

Endocytosis rate increase provoked by bipolar asymmetric electric pulses

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ABSTRACT

It has been reported that electric and electromagnetic pulses can increase the endocytotic rate, showing either an all-or-nothing response above a threshold of the field strength or linear responses as a function of the field strength and the treatment duration – using bipolar symmetrical pulses and monopolar pulses, respectively. We have investigated the reasons of such a discrepancy. To this end, cells were suspended in either low conductive or physiologically conductive medium, and exposed to bipolar asymmetric electric pulses (EPs) for different exposure times (2 to 20 min). These EPs had low amplitude (5.7 to 18 V/cm) with a duration varying from 100 to 500 μ s and a frequency of 100 to 500 Hz. The effects of these parameters on cell viability and field-induced uptake of molecules were analyzed by spectrofluorometric measurements using either the fluorescent dye Lucifer Yellow (LY) or BSA-FITC. The former acted as an indicator molecule for the fluid-phase endocytosis and the latter for detecting receptor-mediated endocytosis. An increase of 1.3 to 1.5-fold in the fluid-phase and receptor-mediated endocytosis rate was observed using the physiologically conductive medium (10 mS/cm). This increase was an all-or-nothing type of response that occurred above a threshold value of the electric field intensity (between 9.3 and 9.8 V/cm). Variations of the repetition frequency or pulse duration did not modify this threshold. The observed increase in either LY or BSA-FITC uptake was not a thermal effect. We examined electric pulses different from that previously published by other researchers to answer the question of which pulse type was more potent in enhancing endocytotic rate.

Keywords: Bipolar asymmetric pulsed electric fields, fluid-phase endocytosis, Lucifer yellow, DC-3F cells.

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Abbreviations: PEF, Pulsed electric field; EP, electric pulses; LY, Lucifer yellow; BSA, bovine serum albumin; FITC, fluorescein-5-isothiocyanate; E_{th} , threshold field intensity.

INTRODUCTION

During the last decades, different approaches have been developed to incorporate macromolecules into cells. *In vitro* there are four main types of physical methodologies that lead to incorporation of molecules into a population of cells. The first method, so-called magnetofection, employs magnetic forces to enhance gene delivery to the cells (Huth et al., 2004; Gersting et al., 2004). The second method uses the “ballistic gun” (Taylor and Fauquet, 2002; Mor and Eliza, 2001) where cells are exposed to ballistic bombardment by microparticles

coated with the molecules of choice (e.g. DNA). The third method is based on exposing cells to ultrasound waves leading to increased transmembrane transport (Sundaram et al., 2003). The fourth approach is based on an electrically driven process (electroporation) where cells are exposed to high intensity PEF for short durations of micro- to milliseconds. This exposure leads to induction of short-lived permeability changes in the membrane due to transient membrane structure defects termed “electropores”, which enables the diffusion of

small molecules across the membrane along their electrochemical gradients or the uptake of large molecules (e.g. electrically-mediated gene delivery) through mechanisms including electrophoresis (Henshaw et al., 2007). The mechanism of pore formation was investigated by direct molecular dynamic simulations of phospholipid bilayers. The simulations suggested that pore formation is driven by local electric gradients at the water/lipid interface in conjunction with the movement of water molecules in these field gradients. Such movements increase the probability of water defects penetrating into the bilayer interior. Water defects cause a further increase in the local electric field, accelerating the process of pore formation. The resulting pores are hydrophilic lined by phospholipid head groups (Tieleman, 2004). If no increase in membrane permeability is wanted, the other method to increase the uptake of molecules must follow an increase in the main cellular system responsible for exchanges between the outside and the inside of the cells, namely the endocytosis. Endocytosis is a basic cellular mechanism contributing to the continuous exchange of molecules between the environment and the cell. During endocytosis, a small portion of the plasma membrane invaginates and forms an endocytotic vesicle called early endosome. It encloses macromolecules bound to transmembrane receptors (receptor mediated endocytosis) as well as a small portion of extracellular fluid (fluid phase endocytosis, also termed pinocytosis). Cellular membrane area is restored by the reciprocal mechanism, called exocytosis (Burgoyne and Morgan, 2003).

Exposure of cells to low pulsed electric fields may lead to at least two distinct primary effects at the level of the cell membrane.

The first possible primary effect induced in cells exposed to low electric fields is that of electrophoretic induced segregation of labile charged lipids and proteins in the plane of the cell membrane. This phenomenon was previously explored both theoretically and experimentally (Poo, 1981; Poo and Robinson, 1977; Poo et al., 1979; Jaff, 1977; McLaughlin and Poo, 1981; Sowers and Hackenbrock, 1981). It has been pointed out that the external electric field tangential to the cell surface E_{θ} is the driving force (Equation 1):

$$E_{\theta} = 1.5 E \sin\theta \quad (1)$$

where E_{θ} will induce electrophoretic mobility toward the anodic or cathodic sides of the cell, either by direct electrophoretic mobility of the negatively and the positively charged components or by electroosmosis, respectively (McLaughlin and Poo, 1981). It emerges from previous studies that global segregation of membrane receptors, reflected by their asymmetric distribution between the cell hemispheres facing the cathode's or anode's sides, occurs only after relatively long exposure time (McLaughlin and Poo, 1981; Orida

and Poo, 1978; Zhao et al., 1999). Therefore, in the case of a brief exposure we may expect the induction of limited short-range electrophoretic mobility leading to localized segregation of membrane proteins and lipid. However, the absence of correlation with the number of coulombs and/or the current delivered limits the implication of this mechanism.

The other primary effect is the electric polarization of the membrane, leading to alteration of the transmembrane potential (Schwan, 1957). For an exposure of a cell to a homogeneous electric field, the transmembrane potential difference induced by the electric field, $\Delta\Psi_i$ is a complex function $g(\lambda)$ of the specific conductivities of the membrane (λ_m), the pulsing buffer (λ_o) and the cytoplasm (λ_i), the membrane thickness and the cell size (r). Thus,

$$\Delta\Psi_i = f g(\lambda) r E \cos\theta \quad (2)$$

where f , is a shape factor (a cell in suspension being a spheroid), E is the electric field in the region where the cell is situated, r is the cell radius, and θ is the polar angle measured from the center of the cell with respect to the direction of the field. This formula tells that the maximum voltage is induced at the points where the electric field is perpendicular to the membrane, that is, at $\theta = 0^\circ$ and $\theta = 180^\circ$ and these physical predictions were checked experimentally by video microscopy by using potential difference sensitive fluorescent probes (Gross et al., 1986; Lojewska et al., 1989; Hibino et al., 1991). The above equation was simplified by Schwan (1957):

$$\Delta\Psi_i = 1.5 r E \cos\theta \quad (3)$$

Thus, the exposure of a cell possessing radius of $\sim 8 \mu\text{m}$ to electric field strength of the positive part the pulses varied from 5.4 to 10 V/cm (used in our studies when employing physiologically conductive media) will result in an induced potential difference 6.5 to 12 mV across the plasma membrane. Therefore, the membrane region facing the anode will be hyperpolarized by 6.5 to 12 mV; that facing the cathode will be depolarized to the same extent by using either unipolar pulsed electric pulses as used by Antov et al. (2005) or bipolar asymmetric electric pulses as used in our study. It is well known that these changes in the transmembrane potential do not lead to electroporation (Rosemberg and Korenstein, 1997; Antov et al., 2004).

Pulsed low electric fields-induced endocytosis has been reported by two groups (Antov et al., 2005; Mahrour et al., 2005). The study of Mahrour et al. (2005) was performed using bipolar symmetrical square pulses with field strength of the positive part of the pulses varied from 1.2 to 8 V/cm, pulse duration from 75 to 580 μs , frequency from 50 to 400 Hz and total exposure time from 5 to 90 min. A bipolar signal was chosen to avoid unidirectional electrophoresis and to minimize

electrochemical reactions at the electrodes. With exposures that could exceed 10 min, an increase of the fluid phase endocytosis rate was observed by ~1.5-fold (with respect to the controls with same exposure duration and without electric pulses), on three different cell types. This increase was reported to be an all-or-nothing type of response that occurred for threshold values of the electric field intensities comprised between 1.6 and 2.6 V/cm depending upon the cell type. Variations of repetition frequency or pulse duration did not modify the cell response to the PEF (Mahrouf et al., 2005). The study of Antov et al. (2004) was performed using unipolar rectangular pulses with field strengths from 2.5 to 20 V/cm, pulse durations from 50 to 250 μ s, repetition frequency of 500 Hz, at temperatures from 4 to 37°C, and in media with conductivities from 6.4 to 18.6 mS/cm. Total exposure time was 1 min in all cases. The uptake of BSA-FITC by two different cell types displayed a linear dependence of the electrically-induced increase of uptake with no sign of a threshold value of the field strength. Maximal uptake could be up to ~7.5-fold higher than in controls, at 20 V/cm. The dependence of the uptake on pulse duration and medium conductivity was also linear (Antov et al., 2005). These two sets of data were markedly different.

The present study analyzes the effects of asymmetric bipolar EPs on the fluid-phase and receptor-mediated endocytosis of DC-3F cells including the influence of the various electrical parameters. For DC-3F cells, an increased fluid-phase and receptor-mediated endocytosis were 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 the exposure process.

MATERIALS AND METHODS

Cell culture

Chinese hamster DC-3F lung fibroblasts were cultured in modified Eagle's medium 1x (MEM 1x) (Life technologies, Grand Island, USA) supplemented with L-glutamine, 10% fetal calf serum (FCS) (Life technologies), 100 units/ml of penicillin and 125 μ g/ml of streptomycin (Invitrogen, Grand Island, USA). The cells were grown in number by adding 1 ml of MEM 1x containing $\sim 10^6$ cells with 9 ml of MEM 1x free of cells in 75 cm² tissue culture flasks at 37°C, in a humid atmosphere of 5% CO₂ in CO₂ incubator for 3 days. Cells attached at the lower surface of the flask were harvested at the log phase of growth by adding 1 ml of 0.05% trypsin-EDTA (Invitrogen Corporation) for 5 min at 37°C to the flask containing the attached cells without culture medium. After detaching the cells 9 ml of fresh MEM 1x was added to avoid the trypsin destruction capability of membrane proteins (Mahrouf et al., 2005; Pron et al., 1999). After centrifugation (210 g, 5 min, room temperature) the culture medium MEM 1x was removed as a supernatant and cells were resuspended (5 to 10 $\times 10^6$ cells/ml) in exposure medium: either S-MEM (product 21385, Invitrogen), a calcium depleted modification of EMEM which has a physiological conductivity (10 mS/cm) or STM [sucrose 0.25 M (Sigma, St. Louis, USA), Tris hydroxymethyl aminomethane 10 mM (Sigma), magnesium chloride hexahydrate 1 mM, (Merck, Darmstadt, Germany), pH = 7] which has a low

conductivity (1 mS/cm) to examine the effect of conductivity on the electroendocytotic process in 2 independent group of experiments. All the exposure media were degassed with a vacuum pump (Fisher Bioblock Scientific pump, Freiburg, Germany) for 10 min at room temperature $\sim 21^\circ\text{C}$ to reduce the volume of gases produced by the electrochemical reactions occurring at the electrodes during the exposure process.

Molecular probes

The green fluorescence water-soluble double negative dye ($Z = -2$), Lucifer yellow CH di-lithium salt (LY, 457.2 Da) (Sigma, St. Louis, USA) was used at a final concentration of 2 mM to detect the fluid-phase endocytosis (Mahrouf et al., 2005).

Bovine serum albumin (BSA) conjugated to fluorescein-5-isothiocyanate (FITC) (66 kDa, 4 mol FITC/mol albumin) (Invitrogen, Paisley, UK) was used at final concentration of 6.8 μ M to detect the receptor-mediated endocytosis (Antov et al., 2005; Antov et al., 2004).

Detection of the electrochemical reactions

Electrochemical reactions were observed at high field strength values and long exposure times. To explore the exposure limits below those of which no major electrochemical reaction was detected; 100 μ l of either low (1 mS/cm) or physiologically (10 mS/cm) conductive media were exposed to different exposure conditions (field intensity 7.7 to 14 V/cm, pulse duration 100 to 500 μ s, frequency 100 to 500 Hz, and total exposure time 2 to 10 min).

The analysis of the LY fluorescence readings caused by the electrochemical contamination was performed by exposing DC-3F cells suspended in low conductive medium to electric field parameters higher than the exposure limits previously determined. Two different conditions were studied by two different techniques. First, LY uptake by the cell suspension was compared for cells exposed to 13 V/cm field intensity, 400 Hz repetition frequency, and 400 μ s pulse duration with the same number of cells but zero electric field using different exposure times (independent variable). Secondly, the pellets of cells exposed for 10 min to 18 V/cm field intensity, 500 Hz frequency, and 500 μ s pulse duration, in the absence and in the presence of LY, were observed by normal and fluorescence microscopy. After the treatment, the cells suspensions were diluted in 10 ml washing buffer (PBS) and centrifuged two times to get rid of the LY remaining in solution outside the cells. The final pellet was re-suspended in 1 ml lysis buffer, of which 5 μ l were deposited on a microscopic slide. Pictures were taken using an Axiovert S100 microscope (Zeiss, Goettingen, Germany) either under phase contrast microscopy or with the filters set to observe the LY fluorescence.

Exposure of cells to bipolar asymmetric pulsed electric fields

Cells in suspension were exposed to a train of low intensity bipolar asymmetric rectangular voltage pulses with the area of the positive part of the pulse (above the base line) nearly equal to the area of the negative part of the pulse (below the base line) in the presence or absence of LY or BSA by employing a 50 MHz pulse generator (Model 801, Wavetek, San Diego, USA). The exposure of 100 μ l of medium containing 0.5 to 1.0 $\times 10^6$ cells and either 2 mM LY or 6.8 μ M BSA-FITC was performed between two parallel stainless steel electrodes in a vertical position, separated by 0.2 cm, resulting in a medium to electrode contact area of 0.5 cm², and yielding a quasi uniform electric field. The exposure was done either in low conductive medium (1 mS/cm) or physiologically conductive medium (10 mS/cm) in 2 separate set of experiments. The electric

field parameters were monitored on line using a digital storage oscilloscope (VC-6025, Hitachi Denshi Ltd., Japan). The range of electrical parameter included electric field strengths of 5.4 to 18 V/cm, pulse individual duration of 100 to 500 μ s, pulse repetition frequencies of 100 to 500 Hz and a total exposure time of 2 to 20 min (independent variables). All experiments were run at \sim 21°C (room temperature), similar dye uptake being observed at 4 and 37°C with no significant differences than that observed at room temperature (data not shown). The temperature of the samples at the end of the exposure was measured using a digital thermometer. For each set of exposures, a control zero electric field sample with similar cell suspensions was placed between the same electrodes.

Determination of the fluorescent probe uptake using spectrofluorometry

Both the control and the treated samples were washed and centrifuged (210 g, 10 min, room temperature) two times in PBS. The pellet was resuspended in 1 ml Lysis buffer (Cell culture lysis reagent 5x, Promega, Charbonnières, France) diluted to 1/100 with water then vortexed to release the cell lysate. 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 emission wavelengths used for fluorescence measurements were 423 and 525 nm, or 494 and 520 nm, respectively for LY or BSA-FITC. Using a fluorescence standard curve and the number of cells per sample, the fluorescence values were converted into number of molecules incorporated per cell as reported in the figures. The fluorescent probe uptake by the pulsed electric field-exposed cells was calculated with respect to the average uptake of control cells (considered to be 100%) measured the same day on parallel samples exposed to LY or BSA-FITC for the same duration, in the absence of the electric pulses. The dependence of fluid phase or receptor-mediated endocytosis on the electric pulses parameters was reported using histograms.

Determination of the number of cells using spectrophotometry

The colorimetric detection and quantification of total protein in dilute aqueous solutions was done using (Micro BCATM Protein Assay Kit, Pierce, Rockford, USA) which is a detergent compatible bicinchononic acid (BCA) as the detection reagent for Cu⁺, which is formed when Cu²⁺ is reduced by protein in an alkaline environment. The purple colored reaction product is formed by the chelation of two molecules of BCA with one Cu⁺. The water soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations. To avoid fluctuations in cell counts eventually resulting of cell damages caused by the electric pulses, and also to determine cell content in each of the treated samples, the measurement of the total protein content was measured in all the samples. Protein standards were prepared by diluting 2.0 mg/ml Bovine Serum Albumin BSA stock standard with water to set the standard curve. Working reagent was prepared according to manufacturer's instructions. 40 μ l of cell lysate for each sample (including unknown samples treated with different electrical parameters and untreated samples of known number of cells) were added to 110 μ l of water in the appropriate microwell plate wells. Blank wells only contained water (150 μ l). Then 150 μ l of the working reagent were added to each well. The plate was covered and incubated at 37°C for 2 h. After incubation the plate was cooled at room temperature and the absorbance was measured at 562 nm on a plate reader. The average reading for the blanks was subtracted from the reading for each standard or unknown sample. A standard curve was prepared by plotting the average blank corrected reading for each BSA standard vs. its

concentration in μ g/ml. Another standard curve was prepared by plotting the protein concentration of the unknown untreated samples vs. its corresponding cell number. The standard curve was used to determine the number of cells for each electrically treated sample.

Statistical analysis

In Figures 1 to 6, each data point represents the mean \pm standard error S.E. of two to four 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 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).

RESULTS

Analysis of the fluorescent products in the presence of the electrochemical reactions

Electrochemical reactions between the inner surfaces of the stainless steel electrodes and the exposure medium were observed in preliminary experiments exploring a large range of field strengths and exposure times. These reactions imposed strict limits to the electrical parameters used in our experiments (Table 1). The first detectable sign of the electrochemical reactions was the formation of air bubbles at the anodic side of the drop of cells suspension. The number and size of the bubbles increased with the time. The second sign was the change in the colourless drop to yellowish green. The use of bipolar equilibrated asymmetric pulses, that had been chosen to reduce as much as possible the electrochemical reactions at the electrodes surface, could not completely suppress them using electrical parameters higher than that mentioned in Table 1.

The exposure media were first degassed before the experiments under a partial vacuum for 10 min at room temperature. While bubbles formation could be largely decreased by media degassing, changes in colour could not be reduced as they directly result from the electrochemical reactions at the electrodes surface. The continuation of the pulses delivery led not only to changes in colour but also to changes in the other physical characters of the drop which became more viscous and turbid. The temperature measurements at the end of the exposure revealed that there was no elevation due to the exposure to the different tested pulse parameters. Table 1 shows the limits to expose the cells without apparent electrochemical reactions, the cells being suspended in either low (Table 1A) or physiologically (Table 1B) conductive medium and the pulses being delivered through the stainless steel electrodes described in the materials and methods. All these values were achieved using degassed media.

Experiments were also conducted under conditions in

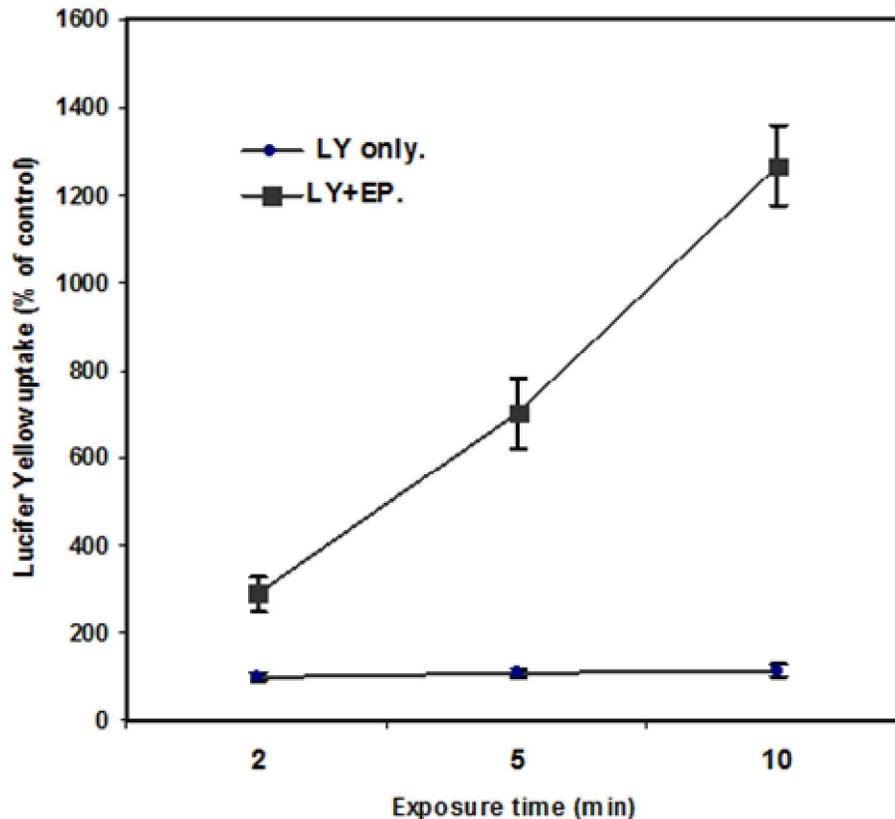


Figure 1. The electrochemical contribution in LY readings under exposure conditions generating detectable electrochemical reactions. Different exposure times, 13 V/cm field intensity, 400 μ s pulse duration, 400 Hz repetition frequency, and 1 mS/cm conductivity of the exposure medium.

which electrochemical reactions were detectable. The dependence of LY fluorescence readings on the electrochemical contamination was examined by exposing DC-3F cells suspended in low conductive medium (1 mS/cm) between the same stainless steel electrodes used in the previous experiments to electric field parameters higher than indicated in Table 1A. The uptake of LY was first analyzed using the procedures employed in the experiments with fields below 10 V/cm. Cells were thus exposed to 13 V/cm field intensity, 400 Hz repetition frequency, and 400 μ s pulse duration in the presence of 2 mM LY. LY readings increased significantly, from 2- to 12-fold compared to the control curve. This increase was linear as a function of the increase in the exposure time (Figure 1). To analyze this behaviour, which contrasts with the data obtained in the absence of electrochemical reactions, cells were also exposed for 10 min to PEF of 18 V/cm field intensity, 500 Hz repetition frequency, and 500 μ s pulse duration in low conductivity medium in either the absence or the presence of LY. The cells suspensions were diluted in 10 ml washing buffer (PBS) and centrifuged two times to get rid of LY outside the cells. The pellets were then suspended in a very small volume and observed under

phase contrast and fluorescence microscopy. Electrochemical aggregates as large as the cells were observed under phase contrast microscopy (Figure 2A and B). If the exposure to PEF was made in the presence of the LY, these aggregates were fluorescent, as shown in the images taken under fluorescence microscopy (Figure 2C and D).

Electroendocytosis in the absence of electrochemical reactions

Effect of field intensity

The influence of field intensity was studied at room temperature $\sim 21^\circ\text{C}$ by exposing DC-3F cells suspended in the physiologically conductive medium (10 mS/cm) with either 2 mM LY or 6.8 μ M BSA-FITC to PEF (of 200 Hz and 200 μ s) of different electric field strengths (5.7 to 10 V/cm, for 2 min followed by a 8 min of incubation without pulses). A significant increase in either LY or BSA-FITC uptake was detected only for PEF of at least 9.8 V/cm (by ~ 35 to 50%) while exposure to field intensities lower than 9.3 V/cm had no significant effect

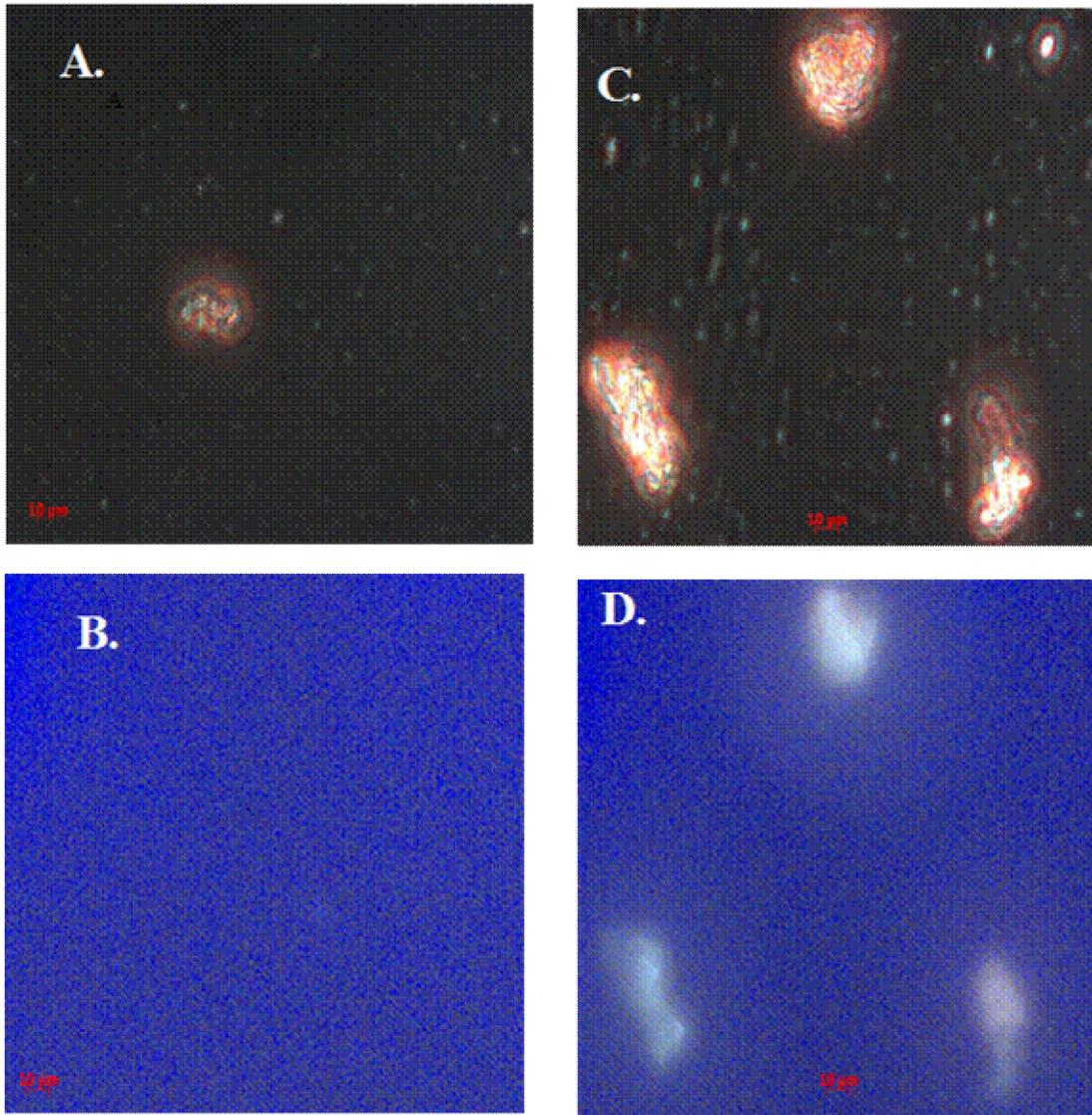


Figure 2. The electrochemical deposits under the fluorescence microscope (objective lens = 40x) using 18 V/cm field intensity, 500 Hz repetition frequency, 500 μ s pulse duration, 10min exposure time. (A, B) Contrast phase and the green fluorescence phase for the cell lysate of DC-3F cells suspended in low conductive medium (1 mS/cm) without LY, respectively. (C, D) Contrast phase and the green fluorescence phase for the cell lysate of DC-3F cells suspended in low conductive medium in the presence of 2 mM LY, using the above electrical parameters.

(Figure 3A and B).

Cells were also suspended in the low conductive medium (1 mS/cm) and exposed to pulsed bipolar asymmetric trains of (10 to 14 V/cm, 300 Hz, 300 μ s, for 2 min followed by 8 min of incubation without pulses) at room temperature (\sim 21°C) in the presence of 2 mM LY in the extracellular medium. In the low conductive medium, no uptake increase was observed. Actually, exposure conditions were limited by the electrochemical reactions (Table 1A). The total uptake did not change with respect to the controls (Figure 3C).

Therefore, the increase in LY or BSA-FITC uptake was dependent on both the conductivity of the exposure

medium and the pulsed electric field intensity, with a threshold value between 9.3 and 9.8 V/cm for DC-3F cells under which no effect was detected. Field intensities above 10 V/cm could not be tested in the physiologically conductive medium because of the electrochemical reactions at the surfaces of the stainless steel electrodes (Table 1B). There was no cell loss at all the tested values of field intensities, and no temperature increase.

Effect of frequency

To evaluate the effect of frequency on fluorescent probes

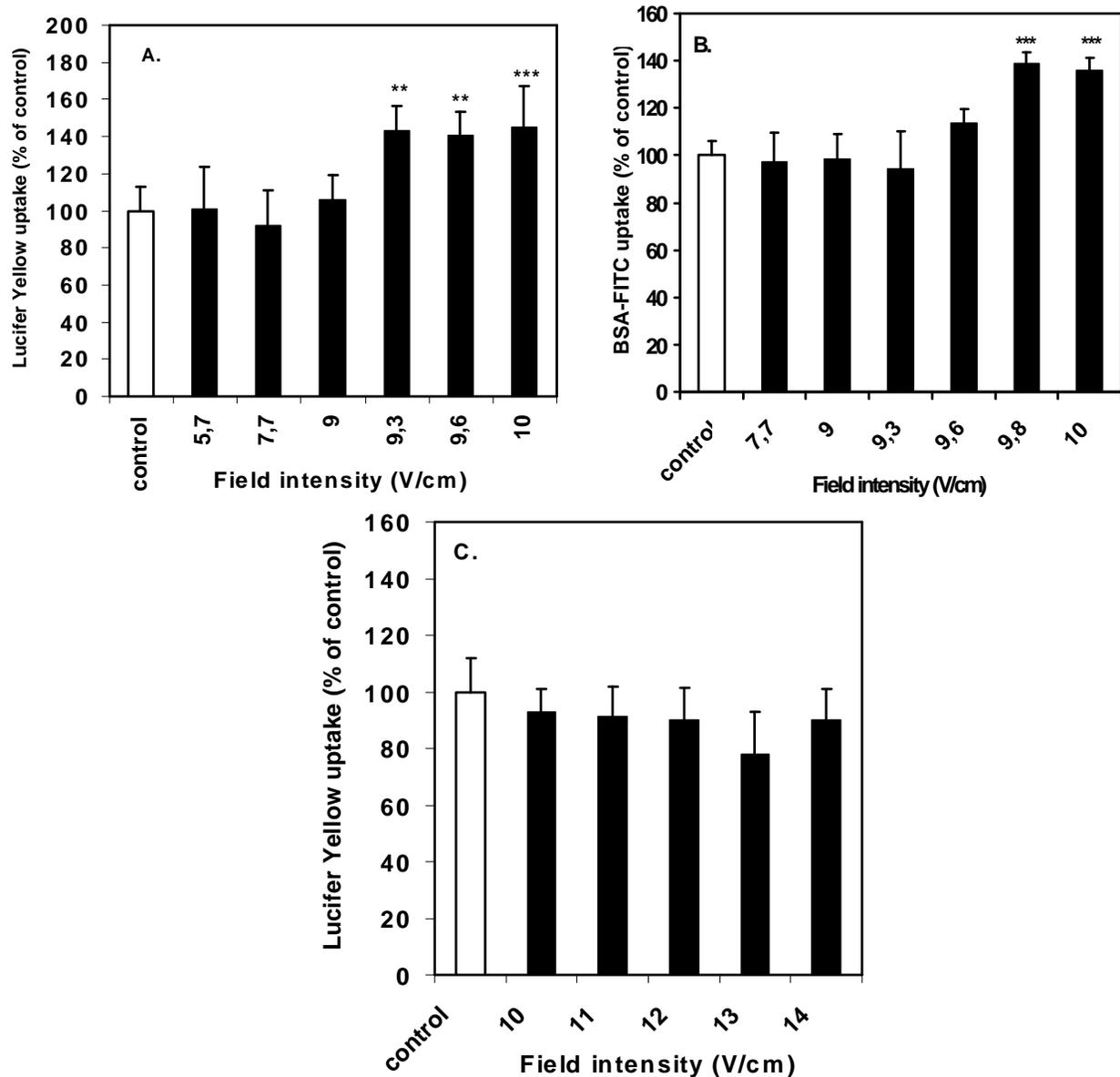


Figure 3. Uptake of the fluorescent probe by DC-3F cells as a function of field intensity. (□) Control cells exposed to 2 mM LY or 6.8 μ M BSA-FITC only. (■) Cells exposed to LY or BSA-FITC and electric pulses (EP). (A) Cells suspended in physiologically conductive medium (10mS/cm) exposed to 200 Hz and 200 μ s PEF for 2 min in the presence of LY followed by an exposure of 8 min to the LY only. (B) Cells suspended in physiologically conductive medium (10 mS/cm) exposed to 200 Hz, 200 μ s, 2 min in the presence of BSA-FITC followed by an exposure of 8 min to the BSA-FITC only. (C) Cells suspended in low conductive medium (1 mS/cm) exposed to 300 Hz and 300 μ s for 2 min in the presence of LY followed by an exposure of 8 min to the LY only. One hundred percent corresponds to control cells uptake in 10 min. Statistical significance: unpaired Student's t-test, ** P < 0.01, *** P < 0.001.

uptake, cells were exposed in physiologically conductive medium to PEF using 10 V/cm field intensity (above the threshold determined in the previous set of experiments) and 300 μ s pulse duration. The exposure time was 2 min (PEF+LY or PEF+BSA-FITC) followed by 8 min incubation in the medium without pulses. The controls were incubated for 10 min with the LY or BSA-FITC only. In this group of experiments, a significant increase (** P <

0.001) in LY or BSA-FITC uptake was found. The increase was almost identical for all the repetition frequencies tested, at a value approximately 40% higher than the unexposed controls (Figure 4A and B). These results confirmed those obtained by changing the field intensity. The values above 400 Hz could not be tested because of the electrochemical reactions at the surfaces of the stainless steel electrodes (Table 1B) at 10 V/cm.

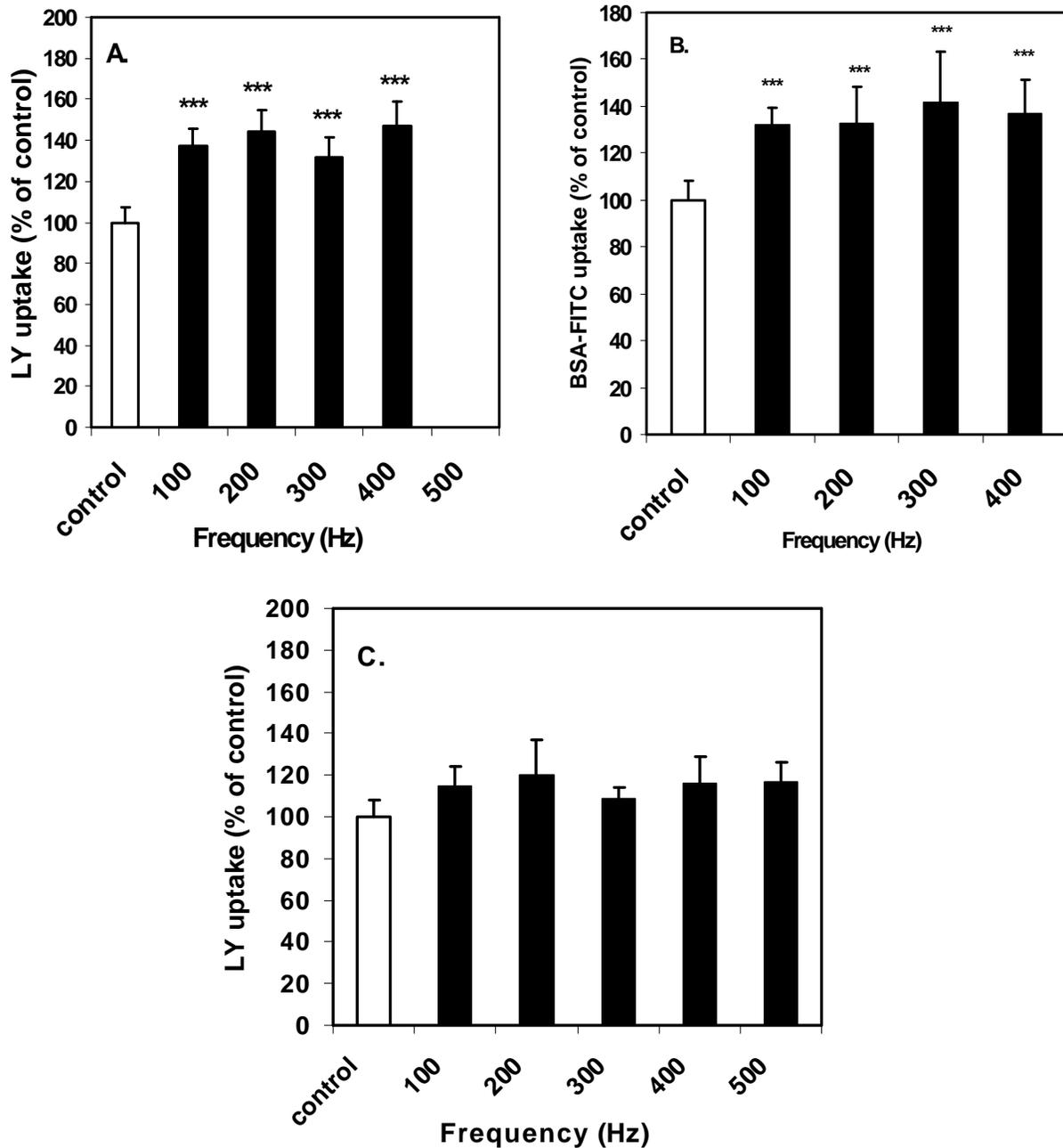


Figure 4. Uptake of the fluorescent probe by DC-3F cells as a function of frequency. (□) Control cells exposed to 2 mM LY or 6.8 μ M BSA-FITC only. (■) Cells exposed to LY or BSA-FITC and electric pulses (EP). (A) Cells suspended in physiologically conductive medium (10 mS/cm) exposed to 10 V/cm and 300 μ s for 2 min in the presence of LY followed by an exposure of 8 min LY only. (B) Cells suspended in physiologically conductive medium (10 mS/cm) exposed to 10 V/cm and 300 μ s for 2 min in the presence of BSA-FITC followed by an exposure of 8 min to BSA-FITC only. (C) Cells suspended in low conductive medium (1 mS/cm) exposed to 10 V/cm and 300 Hz for 2 min in the presence of LY followed by an exposure of 8 min to the LY only. One hundred percent corresponds to control cells uptake in 10 min. Statistical significance: unpaired Student's t-test, *** $P < 0.001$.

Cells were also exposed in low conductive medium to PEF with the same electric parameters. No significant increase in LY uptake was found (Figure 4C). No change in the cell amount was found using different repetition frequencies.

Effect of pulse duration

To study the effect of pulse duration on LY or BSA-FITC uptake, cells were exposed in physiologically conductive medium to PEF using 10V/cm field intensity and 300 Hz

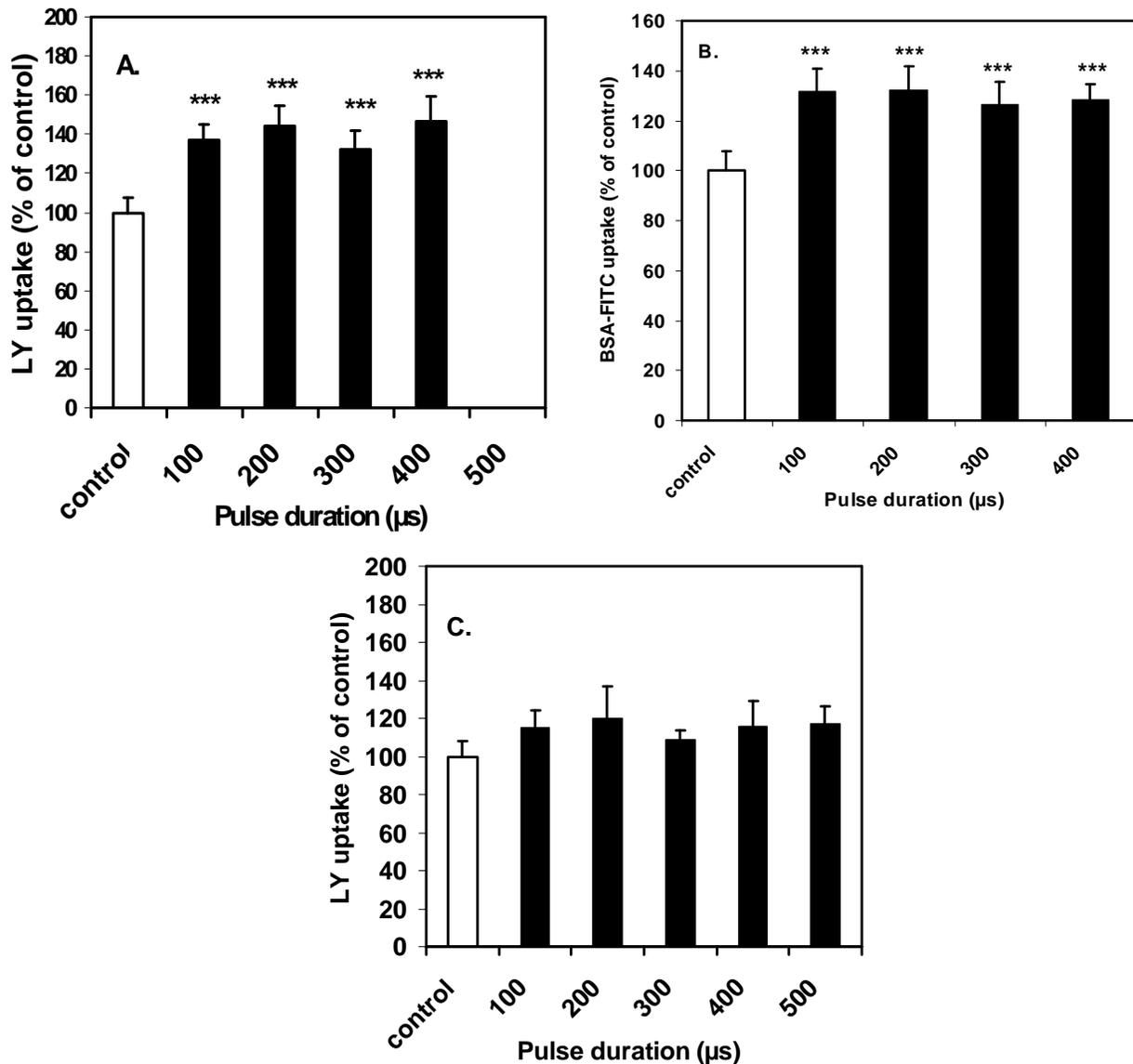


Figure 5. Uptake of fluorescent probe by DC-3F cells as a function of pulse durations. (□) Control cells exposed to 2 mM LY or 6.8 μ M BSA-FITC only. (■) Cells exposed to LY or BSA-FITC and electric pulses (EP). (A) Cells suspended in physiologically conductive medium (10 mS/cm) exposed to 10 V/cm and 300 Hz for 2 min in the presence of LY followed by an exposure of 8 min LY only. (B) Cells suspended in physiologically conductive medium (10 mS/cm) exposed to 10 V/cm and 300 Hz for 2 min in the presence of BSA-FITC followed by an exposure of 8 min BSA-FITC only. (C) Cells suspended in low conductive medium (1 mS/cm) exposed to 10 V/cm and 300 Hz for 2 min in the presence of LY followed by an exposure of 8 min to the LY only. One hundred percent corresponds to control cells uptake in 10min. Statistical significance: unpaired Student's t-test, *** $P < 0.001$.

repetition frequency. The exposure time was 2 min (PEF+LY or PEF+BSA-FITC) followed by 8 min incubation in the same medium without the PEF. The controls were incubated for 10 min with the LY or BSA-FITC only. A significant increase (*** $P < 0.001$) in LY or BSA-FITC uptake was found. The increase was almost identical for all the pulse durations tested, at a value approximately 40% higher than the unexposed controls (Figure 5A and B). The values above 400 μ s could not be tested because of the electrochemical reactions at the

inner surfaces of the electrodes (Table 1B). Cells were also exposed in low conductive medium to PEF with the same electric parameters. No significant increase in LY uptake was found (Figure 5C). In no case a change in the cell amount was found using different pulse durations.

Effect of exposure time

DC-3F cells suspended in either low or physiologically

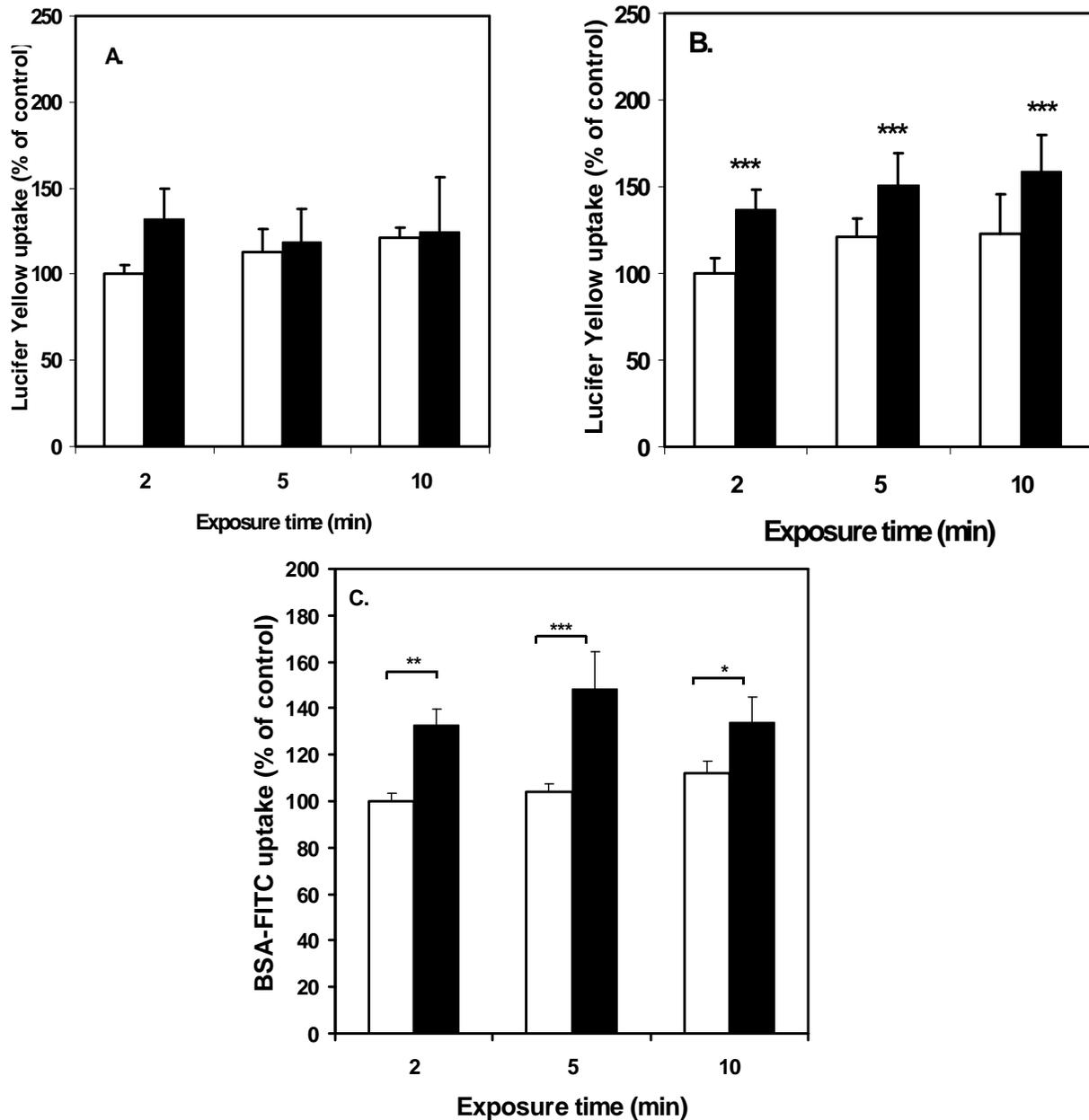


Figure 6. Uptake of fluorescent probes by DC-3F cells as a function of the exposure time, using pulses of 10 V/cm field strength, 300 Hz repetition frequency and 300 μ s pulse duration in either low or physiologically conductive medium (1 mS/cm and 10 mS/cm respectively). (□) Control cells exposed to either LY or BSA-FITC only. (■) Cells exposed to LY or BSA-FITC and electric pulses (EP). (A) LY uptake by cells suspended in low conductive medium. (B) LY uptake by cells suspended in physiologically conductive medium. (C) BSA-FITC uptake for cells suspended in physiologically conductive medium. One hundred percent corresponds to control cells uptake in respectively 2 min. Statistical significance: unpaired Student's t-test, *** $P < 0.001$.

conductive medium (electrical conductivity $\sigma = 1$ or 10 mS/cm respectively) were incubated at room temperature ($\sim 21^\circ\text{C}$) for various durations with either 2 mM LY or 6.8 μM BSA-FITC and concomitantly exposed or not to bipolar asymmetric PEF of 10 V/cm field intensity, 300 Hz repetition frequency, and 300 μs pulse duration. The cells

exposed in the low conductive medium displayed no significant increase in dye uptake compared to the control cells (Figure 6A). The cells exposed in the physiological conductive medium displayed a significantly increased dye uptake compared to the control cells (Figure 6B and C), of about to 40%. There was no cell loss in the

Table 1A. Limits of the electrical parameters used to expose the cells for 10 min in low conductive medium (1 mS/cm) in the absence of detectable electrochemical reactions.

Frequency Hz	Pulse duration μ s				
	100 μ s	200 μ s	300 μ s	400 μ s	500 μ s
100 Hz	14 V/cm	14 V/cm	13 V/cm	13 V/cm	13 V/cm
	0.042 C	0.084 C	0.117 C	0.156 C	0.195 C
200 Hz	14 V/cm	13 V/cm	13 V/cm	13 V/cm	12 V/cm
	0.084 C	0.156 C	0.234 C	0.312 C	0.36 C
300 Hz	13 V/cm	13 V/cm	12 V/cm	12 V/cm	11 V/cm
	0.117 C	0.234 C	0.324 C	0.432 C	0.495 C
400 Hz	13 V/cm	13 V/cm	12 V/cm	11 V/cm	10 V/cm
	0.156 C	0.312 C	0.432 C	0.528 C	0.6 C
500 Hz	13 V/cm	12 V/cm	11 V/cm	10 V/cm	9 V/cm
	0.195 C	0.36 C	0.495 C	0.6 C	0.675 C

The corresponding, number of coulombs delivered under these conditions was also reported.

Table 1B. Limits of the electrical parameters used to expose the cells for 2 min in physiologically conductive medium (10 mS/cm) in the absence of detectable electrochemical reactions.

Frequency Hz	Pulse duration μ s				
	100 μ s	200 μ s	300 μ s	400 μ s	500 μ s
100 Hz	11 V/cm	10.6 V/cm	10.4 V/cm	10.4 V/cm	10.4 V/cm
	0.066 C	0.127 C	0.187 C	0.25 C	0.312 C
200 Hz	10.5 V/cm	10.4 V/cm	10.3 V/cm	10.2 V/cm	10.2 V/cm
	0.126 C	0.125 C	0.371 C	0.49 C	0.612 C
300 Hz	10.3 V/cm	10 V/cm	10 V/cm	10 V/cm	9.7 V/cm
	0.185 C	0.36 C	0.54 C	0.72 C	0.873 C
400 Hz	10 V/cm	9.8 V/cm	10 V/cm	9 V/cm	8.3 V/cm
	0.24 C	0.47 C	0.72 C	0.86 C	0.996 C
500 Hz	9.5 V/cm	9.2 V/cm	8.6 V/cm	8.3 V/cm	7.7 V/cm
	0.285 C	0.552 C	0.774 C	0.996 C	1.16 C

The corresponding, number of coulombs delivered under these conditions was also reported.

exposed samples with respect to the controls.

Electroendocytosis occurs only during EP exposure

In this experiment, no LY or BSA-FITC was present in the medium at the time of the PEF delivery. Thus, just after DC-3F cells exposure (or not) to PEF of 10 V/cm field strength, 300 Hz repetition frequency, and 300 μ s pulse duration in physiologically conductive medium cells were

incubated at room temperature ($\sim 21^\circ\text{C}$) for various durations in the presence of either 2 mM LY or 6.8 μM BSA-FITC. The exposed cells displayed no increased dye uptake compared to the control cells (Figure 7A and B), under conditions (previous figures) under which there is an increase in LY or BSA-FITC if the fluorescent probe is present in the medium during the delivery of the PEF. There was no cell loss in the exposed samples with respect to the controls. One hundred percent corresponds to control cells exposed for 2 min to LY or

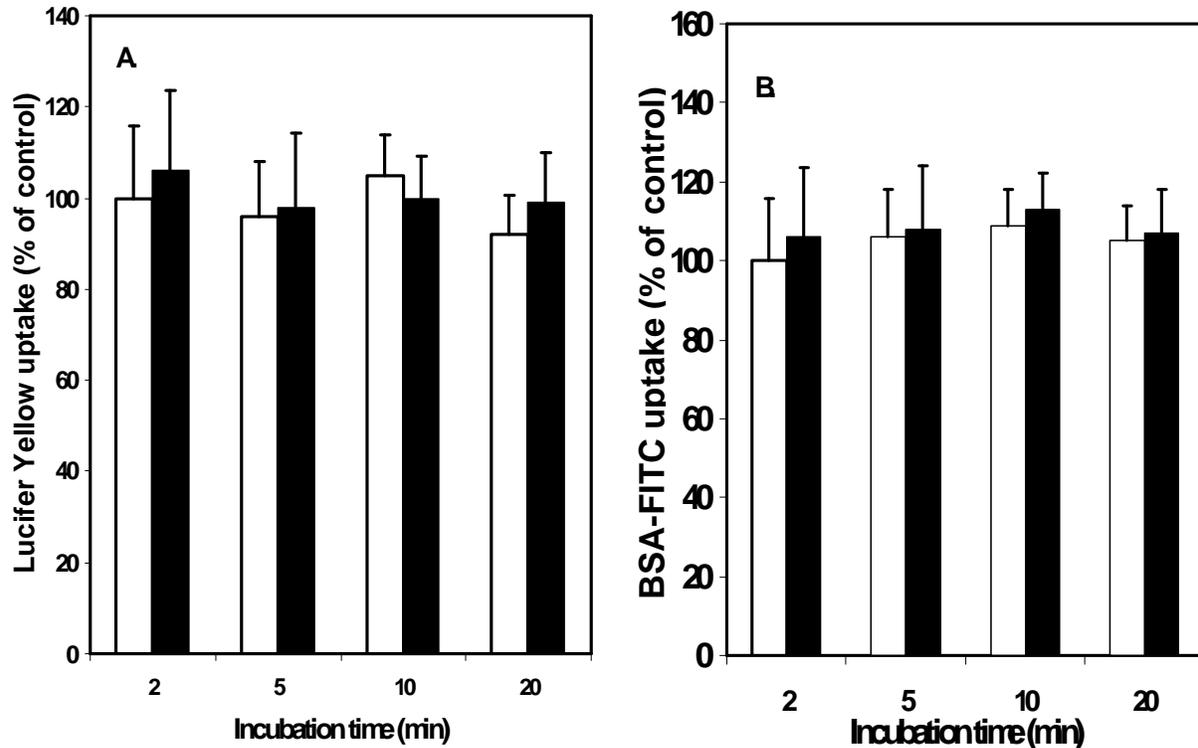


Figure 7. Uptake of the fluorescent probe by DC-3F cells, using different incubation times, physiologically conductive medium (10 mS/cm), 10 V/cm field intensity, 300 Hz repetition frequency, 300 μ s pulse duration, and 2 min exposure time to the electric pulse only. (□) Incubation in the presence of LY or BSA-FITC without EP. (■) Incubation in the presence of LY or BSA-FITC just after the exposure to EP. (A) 2 mM LY uptake. (B) 6.8 μ M BSA-FITC uptake.

BSA-FITC only.

DISCUSSION

A bipolar asymmetric signal with the area of the positive part of the pulse (above the base line) nearly equal to the area of the negative part of the pulse (below the base line) (Figure 8) was chosen to perform the present study in order to have a sort of “net” pulse in one direction (one short and “relatively “intense pulse “above” the threshold in one polarity and one long and much less intense pulse “below” the threshold in the other polarity) trying to approach the unipolar condition (pseudomonopolar) of Antov et al. (because the long and low pulse has a field strength below the threshold defined by the study of Mahrour et al.) with minimization of potential electrophoresis and electrochemical reactions at the electrodes (because the product duration by field strength is similar between the positive and the negative part of this bipolar asymmetric pulse).

The experimental findings here reported demonstrate a statistically significant increase of ~1.3 to 1.5-fold of a non-permeant fluorescent probe uptake concomitant to the exposure to the bipolar asymmetric low intensity square pulses for 2 min total exposure time using

physiologically conductive medium (10 mS/cm). Interestingly, this increase occurs above a threshold value of the field intensity 9.3 to 9.8 V/cm independent on the pulse duration, frequency, and the temperature during the exposure. These results were in agreement with those obtained by Mahrour et al. and in disagreement with those obtained by Antov et al. We found that there was no effect using the low conductive medium (1 mS/cm) which shows the influence of medium conductivity on the fluorescent probe uptake. Conductivities as low as 1 mS/cm were not tested in the previous studies. The effect of conductivity may lead us to the fact that uptake increase could be dependent on the electric current flowing through the cells suspension. The calculated current flowing through the cells suspended in S-MEM (10 mS/cm) was 50 mA at 10 V/cm compared to 5 mA with that obtained by the low conductive medium (1 mS/cm) using the same field intensity. The number of coulombs liberated in the conductive medium using 10 V/cm, 200 Hz, 200 μ s, and 2 min total exposure time, were 0.24 coulombs compared to 0.024 coulombs liberated in the low conductive medium. The results obtained by changing either the repetition frequency or the pulse duration demonstrated an increase in both LY and BSA-FITC uptake at 0.06 coulomb which was corresponding to 10 V/cm, 100 Hz,

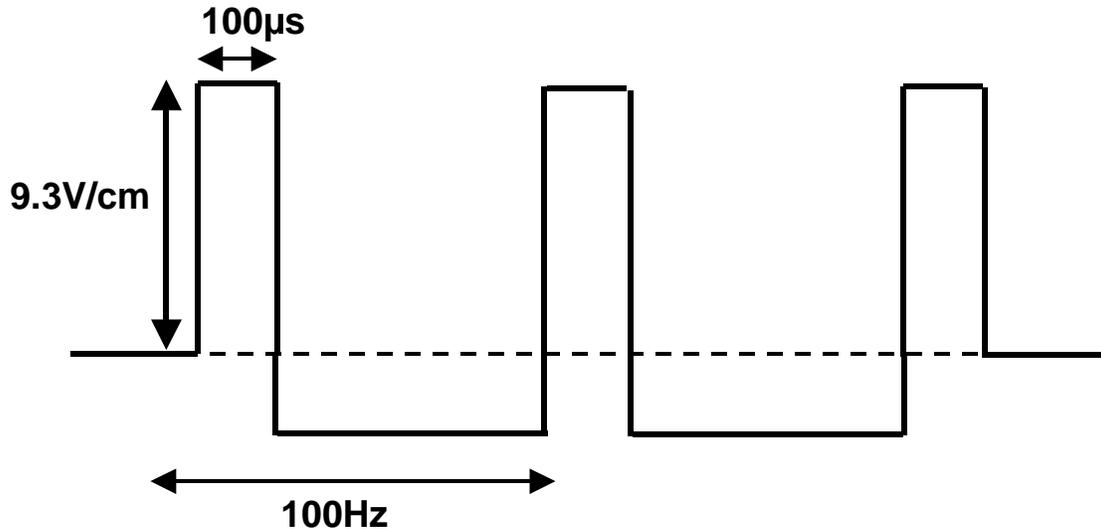


Figure 8. Bipolar equilibrated asymmetric electric pulses used in the present study.

100 μ s, 2 min total exposure time. Thus the number of coulombs (and the current intensity) in the low conductivity medium are not the limiting factor. We can thus exclude a direct effect of the electric current flowing through the cell suspension. This conclusion is reinforced by the presence of a clear plateau for frequencies above 100 Hz (Figure 4) or pulse durations longer than 100 μ s (Figure 5) at 10 V/cm as the number of coulombs is very different but the cell response is very similar. Moreover this “plateau” confirms that this increase is as an all-or-nothing response independent on the energy liberated in the medium as reported by Mahrour et al. The absence of cell response in the low conductivity medium can simply result from the fact that, contrary to the S-MEM, a cell culture medium at 10mS/cm, the low conductive medium at 1 mS/cm is not a “physiological” medium: osmolarity is preserved by the presence of 250 mM sucrose, and ions content (in particular Na⁺ and Cl⁻) is very low. Thus, to increase their endocytosis rate, cells must be in “physiological” conditions.

Our results also confirm that the receptor-mediated and fluid-phase endocytosis increase in the cells exposed to PEF results of a very rapid change: it is detectable after the beginning of PEF exposure and is not detected if the fluorescent probe is added immediately after the end of PEF delivery.

The similarity between our results and the results of Mahrour et al. was the all-or-nothing type of response that is occurring for values above a threshold value for the field intensity. Other set of electrical parameters were also explored (data not shown), all of them supporting the fact that the threshold value for the field intensity was 9.3 to 9.8 V/cm. The threshold of the field intensity obtained in this study was higher than that obtained by Mahrour et al., also in a physiological conductive medium (10 mS/cm). The reason behind this may be due to the fact

that cells used in our study were in suspension while the cells used in the study of Mahrour et al. were adherent cells. It is important to consider that the geometry of attached cells and of cells in suspension is very different. While the latter are spherical, the former have their cell content spread over a large surface on the cell culture support. Because Mahrour et al. also used the DC-3F cells, we can compare the radius “R” of the attached cell (for the simplicity, a disk-shape cell of an average height of 1 μ m) cell (in the study of Mahrour) with the radius “r” of the same cell in suspension (spherical) (in our study) (Figure 9).

It is obvious that R (sphere) is smaller than r (disk-shape). Moreover, assuming that the volume of the cell in both cases is the same (that is, volume of the attached cylindrical cell = volume of the suspended rounded cell), then

$$\begin{aligned} \pi R^2 h &= 4/3 \pi r^3 \\ R &= \sqrt[3]{(4)/(3h)} r^3 \end{aligned} \quad (4)$$

Knowing that the radius “r” of the spherical DC-3F cell is 8 μ m and supposing that the height “h” of the cylindrical cell is 1 μ m and using Equation 1, the radius of the attached cell could be R = 26 μ m (possible, taking into account that the a spindle geometry is more frequent than a disk-shape geometry in these cells). With respect to Equation 2, considering f and g are similar in both cases (for the first approximation), we found that the ratio $E_{th,1}$ of Mahrour (2.6 V/cm) to $E_{th,2}$ in our study (9.3 V/cm) equal to the ratio r/R (that is, $E_{th,1}/E_{th,2} = r/R = 0.3$). This confirms that the transmembrane potential induced by our threshold (maximal value at the pole of the cell, about 11.2 mV) and the threshold of Mahrour (maximal value at the pole of the cell, about 10.1 mV) were nearly the same. It is also important to note that Mahrour et al. did

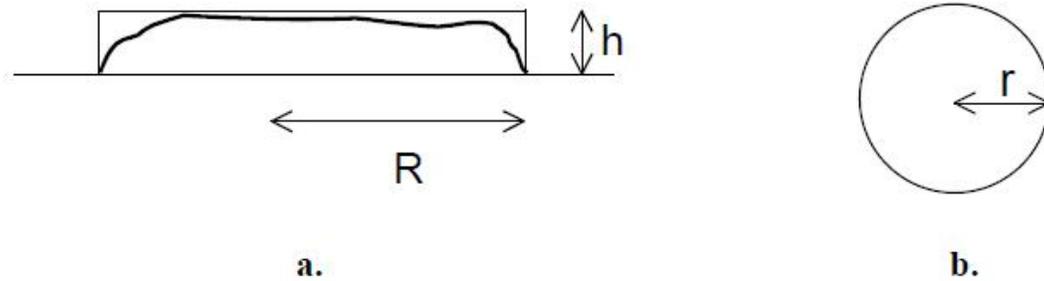


Figure 9. Schematic diagram showing the difference between the attached cell with its simple form (a) and the suspended cell (b).

not reported limitations due to electrochemical reactions. The absence of the electrochemical reactions in the study of Mahrour et al. might be due to (i) the low field strength of the PEF applied (less than 6 V/cm), (ii) the symmetric bipolar character of the electric pulses as mentioned before, or (iii) as well as the experimental set up used in the exposure process as the adherent cells were exposed in petri dishes with electrodes having a small surface of contact with the medium, and moreover the total volume of medium being much larger (2 ml in the Petri dish instead of 100 μ l between the stainless steel plate electrodes).

Electrochemical reactions at the surface of the electrodes

The linear dependence of LEF-induced uptake by Antov et al. on electric field strength with no signs of a threshold value of the field strength, with maximal uptake \sim 7.5-fold higher than in controls at 20 V/cm and with a significant loss in viability by increasing the electric field strength could be explained as a non physiological increase obtained due to the electrochemical contamination. Therefore we investigated how the consequences of the electrochemical reactions occurring at the surface electrodes could interfere with the “biological” data in this type of experiments.

To date, several reports have been published on the detrimental effects of metal ions released from the electrodes (Loomis-Husselbee et al., 1991; Friedrich et al., 1998; Stapulionis, 1999). Loomis-Husselbee et al. (1991) have reported that Al^{3+} released from aluminium electroporation cuvettes can significantly affect biochemical processes involving inositol phosphates. Friedrich et al. (1998) have measured the release of Al^{3+} in concentrations up to 1 mM, and have observed that this release also affects the pH of the suspension. Similarly, Stapulionis (1999) has measured the release of $\text{Fe}^{2+}/\text{Fe}^{3+}$ from stainless steel electrodes in concentrations up to 1.2 mM, as well as the release of Al^{3+} from aluminium electrodes and of Cu^{2+} from copper

electrodes, and has reported that the release of each of these ions can cause substantial precipitation of nucleic acids and proteins from the solution. The release of $\text{Fe}^{2+}/\text{Fe}^{3+}$ from stainless steel electrodes has also been confirmed by Tomov and Tsoneva (2000). Kotnik et al. (2001) have measured concentrations of metal ions released into DC-3F cells suspension from aluminium electroporation cuvettes (release of Al^{3+}) and stainless steel electrodes (release of $\text{Fe}^{2+}/\text{Fe}^{3+}$) during cell membrane electropermeabilization by unipolar and symmetrical bipolar rectangular electric pulses. They showed that in both cases, the concentrations of ions released by bipolar pulses are more than an order of magnitude lower than those released by unipolar pulses of the same amplitude and duration, and thus, the detrimental effects of electrolytic contamination on cells (the most obvious detrimental effect of the released metal ions would be the loss cell viability) and the electrolytic erosion of the electrodes can be largely reduced by the use of bipolar instead of unipolar pulses.

The electrochemical reactions put constrains for the electrical parameters used during the exposure of the cells suspended either in low (1 mS/cm) or physiologically (10 mS/cm) conductive medium by the bipolar asymmetric PEF. The data obtained in the Table 1A and B indicates that by increasing either the pulse duration or the frequency, the threshold voltage required to avoid the electrochemical reactions decreases. Also, the higher the conductivity of the suspended medium the lower the threshold required to obtain electrochemical reactions. This could be explained by the fact that the electrochemical reactions are mainly voltage dependent (and secondary to the other parameters) not current dependent as the electroendocytosis (Table 1A and B). The voltage acts as a driving force for the extraction of atoms from the electrodes and thus release of the electrochemical deposits in the exposure medium. The data concerning the number of coulombs liberated in the medium give us an evidence that the number of coulombs was not the key parameter to get the electrochemical reactions. For example, we obtained electrochemical reactions at 0.042 coulombs

(corresponding to 14 V/cm, 100 Hz, 100 μ s, 10 min total exposure time, 1 mS/cm) while, we did not obtain the electrochemical reactions at 0.96 coulombs (corresponding to 8 V/cm, 500 Hz, 400 μ s, 2 min total exposure time, 10 mS/cm). Both of the previous values were below the threshold voltage required to initiate the electroendocytosis. Interestingly, we found that the electrochemical aggregates $\text{Fe}^{2+}/\text{Fe}^{3+}$ combined with LY molecules outside the cells, forming heavy aggregates (Figure 2). The aggregates could be precipitated in the cells pellet causing an artefact in the readings of LY uptake inside the cells obtained after putting lysis buffer with the pellet to release the cellular contents. The pictures were taken under the fluorescence microscope of 5 μ l cells lysate containing the aggregates in two phases for each, the contrast phase (Figure 2A and C) and the green fluorescence phase (Figure 2B and D). The LY molecules combined with the electrochemical deposits causing a huge increase in the fluorescence readings by more than 10 times interfering with LY uptake readings corresponding to the electroendocytotic activity. So, it is necessary to respect Table 1A and B to avoid massive electrochemical reactions to have LY uptake values indicating the real electroendocytosis.

There was no change in both the temperature and cell loss in the lower levels of electrochemical reactions obtained in the range from 0.042 coulombs to 0.624 coulombs (corresponding to 13 V/cm, 400 Hz, 400 μ s, 10 min total exposure time, 1 mS/cm). It should be stressed that degassation for 10 min at room temperature of the exposure medium before the exposure process is necessary to reduce the effect of electrochemical reactions (that is, without degassation the threshold values obtained in Table 1A and B would be lower).

The generation of fluorescent aggregates collected with the cells by centrifugation is in agreement with the linear curve obtained in this study (Figure 1) as a result of the electrochemical reactions due to exposing the cells in low conductive medium 1 mS/cm using 13 V/cm, 400 Hz, 400 μ s of pulse duration, and different exposure times (Table 1A). It could be a way to explain the differences between the studies of Mahrouf et al. and Antov et al., as the monopolar pulses should maximize the release of metal ions from the electrodes, and therefore the generation of fluorescent aggregates.

CONCLUSION

Analysis of the possible mechanisms for enhancement of electroendocytosis by low intensity pulsed electric fields

The characteristics of the electrical parameters used and the results of the experiments reported in this study allow showing some suggestions about the underlying biological mechanisms:

1. The hypothesis of thermal effects can be eliminated.
2. 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 seconds and 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 gene expression regulation mechanisms.
3. Since a similar increase was observed using different PEF frequencies, there is almost not possible to consider 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 (Tsong and Astumian, 1987), are also unlikely.
4. More probably, the induced fluid-phase endocytosis stimulation could result from a modification of the physiological endocytotic process rather than from a non-physiological perturbation. Normal constitutive endocytosis seems required in order to observe the PEF-induced increase. Preservation of physiological conditions are important and this could explain the absence of increase of the endocytosis rate in the low conductive medium.
5. The effects of a variation of the transmembrane potential difference cannot be excluded at all from our studies. The parallelism of the results reported here on spherical cells with those of Mahrouf et al. on attached cells argue in favour of a role of the transmembrane potential difference.

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