2-NBDG, a Fluorescent Analogue of Glucose, as a Marker for Detecting Cell Electropermeabilization In Vitro

Elham Raeisi · Lluis M. Mir

Abstract This study investigated whether molecules spontaneously transported inside cells, like glucose derivatives, can also be used as electropermeabilization markers. Uptake of a fluorescent deoxyglucose derivative (2-NBDG) by normal and electropermeabilized cells in culture was analyzed. 2-NBDG was added to DC-3F cell suspensions and cells, exposed or not to eight square-wave electric pulses of 100-μs duration and of appropriate field amplitude at a repetition frequency of 1 Hz or 5 kHz, were incubated at 37 °C. 2-NBDG uptake was temperature-, concentration- and time-dependent in cells submitted or not to the electric pulses. In spite of significant uptake of 2-NBDG mediated by GLUT transporters into nonpermeabilized cells, the electric pulses significantly increased about ten to hundred times the 2-NBDG uptake into the cells. The increase in the field amplitude from 900 to 1,500 V/cm resulted in a progressive increase of 2-NDBG. Our results show that under the conditions of in vivo exposure duration to FDG and the physiological concentration of D-glucose, electric pulses increased 2-NBDG uptake into electropermeabilized cells. Under our experimental conditions, the percentage of permeabilized cells within the population of cells exposed to electric pulses remained at the same level regardless of the pulse frequency used, 1 Hz or 5 kHz. The findings showed that glucose derivatives can also be used to detect electropermeabilized cells exposed to electric pulses.

Keywords 2-NBDG · FDG · PET scan · Electropermeabilization · Flow cytometry · Tumor cell

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Introduction

Cell electropermeabilization or electroporation is a physical method that uses short and intense electric pulses to increase cell membrane permeability (Neumann et al. 1982) and, therefore, increase the uptake of molecules such as DNA, antibodies and oligonucleotides into the cells (Mir 2001).

Electroporation can also facilitate the crossing of the cell membrane and the cellular uptake of drugs, for molecules that are hydrophilic and lack transport systems or for low-permeant molecules that poorly cross the plasma membrane (Orlowski et al. 1988; Sersa et al. 2003; Silve and Mir 2011). Several chemotherapeutic drugs were tested in vitro for potential application in combination with cell electroporation, and an increase of thousands of times was demonstrated for bleomycin and one of several times for cisplatin (Melvik et al. 1986; Orlowski et al. 1988; Sersa et al. 1995; Kambe et al. 1996; Cemazar et al. 1998, 2001;

Gehl et al. 1998; Jaroszeski et al. 2000; Kuriyama et al. 2000; Breton and Mir 2012). Therefore, the selection is limited to those drugs that are hydrophilic and lack transport systems in the membrane.

To monitor in vitro the permeability of cells, classical approaches rely on the uptake of fluorescence markers such as Lucifer yellow (Mir et al. 1988) and propidium iodide (Djuzenova et al. 1996) or on the release of previously loaded fluorescent markers, all of them being nonpermeant molecules that cannot diffuse through the membrane of intact cells (Silve and Mir 2011).

Glucose is a permeant molecule taken up by cells through different glucose transporters that specifically internalize the glucose molecules. Once in the cells, glucose is phosphorylated by the hexose kinases and the phosphorylated molecules cannot be transported back to the extracellular medium. We analyzed whether glucose derivatives can be used to detect cell electropermeabilization. Indeed, our final purpose was to evaluate whether ^{18}F-fluorodeoxyglucose (FDG), a radioactive glucose derivative used for clinical investigations and for diagnosis, staging and monitoring of the treatment of cancers by positron emission tomography (PET) (Hoh et al. 1993; Bomanji et al. 2001; Reske and Kotzerke 2001; Chin and Chang 2006), could have the potential for imaging permeabilized cells and mapping electropermeabilized tissues in vivo by PET. To survey the ability of FDG PET for monitoring the efficacy of electric pulses in experiments in vivo, the first mandatory step was to establish the feasibility of this approach in in vitro studies.

Because of limitations to the use of FDG in vitro, like the very short half-time of [18]Fluor, the need for protection in wet labs and the high cost, the use of this molecule is not easy to implement. Fluorescence-labeled deoxyglucose molecules (like 2-NDBG) can replace radioactive glucose derivatives (Nitin et al. 2009). 2-NBDG has been used to evaluate glucose metabolism in different model systems like *Escherichia coli* (Natarajan and Srienc 1999), yeast (Oh and Matsuoka 2002), pancreatic islet cells and neurons (Itoh et al. 2004) as well as in various cancer cell lines (O'Neil et al. 2005; Cheng et al. 2006). As 2-NBDG is a fluorescent analogue of D-glucose, it is transported like FDG into cells by glucose transporters (GLUTs), which are located at the cell membrane and cause facilitated transport of glucose across the membrane (Yoshioka et al. 1996a, 1996b, 1996c; Lloyd et al. 1999; Yamada et al. 2000; Zhang et al. 2004; Cheng et al. 2006; Iori et al. 2006; Nitin et al. 2009). Although six isoforms (GLUT1–GLUT5 and GLUT7) of these transporters have been identified (Pauwels et al. 2000), 2-NBDG is incorporated into the pancreatic ß-cells through GLUT1 and GLUT2 (Yamada et al. 2000). GLUT1 is widely expressed in cancer cells (Younes et al. 1996) such as MCF-7 and HepG2 cells

(Chen et al. 2002). Consequently, 2-NBDG uptake in tumor cells should be facilitated by this glucose transporter (O'Neil et al. 2005). Therefore, there is spontaneous uptake of 2-NBDG in the absence of any cell membrane permeabilization. As electroporation-based therapies, like electrochemotherapy (ECT), are based on the increase of drug uptake into tumor cells caused by transient permeabilization of the cell membrane, we studied the effect of electric pulses on the uptake of 2-NBDG in DC-3F cells. In particular, we explored whether the uptake by transiently electropermeabilized cells could be larger than the spontaneous uptake for a period of time compatible with those of the usual PET investigations.

Materials and Methods

Cells

DC-3F cells are spontaneously transformed Chinese hamster fibroblasts (Biedler and Riehm 1970). They were grown in minimum essential medium (SMEM) supplement with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin solution (all from Life Technologies, Carlsbad, CA). Cells were detached with trypsin/EDTA (life technologies), counted and seeded in new flasks using standard protocols.

Cell Electropermeabilization

After trypsinization, cells were centrifuged for 10 min at 1,000 rpm at 4 °C and suspended in phosphate-buffered saline (PBS) to obtain 5×10^5 cells per 45 µl ($\sim 10^7$ cells/ml). For each experiment, 5 µl of NBDG (MW 342.26) (Molecular Probes, Eugene, OR) were added to the 45 µl of cell suspension. Immediately after, the 50 µl of cells in the presence of NBDG were placed in a sterile electroporation cuvette (Cell Projects, Kent UK; with a 1-mm gap between the electrodes). Eight square-wave electric pulses of appropriate voltage and of 100-µs duration were delivered at a repetition frequency of 1 Hz or 5 kHz using a Cliniporator (IGEA, Carpi, Italy).

In competition experiments, after trypsinization, cells were suspended in PBS containing 5.5, 11 or 27.5 mM D-glucose (Merck, Darmstadt, Germany). All experiments were performed at 22 or 37 °C as explained in the text.

Flow Cytometry

In all experiments, 2-NBDG uptake was stopped by diluting the cells with 15 ml of precooled PBS. Cells were subsequently resuspended in 500 µl precooled PBS and maintained at 4 °C before flow cytometric analysis.

For each measurement, fluorescence was measured with a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cells were excited at 488 nm, and the emitted 2-NBDG fluorescence was collected at 640 nm. Data from each flow cytometric measurement (relative scatter light or fluorescence intensity of each event) were stored under flow cytometry standard format. Controls (no 2-NBDG and no pulse in the presence of 2-NBDG) were included in all experiments.

Data Processing

All experiments were repeated at least three times on different days. Results are presented as the relative difference in the peak channel of the fluorescence intensity between each group and the corresponding control performed in the same experiment. Results are also presented as the percentage of events in the most fluorescent part of the cell population exposed to 2-NBDG and to the electric pulses (which should correspond to the percentage of reversibly permeabilized cells detected by 2-NBDG uptake). ANOVA and paired Student's t-test were used to evaluate the statistical significance of differences between the experimental and control groups. $p < 0.05$ was considered significant.

Results

Temperature Dependence of 2-NBDG Uptake

As spontaneous uptake is mediated by the activity of glucose transporters, we first analyzed the uptake temperature dependence with and without pulses, using typical electrical parameters for the reversible electropermeabilization of DC-3F cells (eight electric pulses of 1,200 V/cm and 100-μs duration delivered at a repetition frequency of 1 Hz). Cells were put in the presence of 300 μM 2-NBDG, immediately subjected to electric pulses (or not in the controls) and incubated for 5 more min at 22 or 37 °C (Table 1). Uptake of 2-NBDG was sensitive to temperature. In nonelectroporated cells, 2-NBDG uptake was larger at 37 °C than at 22 °C

(Table 1). This difference was significant ($p < 0.01$). In samples treated with electric pulses a large increase in 2-NBDG uptake was observed for incubations of just 5 min, the time for the cells to reseal according to previous experience with these cells (Orlowski et al. 1988). Actually, a biphasic distribution was found, corresponding to the fluorescence of the transiently permeabilized cells and of the nonpermeabilized cells (in spite of their exposure to the electric pulses). Uptake in permeabilized cells was much more important than that in nonpermeabilized cells. Moreover, fluorescence was significantly ($p < 0.001$) higher at 37 °C with respect to uptake at 22 °C (mean peak channel increased from 2,951 to 3,918). Because in all cases uptake at 37 °C was higher than at 22 °C and since 37 °C is close to the physiological body temperature in most mammals, it was decided that all further experiments would be performed at 37 °C.

It must also be noted that at 22 °C 2-NBDG uptake was very low as there was almost no increase in cell fluorescence compared to the autofluorescence of those cells (Table 1, Fig. 1). At 37 °C, a net increase in fluorescence was found, which was the consequence of the increase in the cell basal metabolism as a result of exposure of cells at 37 °C physiological temperature.

2-NBDG Uptake Dependence on Its Concentration

To assess the optimal 2-NBDG concentration to be used in further experiments, cells were incubated in the presence of 10, 20, 40, 80, 160, 240 and 300 μM of 2-NBDG for 10 min after applying electric pulses at zero (controls no pulse), 1,000 or 1,200 V/cm (Fig. 2). In all cases, cell fluorescence increased with respect to the controls incubated in the absence of 2-NBDG ($p < 0.001$). In samples exposed to electric pulses, there was a large increase in uptake ($p < 0.001$), by two orders of magnitude, in electropermeabilized cells (Fig. 2). The highest 2-NBDG uptake was obtained at 300 μM, but the working concentration of 240 μM was chosen because the percentage of events in the part of the cell population that was electropermeabilized was higher in the presence of 240 μM 2-NBDG (data not

Table 1 Effect of electric pulses on uptake of 2-NBDG at an external concentration of 300 μM

Group	Peak channel of M_1 or M_2 region (means ± SD)		
	22 (°C)	37 (°C)	37/22 (°C)
No 2-NBDG (Autofluorescence)	18 ± 8	18 ± 8	1.0
2-NBDG alone	47 ± 30	76 ± 1.5[a]	1.6
2-NBDG + electric pulses	2,952 ± 501	3,918 ± 104[a]	1.3

The changes in the value of the peak channel of the high-fluorescence cell population (M_2) are reported in the table. Cells were incubated with 300 μM 2-NBDG for 5 min at room temperature (22 °C) or at 37 °C and exposed to eight pulses of 100 μs and 1,000 V/cm at a repetition frequency of 1 Hz

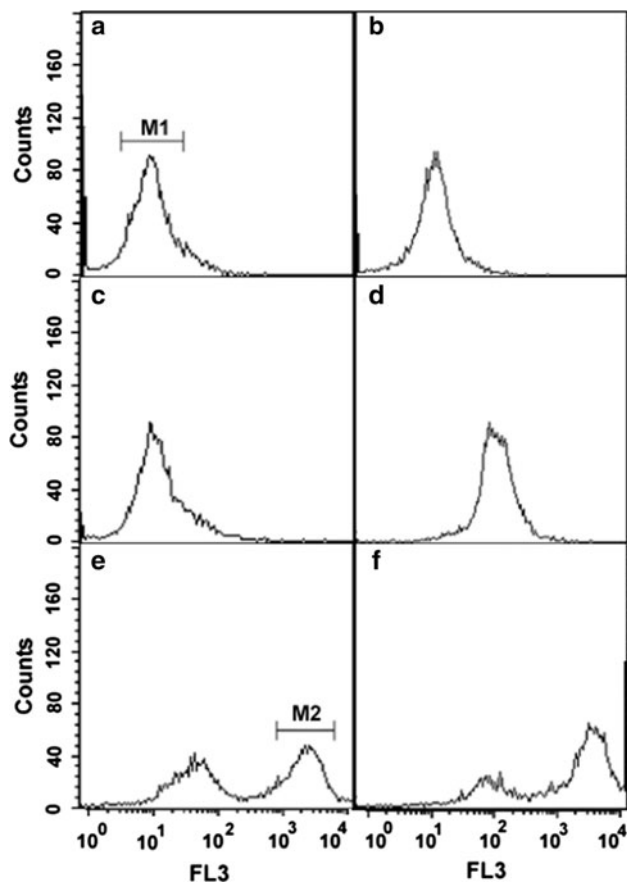[a] Difference statistically significant for 2-NBDG uptake at 37 °C with respect to 22 °C, $p < 0.05$

Fig. 1 Flow cytometric histograms of DC-3F cells: autofluorescence (**a**, **b**), 2-NBDG alone (**c**, **d**) and 2-NBDG together with electric pulses (**e**, **f**) of cells incubated at 22 °C (**a**, **c** and **e**) or at 37 °C (**b**, **d** and **f**) during incubation with 2-NBDG (**c–f**) or without 2-NBDG (**a**, **b**, autofluorescence, control). The effect of eight electric pulses of 100 μs, 1 Hz at 1,200 V/cm on uptake of 2-NBDG was studied (at an external concentration of 300 μM) into DC-3F cells exposed or not to the electric pulses

shown). This could mean that uptake in the presence of 300 μM 2-NBDG is toxic to electropermeabilized cells, and therefore, this high concentration was not used in subsequent experiments, to avoid such potentially toxic effects. It can also be noted that uptake with pulses of 1,200 V/cm was higher than uptake with pulses of 1,000 V/cm and that at the lowest concentration tested (10 μM 2-NBDG) spontaneous uptake by transporters was equally intense as uptake by electroporation (data not shown). Interestingly, at concentrations as low as 20 μM, the fluorescence intensity in samples with pulses was already higher than that in the corresponding controls: the electric pulses caused an increased of fluorescence from 12 to 79 and from 8.5 to 117.5 at 1,000 and 1,200 V/cm, respectively ($p < 0.001$). Therefore, the electric pulses moderately enhanced uptake of 2-NDBG at low concentration (20 μM) and highly increased it at higher concentrations.
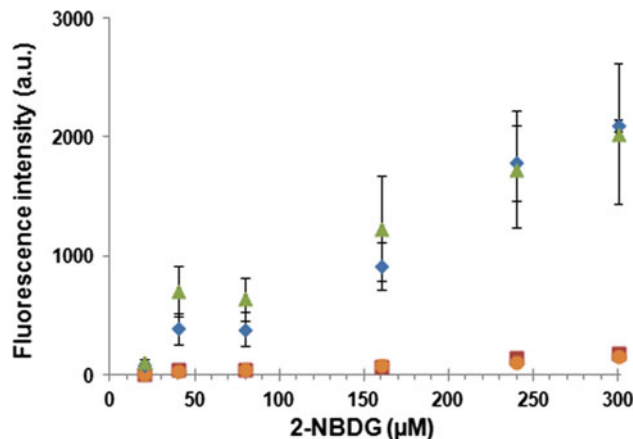

Fig. 2 Changes in the peak channel of monodistributed fluorescent cells not exposed to the electric pulses or changes in the value of the peak channel of the high-fluorescence cell population exposed after electric pulses (eight pulses of 100 μs, 1 Hz at 1,000 or 1,200 V/cm) in cells. Cells were incubated for 10 min at 37 °C. Data are means ± SD of at least three independent experiments. *filled square*, *filled circle* no electric pulses; *filled diamond* electric pulses at 1,000 V/cm; *filled triangle* electric pulses at 1,200 V/cm

2-NBDG Uptake as a Function of Pulse Amplitude

In the analysis of 2-NBDG uptake as a function of pulse amplitude, electric pulses with amplitudes of 900; 1,000; 1,100; 1,200; 1,300; 1,400 or 1,500 V/cm were delivered. Cells were pulsed immediately after the addition of the fluorescent marker and after the pulses; they were incubated at 37 °C for 10 min in a water bath to ensure that they were incubated at 37 °C. Two values were considered: the percentage of actually permeabilized cells and the peak channel of their fluorescence, which indicates the level of 2-NBDG uptake by these cells. 2-NBDG uptake increased with the field amplitude applied (Fig. 3a). Field amplitudes higher than 1,500 V/cm were not tested as it was known that they were highly toxic for the cells (induction of irreversible permeabilization and, thus, of cell death). As expected, the percentage of events in the population of cells permeabilized due to electric pulses started to decrease below this field amplitude (Fig. 3b). The highest convenient uptake was obtained using electric pulses of 1,200 V/cm, but the maximum percentage of highly fluorescent cells was reached at 1,000 V/cm ($p < 0.01$). Thus, 1,000 V/cm was considered to be the optimal electric field amplitude for the next part of this study.

2-NBDG Uptake in the Presence of D-Glucose

The previous experiments were performed in the absence of external glucose competing with 2-NBDG uptake. The effect of the presence of different concentrations of normal D-glucose on 2-NBDG uptake in DC-3F cells was
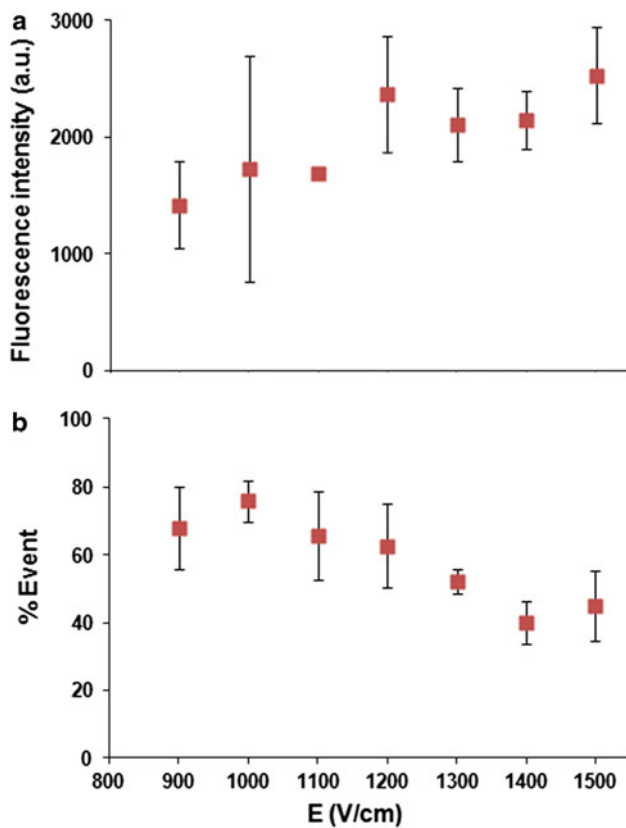
Fig. 3 a Changes in the value of the peak channel of the high-fluorescence cell population as a function of the pulse field amplitude, E (in V/cm). **b** Percentage of highly fluorescent (permeabilized) cells after incubation with 240 μM 2-NBDG for 10 min at 37 °C. Data are means ± SD of at least three independent experiments

examined to evaluate the potential reduction of fluorescence accumulation in the cells, particularly in the presence of the physiological external concentration of glucose (5.5 mM). Cells were thus incubated with 240 μM 2-NBDG or with 2-NBDG and 5.5, 11 or 27.5 mM glucose, together with the delivery or not of eight electric pulses of 100 μs and 1,000 V/cm at a repetition frequency of 1 Hz. Cells were incubated for 10 min at 37 °C after pulse delivery. Glucose, at all concentrations, affected 2-NBDG uptake by cells that were not exposed to electric pulses. However, only partial competition was found at 5.5 mM as the fluorescence of only a fraction of the cells decreased (creating a shoulder in the cell distribution, see Fig. 4c). However, the peak channel value was not affected (Fig. 4). At 11 mM, a small majority of the cells showed reduced fluorescence uptake, while the "fluorescent" cells remained at the control level. At 27 mM, competition was complete as even the more "fluorescent" fraction of the cells showed a decrease in individual fluorescence (peak channel at a value of 118, significantly different [$p < 0.05$] from the peak channel values of the other groups, at about a value of 150).

In cells that were treated with the electric pulses in the presence of different concentrations of external D-glucose, again there was an increase in uptake by two orders of magnitude in the part of the cell population exposed to electric pulses (Fig. 4) ($p < 0.001$).

When cells were exposed to electric pulses, the peak channel value of the highly fluorescent cell population did not significantly increase in the presence of additional external D-glucose ($p = 0.659$ with respect to the control with no added D-glucose). In addition, the percentage of events displaying high fluorescence values decreased at the highest concentrations of D-glucose, 11 or 27.5 mM (Fig. 5). This could reflect partial competition between 2-NBDG and D-glucose to enter the cells, but the difference was not statistically significant ($p = 0.126$).

Time Course of 2-NBDG Uptake

To analyze the time dependence of 2-NBDG uptake, cells were incubated with 240 μM of 2-NBDG at 37 °C for 0, 2, 5, 10, 20, 40 or 60 min in PBS (no D-glucose) after applying (or not) electric pulses at 1,000 V/cm. In the absence of D-glucose and in the absence of pulse delivery, uptake of 2-NBDG increased in a time-dependent manner (Fig. 6). It reached a maximum after 60 min of incubation. In samples exposed to the electric pulses, an increase in uptake by two orders of magnitude in the part of the cell population permeabilized by electric pulses was again observed, even at the maximum incubation time of 60 min (longer times were not tested as cells did not support longer incubations in the PBS buffer) (Fig. 6). Changes in fluorescence intensity showed significant differences during the different incubation times after the application of electric pulses ($p < 0.001$). At 60 min electroporation-based uptake (which does not seem to be modified during the incubation) is completed by the transport-mediated uptake (which increases during this time). In addition, there was always a significant difference in fluorescence intensity between the two experimental groups and between each of the experimental groups and the control without 2-NBDG ($p < 0.001$). This difference remained significant for up to 60 min of incubation ($p < 0.05$).

Time-dependence experiments were also performed in the presence of 5.5 mM of D-glucose (Fig. 6). Cells were manipulated under the same conditions except that after trypsinization they were suspended in modified PBS that contained 5.5 mM of D-glucose. In the absence of electric pulses, fluorescence increased regularly with time. Statistical analysis revealed no significant differences between the uptake of 2-NBDG alone in the presence or in the absence of D-glucose in all periods examined (2–60 min) in the absence of electric pulses.

Fig. 4 Flow cytometric histograms of DC-3F cells treated **a** with no 2-NBDG (absolute control); **b–e** with 2-NBDG alone in the presence of 0, 5.5, 11 or 27.5 mM D-glucose; and **f–i** with 2-NBDG and electric pulses (eight pulses of 100 μs, 1 Hz at 1,000 V/cm) in the presence of 0, 5.5, 11 or 27.5 mM D-glucose, respectively
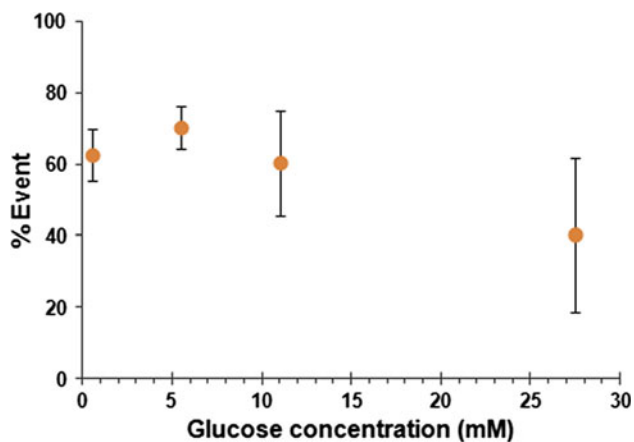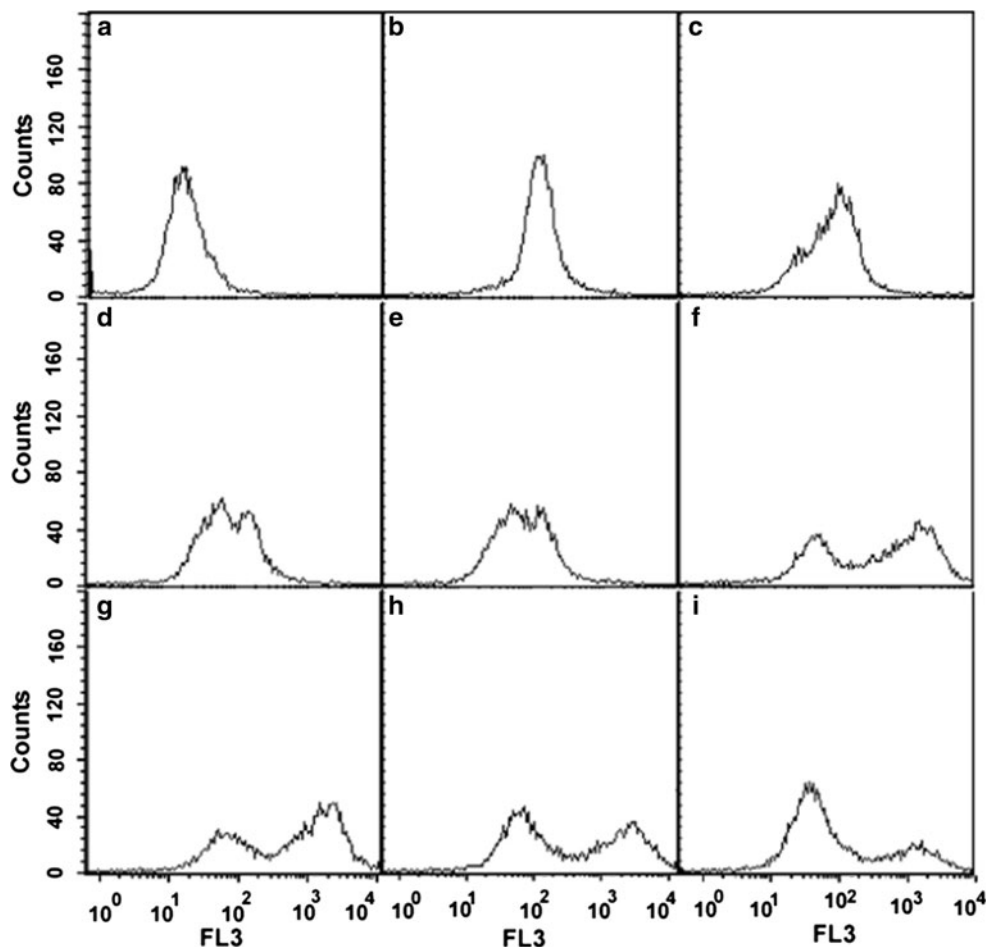

Fig. 5 Percentage of highly fluorescent (permeabilized) cells after exposure of cells to eight pulses of 100 μs and 1,000 V/cm and incubation with 240 μM 2-NBDG for 10 min at 37 °C in the presence of 0, 5.5, 11 or 27.5 mM of D-glucose. Data are means ± SD of at least three independent experiments

In the presence of the electric pulses and 5.5 mM D-glucose, a significant increase in uptake in electropermeabilized cells, by about two orders of magnitude, was again observed, even at 60 min (Fig. 6). Values of fluorescence intensity were similar in the presence and in the absence of D-glucose, except at 20 or 60 min, when significantly higher uptake was recorded in the presence of D-glucose ($p < 0.05$).

2-NBDG Uptake as a Function of Electric Pulses at 1 Hz and 5 kHz Repetition Frequencies

Finally, the effect of a 5-kHz repetition frequency during the delivery of the eight electric pulses on 2-NBDG uptake into electropermeabilized cells was evaluated in comparison to the effects of pulses delivered at the usual 1-Hz repetition frequency. Cells were incubated with 240 μM 2-NBDG for 10 min after applying eight (or no) electric pulses of 100 μs and 1,000 or 1,200 V/cm at repetition frequencies of 1 Hz or 5 kHz. In the absence of D-glucose a difference in uptake by electropermeabilized cells was indeed found (Fig. 7a). Fluorescence intensity was significantly higher when pulses were delivered at a repetition frequency of 1 Hz ($p < 0.001$). However, the same data showed no significant change in the percentage of permeabilized cells ($p = 0.898$) (Fig. 7b).
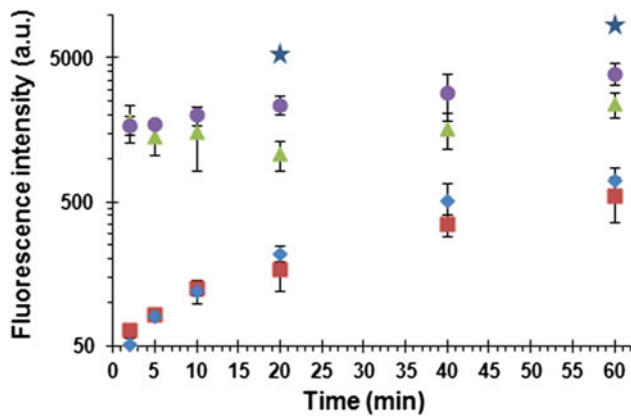
Fig. 6 Changes in the peak channel of monodistributed fluorescent cells not exposed to the electric pulses or changes in the value of the peak channel of the high-fluorescent cell population after electric pulse delivery. Cells were incubated in the absence or presence of a physiological glucose concentration (5.5 mM) for 2–60 min, always in the presence of 240 μM 2-NBDG and with or without the delivery of electric pulses (eight pulses of 100 μs, 1 Hz at 1,000 V/cm). Data are means ± SD of at least three independent experiments. *Stars* denote the only values in which a statistically significant difference was recorded between the absence and the presence of the external 5.5 mM D-glucose. *filled square* 2-NBDG, *filled diamond* 2-NBDG + 5.5 mM D-glucose, *filled triangle* electric pulses, *filled circle* electric pulses + 5.5 mM D-glucose

As the data also show, there was no significant difference between 2-NBDG uptake in the absence or the presence of 5.5 mM D-glucose. Thus, a significant difference was also found in 2-NBDG uptake after application of electric pulses at 1-Hz or 5-kHz repetition frequencies in the presence of 5.5 mM D-glucose ($p < 0.001$) (Fig. 7a), while no significant change was observed in the percentage of cells permeabilized due to the delivery of electric pulses at either 1 Hz or 5 kHz ($p < 0.007$) (Fig. 7b).

Discussion

We have described for the first time the effect of electric pulses on the uptake of 2-NBDG (2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose), a fluorescent derivative of D-glucose.

As stated in the Introduction, 2-NBDG is a marker of glucose uptake in cells of various origins and should have characteristics similar to those of FDG, which has been widely used in PET. In spite of high uptake of 2-NBDG by GLUT transporters into tumor cells, electroporation significantly increases 2-NBDG uptake into the tumor cells used in this study (DC-3F).

Indeed, in the present study, we show that 2-NBDG is rapidly taken up by, and accumulated in, DC-3F tumor cells in the absence of electric pulses. Our results are consistent with previous investigations that have used this
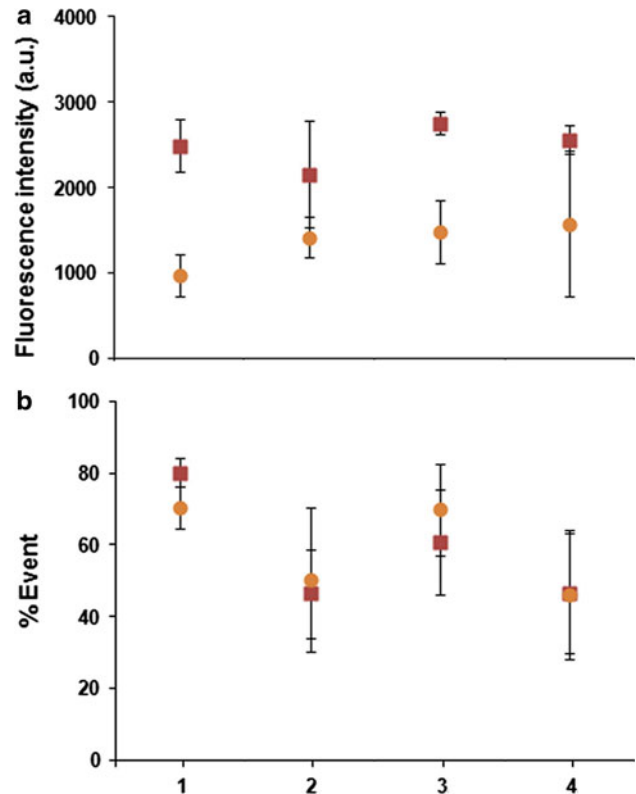

Fig. 7 a Changes in the value of the peak channel of the high-fluorescence cell population. **b** Percentage of highly fluorescent (permeabilized) cells after application of electric pulses (eight pulses of 100 μs, 1 Hz or 5 kHz at 1,000 or 1,200 V/cm). Cells were incubated for 10 min at 37 °C in the absence or presence of physiological glucose solution (5.5 mM). *1* 1,000 V/cm, *2* 1,000 V/cm + 5.5 mM D-glucose, *3* 1,200 V/cm, *4* 1,200 V/cm + 5.5 mM D-glucose, *filled square* electric pulses 1 Hz, *filled circle* electric pulses 5 kHz

molecule as a fluorescent marker for monitoring glucose uptake into malignant tumor cells (O'Neil et al. 2005; Cheng et al. 2006). Because of the aggressive growth patterns of the tumor cells, uptake of 2-NBDG by tumor cells is high. However, enhanced accumulation or trapping of 2-NBDG phosphorylated forms as a result of an increase of hexokinase activity has also been reported (Flier et al. 1987; Aloj et al. 1999).

Our study demonstrates that, after application of permeabilizing electric pulses, uptake of 2-NBDG is actually increased in the fraction of cells that are electropermeabilized by the electric pulses. The fluorescence of these cells is about 10–100 times larger than that of cells not exposed to electric pulses, whatever the experimental conditions used (with external glucose added or not, for example), resulting in the reversible electropermeabilization of a fraction of the treated cells.

In the initial experiments, we determined the experimental conditions. Uptake of 2-NBDG was higher at 37 °C, with or without pulses, than at room temperature

(22 °C). Our results are in agreement with previous studies showing that in the absence of electric pulses and in the presence of 600 μM 2-NBDG, uptake into MIN6 cells was temperature-dependent, the initial velocity of 2-NBDG uptake at 37 °C being twice that at 25 °C (Yamada et al. 2000). We then determined that 240 μM 2-NBDG seemed to be a convenient concentration that results in reproducible experimental results with the cells used in the present study. It can be seen in Fig. 2 that the uptake of 2-NBDG remarkably increased by exposing cells to electric pulses at all concentrations examined except at the lowest one, 10 μM. Membrane electropermeabilization opened new ways for the penetration of 2-NBDG inside cells, aside from its general uptake by the GLUT transporters described in the Introduction. This is the reason for the increased 2-NBDG accumulation inside electroporated cells. Contrary to facilitated transport, the rate of which is driven mainly by the number and activity of the transporters located at the cell surface, uptake through the electroporated membrane is proportional to the external concentration as clearly shown with typical nonpermeant molecules (Orlowski et al. 1988; Silve and Mir 2011). The same rule has been observed with 2-NBDG, the electroporation of the cell membrane leading to the highest uptake per cell at the highest concentrations tested, 240 and 300 μM. However, at 300 μM the percentage of cells in the electropermeabilized fraction slightly decreased, reflecting a potential toxicity on the electroporated cells of 2-NBDG at the high internalized concentration in the presence of 300 μM external 2-NBDG.

As 2-NBDG is normally transported into cells by the glucose transporters, this uptake should be inhibited by a high external concentration of unlabeled D-glucose competing with 2-NBDG to interact with the transporters. Previous studies demonstrated that 2-NBDG uptake was indeed inhibited in the presence of D-glucose. There is competition between 2-NBDG and D-glucose for the facilitated transporters to enter the cell in single vascular smooth muscle cells (Lloyd et al. 1999), E. coli cells (Yoshioka et al. 1996a), yeast Candida albicans (Oh et al. 2002) and tumor cells (MCF-7, HepG2, M-1 and U87MG) (O'Neil et al. 2005; Cheng et al. 2006). Moreover, other studies have reported that a high external concentration of D-glucose partially blocks uptake of 2-NBDG in other cells (Iori et al. 2006). For example, 2-NBDG uptake is inhibited about 70 % in the presence of 22 mM D-glucose in pancreatic ß-cells and 37 and 52 % in the presence of 5.6 and 11.2 mM of D-glucose, respectively (neurons, human erythrocytes) (Yamada et al. 2000). However, the exact mechanism of 2-NBDG transport is not known as it has not been determined whether all six types of glucose transporter can facilitate 2-NBDG uptake or not.

We analyzed the effect of the presence in the medium of D-glucose on 2-NBDG uptake by DC-3F cells, with or without electric pulses. Four different concentrations of D-glucose (0, 5.5, 11 or 27.5 mM) were tested. Competitive inhibition, increasing with the increased concentration of the added glucose, was found; but at 5.5 and 11 mM it affected only some of the cells in the absence of electric pulses. This result is consistent with previous studies that reported partial blocking of 2-NBDG uptake at high concentrations of D-glucose (Yamada et al. 2000; Iori et al. 2006). Nevertheless, our results are extremely relevant as the final aim of the study was to analyze whether, by studying NDBG uptake in vitro, we could determine whether FDG could be used to image electropermeabilized tissues in vivo by a technique like PET. Indeed, we show here that competition between 2-NBDG and glucose is minimal under physiological conditions (that is, with the 5.5 mM concentration) in the absence of electric pulses and that in the presence of the electric pulses there is, as expected, no detectable competition (which is in agreement with the mechanism of diffusion through the electropermeabilized membrane, which is not saturable like transport through transporters). Moreover, at the different concentration of D-glucose, there was increasing 2-NBDG uptake into electroporated cells.

The time course of 2-NBDG uptake into DC-3F cells, in the presence or absence of glucose and with or without pulse application, was also analyzed. Indeed, in PET analysis images are taken some minutes after the injection of FDG into the body. Therefore, it was necessary to ascertain that the differences observed after 10 min of incubation (most of the experiments reported) were also observed for longer incubation times. In the absence of the electric pulses, our results are in agreement with a study that measured 10 μM 2-NBDG uptake at 37 °C for 0, 15, 30, 60, 120 or 180 min in HepG2 human hepatocarcinoma cells and L6 rat skeletal muscle cells (Zou et al. 2005). According to these results, the highest uptake increases were observed from 0 to 60 min after the beginning of incubation. Interestingly, the results of this experiment confirm that in electropermeabilized cells there is no competition between 2-NBDG and glucose at the physiological concentration (5.5 mM). Moreover, the fluorescence taken up by electropermeabilized cells a short time after the pulse delivery (before the cells reseal) is not lost during the prolonged incubation of cells, and the difference from nonpermeabilized cells remains high for all of the times. As some more uptake can also occur in these cells after their resealing by the GLUT transporters, as in the cells not exposed to electric pulses, it is understandable that the difference is relatively maintained with respect to nonpermeabilized cells, at least during the 60 min of

incubation during which changes were followed in the experiments reported here.

In this study, the effect of the 1-Hz and 5-kHz pulse frequencies on 2-NBDG uptake into electropermeabilized cells was also examined. Indeed, the clinical protocols for electrochemotherapy (Marty et al. 2006; Mir 2006) often use trains of eight pulses delivered at a repetition frequency of 5 kHz. This repetition frequency has several advantages. When pulses are delivered close to muscles, a single muscle contraction is provoked at 5 kHz instead of eight muscle contractions when pulses are delivered at 1 Hz. Moreover, treatment is much faster: the train of pulses between a pair of electrodes is delivered in 1.5 ms instead of about 8 s. The results reported here show that, in the absence of externally added D-glucose, the application of electric pulses highly increased 2-NBDG uptake with noticeable differences as a function of the repetition frequency of the electric pulses. Indeed, if the percentage of electropermeabilized cells is the same at 1-Hz and 5-kHz pulse frequencies, the fluorescence intensity of the permeabilized cells was significantly lower at 5 kHz than at 1 Hz. A study by Pucihar et al. (2002) showed that Lucifer yellow uptake into the same type of cells (DC-3F) followed the same trend. Indeed, to maintain the uptake of Lucifer yellow at the same level, the increase of the repetition frequency (from 1 Hz to 8.3 kHz) imposed an increase in the amplitude of the electric pulses applied, which means that the pulses at the highest repetition frequency were less efficient at permeabilizing the cells. If we consider that in our study the effect of the 1-Hz and 5-kHz pulse frequencies was tested at the same amplitude in the absence or presence of physiological D-glucose, our results are indeed in agreement with these previous results. It is interesting that these two markers give the same result as the Lucifer yellow is a completely nonpermeant molecule, while 2-NBDG would also be a nonpermeant molecule if no GLUT transporters actively transported it inside the cells. The reduced effectiveness of the 5-kHz pulses is thus corroborated by molecules with different behaviors, considering their ability to cross the plasma membrane. Simultaneously, these similarities also confirm that the mechanism of drug uptake in electropermeabilized cells is diffusion across the permeabilized membranes.

The results of our in vitro studies highlight the ability of glucose derivatives as markers for monitoring the electropermeabilization of cells exposed to adequate electric pulses. Until now, only strictly nonpermeant molecules like Lucifer Yellow or bleomycin were used as cell electropermeabilization markers (Mir et al. 1998; Pron et al. 1999; Pucihar et al. 2002; Silve and Mir 2011). As FDG is a radioactive glucose derivative used for clinical investigations, the results reported here with 2-NBDG provide a preliminary step toward in vivo studies of FDG PET imaging in combination with electroporation. Considering the common applications of FDG, PET could be a useful means to probe electroporated cells in vivo, which provides a noninvasive way to monitor tissue electropermeabilization in living laboratory animals. FDG PET could then become a very attractive technique for the accurate detection of electropermeabilization efficacy in vivo. Further in vivo studies are thus necessary to assess the potential of this technique.

Conclusions

We investigated the potential of 2-NBDG, a fluorescent analogue of glucose, to reveal the electropermeabilization of cells after delivery of electric pulses in vitro. Based on the results, it can be suggested that FDG PET imaging could have potential to measure the electropermeabilization of cells in vivo.

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