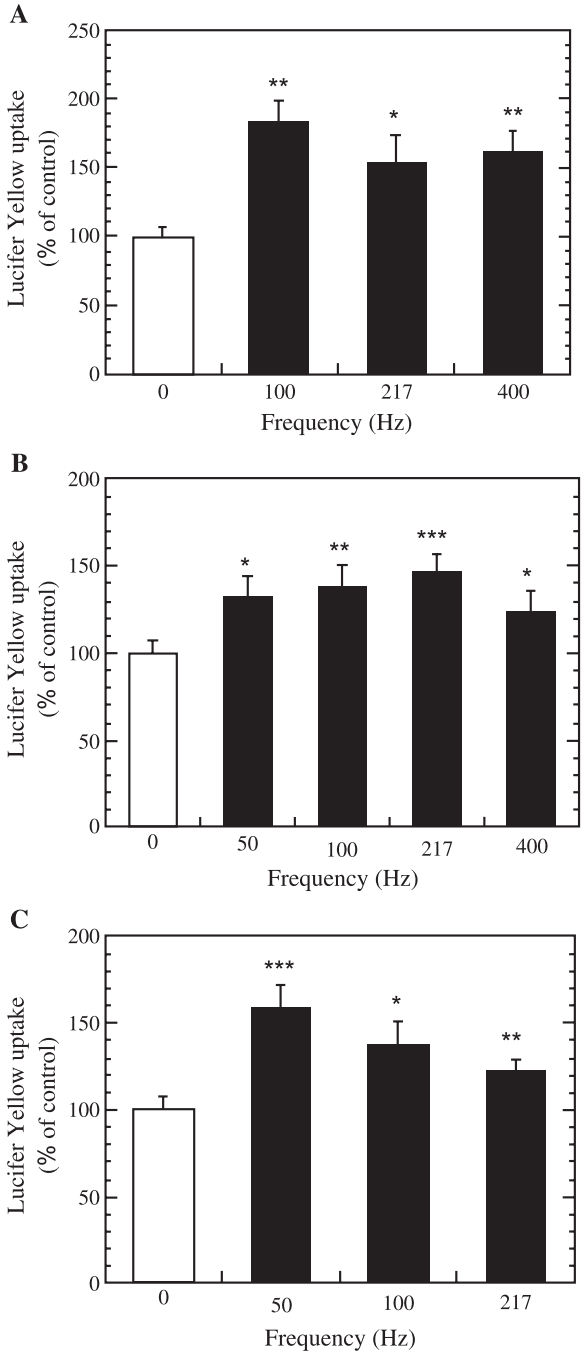

Fig. 6. Increase of LY uptake as a function of the frequency of the applied PEF. (□) Control cells exposed only to LY. (■) Cells exposed to LY and to PEF. (A) B16-F1 cells exposed to electric field (1.9 V/cm, 580 μs) for 10 min. One hundred percent corresponds to control cells uptake in 10 min, i.e., to $(5.7\pm1.0)\times10^6$ LY molecules per cell. (B) The same experiment with a field strength of 2.6 V/cm, where 100% corresponds to $(7.9\pm2.1)\times10^6$ LY molecules per cell. (C) A253 cells exposed to electric field (2.6 V/cm, 580 μs) for 30 min. One hundred percent corresponds to control cells uptake in 30 min, i.e., to $(15.9\pm1.5)\times10^6$ LY molecules per cell. Statistical significance: unpaired Student's *t*-test, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

while exposure to 1.3 V/cm PEF had no effect (Fig. 5A). Therefore, the increase in LY uptake was dependent on the PEF intensity, with a threshold value (between 1.3 and 1.6 V/cm for B16-F1 cells) under which no effect was detected. A similar situation, with a threshold value between 1.9 to 2.3 V/cm, was detected for the A253 cells (Fig. 5B). Above the threshold values, this increase is kept constant at approximately 1.5-fold. Therefore, the PEF-induced increase of LY uptake appears to be an all-or-nothing response.

Taking advantage of the possibilities offered by the PEF generator, the influence of various electrical parameters of the PEF was further tested. At either 1.9 or 2.6 V/cm and for 580-μs pulse duration, cells were exposed to frequencies ranging between 50 and 400 Hz for 10 min (B16-F1 cells) or 30 min (A253 cells). Similar increases were obtained at all the frequencies tested (Fig. 6). The B16-F1 and A253 cells were also exposed to PEF of pulse durations ranging between 75 and 580 μs (at 217 Hz and 2.6 V/cm) for 10 and 30 min, respectively. The same level of increase was obtained (about 1.5-fold) either for B16-F1 cells (Fig. 7A) or for A253 cells (Fig. 7B). Consequently, neither the frequency nor the pulse

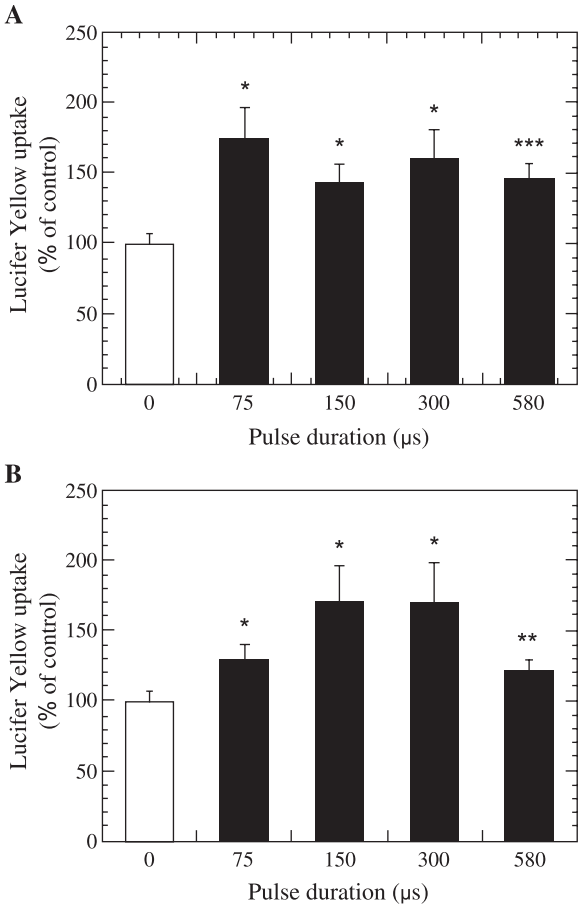

Fig. 7. Increase of LY uptake as a function of the pulse duration of the applied PEF. (□) Control cells exposed to LY only. (■) Cells exposed LY and to PEF (2.6 V/cm, 217 Hz). (A) B16-F1 cells exposed for 10 min. One hundred percent corresponds to control cells uptake in 10 min, i.e., to $(6.4\pm1.1)\times10^6$ LY molecules per cell. (B) A253 cells exposed for 30 min. One hundred percent corresponds to control cells uptake in 30 min, i.e., to $(17.4\pm1.5)\times10^6$ LY molecules per cell. Statistical significance: unpaired Student's *t*-test, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

duration variations lead to any modification of the observed increase in the fluorescent dye uptake, within the range of the tested values.

3.3. Further characterization of the dye uptake increase

Experiments were repeated and extended using a third cell line of different origin, the DC-3F Chinese hamster fibroblasts. The LY uptake by the DC-3F cells is slower comparatively to A253 cells [31]. PEF delivered to DC-3F cells also induced an increase of LY uptake, with a maximum level of 1.6-fold after 60 min of exposure (Fig. 8A). As for the two previous cell types, the increase became increasingly less significant for longer exposures, loosing statistical significance at 90 min. For the DC-3F cells, the

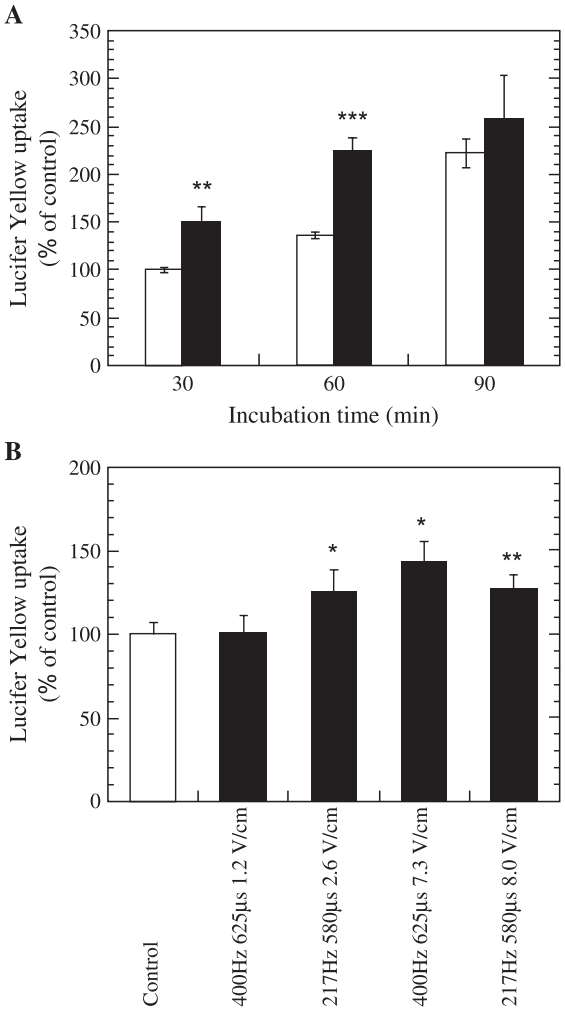

Fig. 8. Dependence of LY uptake increase on the time and on the field strength into DC-3F cell line. (□) Control cells exposed to LY only. (■) Cells exposed to LY and to PEF. (A) DC-3F cells exposed to electric field (7.3 V/cm, 400 Hz, 625-µs pulse duration). One hundred percent corresponds to control cells uptake in 10 min, i.e., to $(9.1\pm1.7)\times10^6$ LY molecules per cell. (B) DC-3F cells were exposed during 60 min. One hundred percent corresponds to control cells uptake in 60 min, i.e., to $(13.8\pm1.5)\times10^6$ LY molecules per cell. Statistical significance: unpaired Student's *t*-test, **P*<0.05, ***P*<0.01, ****P*<0.001.

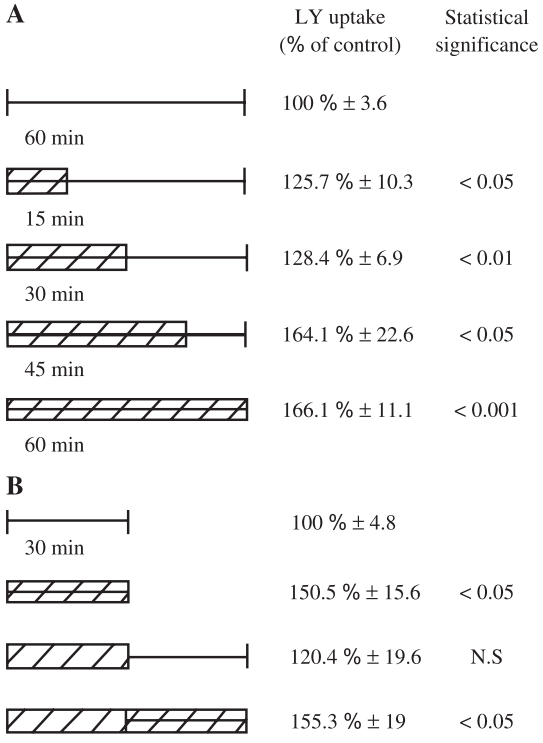

Fig. 9. Analysis of the persistence of the electric-field-induced fluid phase endocytosis increase. ⊢⊣: LY incubation; ▨: exposure to PEF (7.3 V/cm, 400 Hz, 625 µs); ▨: simultaneous LY incubation+exposure to PEF. (A) DC-3F cells were always incubated for 60 min in presence of LY and the electric field was applied during the first 15, 30 or 45 min or during all the 60 min of the incubation. This figure is the result of five independent experiments, performed in triplicate. One hundred percent corresponds to control cells uptake in 60 min, i.e., to $(9.3\pm2.1)\times10^6$ LY molecules per cell. (B) DC-3F cells are incubated in presence of LY for 30 min, the incubation occurring either concomitantly to the electric field exposure of 30 min, or immediately after the 30 min of exposure, or during the 30 last min of a total exposure of 60 min. One hundred percent corresponds to control cells uptake in 30 min, i.e., to $(15.6\pm2.7)\times10^6$ LY molecules per cell. NS: no significance. This figure is the result of three independent experiments, performed in triplicate.

PEF strength threshold was also present, for a value between 1.2 and 2.6 V/cm (Fig. 8B). The presence of a clear plateau up to 8.0 V/cm (a higher electric field intensity than those used in Fig. 5A,B) confirms that this increase is as an all-or-nothing response.

DC-3F cells were incubated for 60 min in the presence of LY but PEF were applied only during the first 15, 30 or 45 min or during all the 60 min of the incubation. Fig. 9A shows that the increase of LY uptake closely followed the duration of the exposure to PEF. Moreover, DC-3F cells were incubated in the presence of LY for 30 min, the incubation being performed either simultaneously to a 30-min exposure to the PEF, or immediately after a 30-min exposure, or during the last 30 min of a total 60-min exposure to the PEF (Fig. 9B). The increase of LY uptake was observed only when LY was present at the time of PEF delivery. The exposure to PEF previously to the LY incubation did not induce a significant increase of LY uptake proving that changes induced by the PEF do not lead

to a persistent cell perturbation or that perturbation is rapidly reversible.

3.4. Uptake increase under suboptimal conditions

All previous experiments were performed under favorable physiological conditions (at 31±1 °C, using cells in exponential growth, 0.7×10^6 cells per cover slip). Exposure of DC-3F cells was also performed under less appropriate conditions, i.e., temperatures ranging between 20 and 24 °C, alkaline pH ranging between 8 and 9, high cell density (cells in the stationary phase) (data not shown). B16-F1 and A253 cells were also tested at high cell density. Under all these suboptimal conditions, the LY uptake rate of control cells was about two times slower than under physiological conditions. Moreover, exposures to PEF under these suboptimal conditions did not result in the increase of LY uptake found under favorable physiological conditions (data not shown).

4. Discussion

Up to now, there is no unique theory to explain the interactions between EMFs and living cells. Therefore, it seems important to try to draw out the relevant parameters that can affect basic mechanisms in the living cells and to make measurements under very strict experimental conditions. In the present study, we have reported the influence of GSM-EMFs on the endocytotic process of various cells in culture.

Exposure to EMFs was performed on three different types of cultured cells, chosen to represent three different histological types of tissues, from three different species: murine melanoma cells, human carcinoma cells and Chinese hamster fibroblasts. Interestingly, the results obtained with these three cell types are mutually consistent. We used a new device for cell exposure to GSM-EMFs specially designed for our type of experiments [32]. This exposure system, the "wire patch antenna", allowed a good matching at 900 MHz, provided a very good homogeneity of the field, and was well characterized by a dosimetry experimentally validated [32,33].

4.1. Characterization of dyes uptake increase

Our findings demonstrate a statistically significant increase of about 1.5-fold of a nonpermeant fluorescent dye uptake concomitant to the exposure to the radiofrequency EMF. Interestingly, this increase occurs above a threshold of the delivered power for all the three cell types examined. The comparison between GSM- and CW-EMFs effects showed that an important parameter to induce this increase is the peak value of the SAR, not the mean value. Alternatively, this could indicate a role for the low-frequency envelope as previously suggested [22]. Therefore, the influence of the low-frequency part of the electric component alone was examined, using a system that allowed a homogeneous exposure of the cells to bipolar symmetrical pulsed electric fields. A similar increase of fluorescent dye uptake was observed in comparison to the GSM-EMF exposure. We found also a threshold value of the electric field strength below which, with respect to control cells, there is no modification in fluorescent marker uptake (or changes are not statistically significant). The calculations of the strength of the electric field component of the used EMFs demonstrate that the pure electric (i.e., resulting from DC pulses at extremely low frequencies) threshold value is similar to the EMF-associated electric field threshold value. This comparison strongly suggests that the electric component of low frequency, alone, is sufficient to reproduce the effects of the GSM-EMFs on fluorescent dye uptake.

Moreover, when cells were exposed to different EMF powers or electric field strengths above these thresholds, and the pulse duration or the repetition frequency of the low strength PEF were modified, the level of the increase of LY uptake was not significantly modified (always ≈1.5-fold above the control uptake). Therefore, the stimulation caused by EMFs or PEF appears to be an all-or-nothing response.

4.2. Link between constitutive and electric field-increased dye uptake

Our experiments show that (i) the hypothesis of thermal effects can be eliminated; (ii) the stimulation occurs only during the PEF application, with a "switch on" of the increase in LY uptake requiring a short time period, in the range of minutes or less; and (iii) the induced cell perturbation is not persistent, with a rapid "switch off" of LY uptake increase after the end of the exposure to the PEF. These observations allow excluding all the mechanisms that take more than few minutes to be activated (and also to be inactivated), such as the gene expression regulation mechanisms.

We compared our observations to three reports on the membrane level effects of cellular exposure to PEFs. It was reported that fluid-phase endocytosis was stimulated after cell electroporation performed with 1 DC pulse of 5 ms at 500 V/cm [27]. Authors proposed that cells respond to the electroporation-induced stress by excising the damaged patches of membranes by means of endocytosis. Other authors observed that up to 60 min after cell electroporation at 800 V/cm (5 DC pulses of 5-ms duration), a long-lived macropinocytosis takes place and that the macropinocytosis vesicles come from the electropermeabilized cell membrane area [28]. In parallel to these observations, our results on LY and FD uptake provide evidence for fluid-phase endocytosis stimulation obtained independently of cell electroporation, with much lower electric field intensities. In addition, the 10-min exposure to electric fields of 60 to 100 V/cm (a strength field still more bigger than those used in our

experiments) has been shown to induce an endocytotic-like process into different cell types (B lymphoma, Lewis lung carcinoma, COS 5–7) [29]. Authors excluded that this uptake involved classical endocytotic or pinocytotic processes because the same results were obtained at 4 °C and on photosynthetic membrane vesicles (thylakoids) or red blood cells, known to be devoid of any endocytotic process. On the contrary, the fluid-phase endocytosis increase obtained in our experiments with extremely low electric field intensities is totally abolished under suboptimal conditions, in particular below 20 °C and the kinetics of this increase are related to constitutive fluid-phase endocytosis rate as suggested by the results obtained using cells with different constitutive fluid-phase endocytosis rates (B16-F1 and A253 cells vs. DC-3F cells).

More probably, the induced fluid-phase endocytosis stimulation could result from a modification of the physiological endocytotic process rather than from a nonphysiological perturbation since, after the exposure to EMFs, the cells kept their viability and no cellular damages were noticed by microscopy. Normal constitutive endocytosis seems required in order to observe the PEF- or EMF-induced increase since exposures under suboptimal physiological conditions did not reproduce the effect.

The fluid-phase endocytosis increase in the cells exposed to the EMFs and PEFs is detectable within the first minutes. In some of our experiments (e.g., Fig. 4A), the relative differences in LY uptake of exposed cells vs. unexposed cells decreased with the increase of the exposure duration. A reasonable supposition would be that a simultaneous increase of the exocytosis rate would also be induced, in order to keep constant the cell surface area (taking into account this physiological compensation observed every time that either endocytosis or exocytosis are stimulated) [34]. Thus, the initial increase of fluid-phase endocytosis uptake could be the marker of a new physiological steady state of the cells, induced by the EMFs or the low-strength PEF, in which endo/exocytosis rates are accelerated.

4.3. Analysis of the potential mechanisms underlying the endocytosis rate increase

The characteristics of the electrical parameters used and the results of the experiments here reported allow to draw some indications about the underlying biological mechanisms:

a. Pulsed magnetic fields (e.g., 0.1 mT, 60 Hz) can affect chemical reactions and some enzymes activity leading to perturbations of free radicals production and probably of membrane lipids peroxidation [21]. However, our results show that antioxidants are not modifying the value of endocytosis-increased rate probably because this is not in relation with an increase of the free radical production. Moreover, this observation is consistent with the fact that the magnetic component of the EMFs does not seem necessary for the fluid-phase endocytosis increase.
b. The bipolar character of the electric fields strongly diminishes the possibility of mechanisms involving electrophoresis over long distances of any charged component, including membrane proteins within the membranes.
c. Since a similar increase was observed using different PEF frequencies, there is less possibility that an acute resonance interaction with a cellular periodic phenomenon, such as intracellular calcium oscillations, is involved. Conformational modifications of enzymes or proteins implied in the endocytotic process induced by electric fields, which can potentially lead to functional perturbations, according, for example, to theoretical descriptions in reference [35], also appears unlikely.
d. In spite of the very low electric field strengths used in our experiments, it is necessary to evoke mechanisms in relation with transmembrane potential variations. Indeed, the value of the transmembrane potential (ΔV_m) induced at a point M on the cell surface by an external electric field can be calculated from the expression: $\Delta V_m = f E_{ext} r \cos \Theta$, where f is a geometrical factor ($f \approx 1.5$ for spherical cells), E_{ext} is the external electric field strength, r is the cell radius and Θ is the angle made by the direction of the electric field and the normal to the cell surface at M point [36]. Considering that $r \approx 10$ μm and $\cos \Theta \approx 1$, for E_{ext}=1.8 V/cm, we can calculate: $|\Delta V_m|_{max} \approx 2.7$ mV. Even this value seems very low; it corresponds nevertheless to a non-negligible fraction of the resting membrane potential. Indeed, particularly in the case of dedifferentiated cells growing rapidly in vitro, the resting potential is in the range of a few tens of mV (between −20 and −40 mV) [37]. Thus, the amplitude of the induced perturbation could be in the range of 10% of the resting value. Further experiments are needed to confirm that such a perturbation can be at the origin of the phenomenon found during our experiments. However, the values of the change in the transmembrane voltage responsible for the observed effects could be quite different from those calculated here, since we used attached cells. Nonconfluent plated cells are neither spherical nor disk-like with a homogeneous thickness: around nucleus, the cell is thicker and even prominent with respect to the very flat cell periphery. Thus, only this thicker part of the cell could be actually exposed to the electric field lines parallel to the cell support.

In the case of the EMF, it has been recently calculated that the value of factor f in the GHz range should not be null. However, it should be very low, about two orders of magnitude lower than the value given by the simplified above-mentioned Schwan's equation ($f \approx 1.5$ is valid only from DC to EMF in the hundreds of kHz range) [26]. As a

consequence, it is only possible to make speculations on the possibility that a membrane potential alteration could be involved in the mechanism of endocytosis stimulation by EMF in the GHz range. Moreover, in the patch wire antenna (as described in Methods), the main direction of the electric field component is perpendicular to the cell support, thus parallel to the shortest dimension of the cell. Nevertheless, as in the case of attached cells exposure to the PEF, cells are not homogeneously thick disks and maximal effects of the exposure could be provoked on the thickest part of the cell (around the nucleus). Actually, the precise local value of the electric field at the level of the membrane is not known and could be higher, due to the unpredictable shape of the adherent cells as well as the consequences of interactions between neighboring cells [38] or between the cells and the dielectric culture support.

4.4. Biological relevance

As a conclusion, we demonstrate that GSM-EMFs, probably through their low-frequency electric component, or low-strength, low-frequency PEF can accelerate in vitro the physiological fluid-phase endocytosis of different cell types. This increase seems to result from a stimulation of the constitutive endocytosis even though we have not identified the molecular mechanisms triggering this stimulation. Anyway, it is highly possible that the primary target of the applied fields is at the level of the cell membrane. As any cellular function characterized by physiological limits compatible with the maintenance of cells' viability, endocytosis increase appears as a saturable effect that is observed as an all-or-nothing response.

If we extrapolate our results to a whole organism, they provide no evidence for acute risks due to exposure to GSM-EMFs or to low-strength, low-frequency PEF since there is no cell damage. However, it cannot be excluded that EMFs might activate the endocytosis in vivo, but within a physiological range. Still, in the brain of healthy young subjects exposed to RF EMF at 900 MHz, typical spatial peak SAR averaged over 1 g tissue was about 0.75–1.55 W/kg depending of the mobile phone antenna [39]. The corresponding electric field is equal to 0.3–0.4 V/cm for a typical value of the conductivity 0.9 S/m at 900 MHz and a density 1000 kg/m^3 [39]. These values are below the threshold values determined in the present study. Moreover, it must be emphasized that our data have been obtained on cells in culture: They should only be considered as an experimental basis for prompting investigations at the in vivo level and they must not be used to draw speculations about possible effects on health. The practical implications of our results are that the various effects of the EMFs that have been reported until now should be examined again under the light of the changes here described because endocytosis/exocytosis is a fundamental physiological function in all living eukaryotic cells.

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