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The Systematic Study of the Electroporation and Electrofusion of B16-F1 and CHO Cells in Isotonic and Hypotonic Buffer

Marko Usaj · Masa Kanduser

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Abstract The fusogenic state of the cell membrane can be induced by external electric field. When two fusogenic membranes are in close contact, cell fusion takes place. An appropriate hypotonic treatment of cells before the application of electric pulses significantly improves electrofusion efficiency. How hypotonic treatment improves electrofusion is still not known in detail. Our results indicate that at given induced transmembrane potential electroporation was not affected by buffer osmolarity. In contrast to electroporation, cells' response to hypotonic treatment significantly affects their electrofusion. High fusion yield was observed when B16-F1 cells were used; this cell line in hypotonic buffer resulted in 41 ± 9 % yield, while in isotonic buffer 32 ± 11 % yield was observed. Based on our knowledge, these fusion yields determined in situ by dual-color fluorescence microscopy are among the highest in electrofusion research field. The use of hypotonic buffer was more crucial for electrofusion of CHO cells; the fusion yield increased from below 1 % in isotonic buffer to 10 ± 4 % in hypotonic buffer. Since the same degree of cell permeabilization was achieved in both buffers, these results indicate that hypotonic treatment significantly improves fusion yield. The effect could be attributed to improved physical contact of cell membranes or to enhanced fusogenic state of the cell membrane itself.

M. Usaj · M. Kanduser (✉)
Faculty of Electrical Engineering, University of Ljubljana, Trzaska cesta 25, 1000 Ljubljana, Slovenia
e-mail: masa.kanduser@fe.uni-lj.si

M. Usaj
e-mail: marko.usaj@fe.uni-lj.si

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Introduction

According to current opinions in cell biology, cell fusion is the beginning and end, the alpha and omega, of all living beings. A human life starts with the fusion of two cells. However, recently published data also indicate that uncontrolled fusion of healthy cells results in cancer (Duelli and Lazebnik 2003, 2007). Furthermore, cell fusion is suggested to be one of the major mechanisms of the metastasis formation (Larsson et al. 2008; Lu and Kang 2009). Cell fusion is of interest not only as a fundamental biological process but also as a useful experimental tool in biotechnology, medicine and biology. For therapeutic purposes, we can use cell fusion to investigate and treat different diseases like diabetes (McClenaghan 2007), to regenerate axons of the central nervous system (Sretavan et al. 2005) and to produce cells with desired properties, such as reprogrammed progenitors for stem cell therapy (Yamanaka and Blau 2010). Even more, cell fusion also holds great promise in transplantation medicine (Sullivan and Eggan 2006). The most-know applications of cell fusion are the production of monoclonal antibodies in hybridoma technology (vor dem Esche et al. 2011; Trontelj et al. 2008) and the production of cell vaccines for cancer immunotherapy (Koido et al. 2010).

However, the success of the methods based on cell fusion depends on the number of fused and functional cells, which is not a trivial task to achieve. For this reason, an universal tool which will reliably produce a high fusion yield has been sought for almost 30 years. Among the physical, viral,

chemical and even genetic methods (Gottesman et al. 2010), cell fusion using electric pulses, known as "electrofusion," is the most promising one. However, its status as a routine tool for cell fusion has yet to be established. The method is relatively simple, is potentially highly efficient and enables a fusion of a large number of cells at the same time. In studies where the chemical method of cell fusion (PEG) was compared to electrofusion, the authors reported that electrofusion was more efficient (Hui and Stenger 1993; Karsten et al. 1988; Yu et al. 2008). Electrofusion also holds the great promise in the clinical environment since it does not include any viral or chemical additives.

The reason electrofusion is not already a universal tool for cell fusion is that all parameters and mechanisms are not yet completely known and optimized. It was shown that, even with modern microfluidic devices developed recently, fusion yields can still be very low (up to 5 %) (Ju et al. 2009). From the literature we can see that there are many factors which affect electrofusion, but no systematic study has been performed to clarify the influential parameters and to suggest directions for further studies.

By definition, electrofusion is a two-condition process: (1) a cell membrane has to be brought into a fusogenic state and (2) close physical contact between two fusogenic membranes has to be established (Teissie and Rols 1986). The fusogenic state of the cell membrane is achieved by electric pulse application, resulting in electroporation that causes a dramatic increase in membrane permeability after the cell is exposed to short and intense electric pulses. The energy for membrane permeability based on rearrangement of lipid molecules in the cell membrane is obtained by induced transmembrane voltage (ITV) (Neumann et al. 1989). In general, it is accepted that at higher ITV higher electroporation efficiency is achieved. The change in cell membrane permeability is not the only consequence of electric pulse application; such a membrane is also brought into a fusogenic state (Teissie and Ramos 1998). Therefore, for effective electrofusion, adequate electric field parameters have to be selected (Trontelj et al. 2008).

Close physical contact between cells is the second condition required for effective cell fusion; it is important to note that the contact has to be established while cell membranes are in the fusogenic state. Electrofusion is a considerably more complex process than electroporation due to the fact that cell contact is crucial and that the physiology of the cell is involved in the postpulse process leading to effective cell fusion. No theoretical descriptions yet exist which would predict a fusion yield. Even more, a fusion yield varies tremendously between different cell lines (Salomskaite-Davalgiene et al. 2009; Usaj et al. 2010). Thus, the mechanisms involved in efficient cell fusion and optimization of parameters involved in the process still require further studies. While part of the difference in electrofusion behavior can be attributed to cell size, an important part is governed by biological characteristics and the response of cells to the treatment (Glaser and Donath 1987; Neil and Zimmermann 1993). One of the earliest approaches proposed to improve electrofusion efficiency was the use of hypotonic buffers (Klock et al. 1992; Schmitt and Zimmermann 1989; Vienken and Zimmermann 1985). How hypotonic treatment improves electrofusion is still not known in detail. In the literature to date (Ahkong and Lucy 1986; Perkins et al. 1991; Reuss et al. 2004; Stenger et al. 1988; Sukhorukov et al. 1993, 2005, 2006; Zimmermann et al. 1990; Zimmermann and Neil 1996) we find only a few hypotheses, which can be divided into two groups: (1) a hypotonic treatment enhances the electroporation itself and (2) a hypotonic treatment improves cell contact and, by that, fusion yield. It is also possible that an improved fusion yield is the consequence of both phenomena. This questions cannot be answered only by analyzing the data already published since there is no systematic study where both phenomena, i.e., electroporation and electrofusion, were investigated in parallel using isotonic and hypotonic buffers with the same cell line, buffer composition, electric field parameters, method for establishing cell contact and temperature.

The aim of our study was to investigate electroporation and electrofusion in isotonic and hypotonic buffers for two cell lines using the same experimental conditions to enable us to separate the effects of cell membrane permeabilization and cell fusion. For electroporation we tested different electric pulse amplitudes. We used electric field amplitudes that resulted in comparable transmembrane potentials in isotonic and hypotonic buffers in order to exclude the influence of cell size (or ITV) on electroporation and electrofusion. Thus, from the data obtained we could separately evaluate the effect of hypotonic treatment on electroporation and electrofusion beyond the effect of the cell size. In our previously study (Usaj et al. 2010) we described a modified adherence method to efficiently perform cell fusion in hypotonic buffer. In this study we focused on the comparison of the electroporation and electrofusion in isotonic and hypotonic buffers, an aspect that was only briefly addressed in our previous work. Besides we described a simple but very effective modification of our electrofusion method, which gives a three times higher electrofusion yield compared to our previously published study.

Materials and Method

Chemicals, Cell Culture Media

Dulbecco's minimal essential medium (DMEM), Ham's Nutrient Mixtures (F-12 HAM), fetal bovine serum (FBS), L-glutamine, sucrose, dipotassium hydrogen phosphate

(K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), magnesium chloride ($MgCl_2$), trypsin and EDTA were obtained from Sigma-Aldrich (Taufkirchen, Germany). Antibiotics (crystacillin and gentamicin) were obtained from Lek (Ljubljana, Slovenia). Propidium iodide, CMFDA and CMRA cell trackers were obtained from Molecular Probes/Invitrogen (Carlsbad, CA).

Cell Culture and Electroporation Buffer

Cell lines were cultured in humidified atmosphere at 37 °C and 5 % CO_2 in the following culture media: mouse melanoma (B16-F1) in DMEM supplemented with 10 % FBS, antibiotics (gentamicin, crystacillin) and L-glutamine; Chinese hamster ovary cells (CHO) in F-12 HAM supplemented with 10 % FBS, antibiotics and L-glutamine. Cells were grown in a 25 cm^2 culture flask (TPP, Trasadingen, Switzerland) to 70–80 % confluence. Iso- and hypotonic potassium phosphate buffer (KPB; 10 mM KH_2PO_4/K_2HPO_4, 1 mM $MgCl_2$) with 250 or 75 mM sucrose corresponding to osmolarities of 260 and 93 mOsm [mOsmol/kg, determined by Knauer vapor pressure osmometer (K-7000; Knauer, Wissenschaftliche Geratebau, Germany)] were used in the experiments. The conductivity of both buffers was 1.62 mS/cm and pH 7.2.

Electroporation

Cell suspensions were prepared on the day of the experiment by 0.25 % trypsin/EDTA solution. Trypsin solution was then removed and replaced by 5 ml of culture medium, and a homogenous cell suspension was prepared. For electroporation we used an electric pulse generator (Cliniporator; IGEA, Carpi, Italy) and 4 mm gap cuvettes (Eppendorf, Hauppauge, NY). The electric field was calculated as $E = U/d$, where U is applied voltage and d is distance between electrodes (4 mm). Aliquots of 1.5×10^5 cells for B16-F1 (or 3×10^5 cells for CHO) were prepared, centrifuged ($290\times g$, 5 min, 4 °C) and kept at 4 °C. Supernatant was carefully removed, and cells were resuspended in 270 µl of hypotonic buffer. Electroporation was performed 2 min after hypotonic buffer was added as it was shown previously that cells are close to their maximal size induced by hypotonic cell swelling (Usaj et al. 2009). Propidium iodide (30 µl, 1.5 mM) was added to the cell suspension 15 s before pulse application, and the cell suspension was then transferred to an electroporation cuvette. The same procedure was used for isotonic buffer. Electroporation was performed by application of eight rectangular pulses with pulse duration 100 µs, repetition frequency of 1 Hz and different pulse amplitudes from 0 V (0 V/cm, negative control) to 640 V (1,600 V/cm, positive control) for hypotonic buffer or 800 V (2,000 V/cm, positive control) for isotonic buffer, in 80 V (200 V/cm) steps. Electroporation efficiency was determined spectrofluorometrically by means of propidium iodide uptake in a microplate reader (Infinite M200; Tecan, Mannedorf, Switzerland) at 535 nm excitation and 617 nm emission wavelength, 3 min after pulse application. The percentage of propidium iodide uptake was then calculated. The value obtained from the negative control was subtracted from the value of the treated sample and then divided by that of the positive control. Mean values (±SD) for given pulse amplitudes were calculated from at least four independent experiments. The differences between electroporation in isotonic and hypotonic buffers at the same applied voltage (electric field amplitude) for each cell line were statistically tested using the independent samples t test (SPSS Statistic; SPSS, Inc., Chicago, IL).

Calculation of Maximal Induced Transmembrane Voltage

For ITV calculation we used an equation for induced transmembrane voltage of spherical cells

$$ITV = -1.5rE\cos\varphi \quad (1)$$

where r is the radius of the cell, E is the strength of the external electric field and φ is the angle between the direction of the external applied electric field and the normal from the center of the cell to the point of interest on the cell surface (Kotnik et al. 1997; Neumann et al. 1989; Pucihar et al. 2009). The hypotonic treatment used in our experiments induces swelling of the cells and, therefore, affects the maximum induced transmembrane voltage (ITV_{max}) and putatively the efficiency of electroporation/electrofusion. The cell radii for B16-F1 and CHO before and 2 min after the start of hypotonic treatment were determined in our previous studies (Usaj et al. 2009, 2010). The cell radii of B16-F1 in isotonic and hypotonic buffers were 8.1 ± 1.1 and 9.3 ± 1.8 µm, while those for CHO were 6.1 ± 0.6 and 7.7 ± 0.4 µm, respectively. An independent samples t test showed us that B16-F1 cells were significantly larger than CHO cells in isotonic buffer ($P < 0.05$). However, this difference was not statistically significant after the cells were maintained in hypotonic buffer for 2 min. A paired samples t test made on the sizes of the same cells in isotonic buffer and after 2 min in hypotonic buffer revealed that the increase in cell size due to hypotonic swelling was statistically significant for both cell lines ($P < 0.05$).

Electrofusion

Fluorescence microscopy was used for the detection and quantification of fused cells as described previously (Trontelj et al. 2010; Usaj et al. 2010). Cells in one flask

were stained with green CMFDA, while cells in the other flask were stained with red CMRA at 7 μM loading solution. The cells were then trypsinized and mixed together at a ratio of 1:1.

Close cell–cell contacts were established by a modified adherence method (Trontelj et al. 2010). Here has been made a simple but efficient improvement of the method since our first publication (Usaj et al. 2010). Instead of plating the whole microplate well with 1 ml of cells in suspension (Usaj et al. 2010), we placed only a 40 μl drop of cells in suspension in the middle of the well. Doing so, the cell contact is much more controllable since cells stay in the area between electrodes and do not distribute to the edge of the microplate well, where they are not exposed to electric pulse treatment. Thus, a 40 μl drop of cell suspension containing 2×10^6 cells/ml for B16-F1 and 4×10^6 cells/ml for CHO was placed in each well of a 24-multiwell plate (TPP). Cells were incubated in 5 % CO_2 at 37 °C for 20 min to slightly attach to the surface of the well. Before electroporation, cells were washed with isotonic buffer and 350 μl of hypotonic or isotonic buffer was added. Two minutes later, electric pulses (8 × 100 μs at 1 Hz) were delivered using two parallel wire electrodes (Pl/Ir = 90/10) with a 5 mm gap. Electric field amplitudes were selected in order to induce the same ITVs in isotonic and hypotonic buffers (see Eq. 1). After delivery of pulses, cells were left undisturbed for 10 min for cell fusion to take place. The fusion yield was determined by dual-color fluorescence microscopy (Jaroszeski et al. 1998; Trontelj et al. 2010). We used two emission filters, the first at 535 nm (HQ535/30 m, for CMFDA) and the second at 510 nm (D510/40 m, for CMRA) (both from Chroma, Brattleboro, VT), and a monochromator (Polychrome IV; Visitron, Puchheim, Germany). Cells were observed under an inverted fluorescence microscope (Axiovert 200; Zeiss, Oberkochen, Germany) with ×20 objective magnification. Three images (phase contrast, red and green fluorescence) were acquired from five randomly chosen fields in each well using a cooled CCD video camera (VisiCam 1280, Visitron) and PC software MetaMorph 7.1 (Molecular Devices, Palo Alto, CA).

For each parameter an image triplet composed of phase contrast, red fluorescent and green fluorescent images was created. The image-processing software ImageJ (NIH Image, Bethesda, MD) was used to create three channel images (Fig. 2) from each image triplet (phase contrast, red and green fluorescence). Cells were manually counted, and the fusion yield was calculated as a percentage of double-labeled fused (or polynucleated) cells: $(N_{double}/N_{total}) \times 100$. The fusion yield is presented as an average value (±SD) for a given cell line and the ITV_{max} obtained from at least three independent experiments. Differences between electrofusion in isotonic and hypotonic buffers at the same ITV for each cell line were statistically tested using the independent samples *t* test.

Results

Electroporation

We investigated the electroporation of B16-F1 and CHO cells in isotonic and hypotonic buffers at different electric field amplitudes. The results are shown in Fig. 1a, b. At first sight the hypotonic buffer enhanced cell electroporation. Propidium iodide uptake was higher in hypotonic buffer than in isotonic buffer at the same electric field amplitudes for both cell lines. Consequently, saturation was achieved at lower electric field amplitudes in hypotonic than in isotonic buffer. The apparent threshold for electroporation was lower in hypotonic buffer, 200–400 V/cm, than in isotonic buffer, 400–600 V/cm; however, the resolution of data points around the electroporation threshold was too low to more accurately confirm this visual observation and to more accurately determine the exact value of the electroporation threshold.

To gain insight into the effect of the hypotonic buffer, we calculated ITV_{max}. By doing so we excluded the effect of cell size on cell electroporation caused by hypotonic cell swelling. The radii of B16-F1 cells in isotonic and hypotonic buffers were 8.1 ± 1.1 and 9.3 ± 1.8 μm, while those for CHO cells were 6.1 ± 0.6 and 7.7 ± 0.4 μm, respectively. Based on these cell sizes, ITV_{max} values were calculated for both cell lines. The percentages of propidium iodide uptake by cells were then plotted against ITV_{max} values and are presented in Fig. 1c, d. No apparent differences in electroporation efficiency at any of the ITV_{max} values were found. More than 50 % of cells were permeabilized at ITV_{max} values of 0.8–0.9 V, while at 1.25–1.5 V all cells were permeabilized. The differences in electroporation efficiencies between the two cell lines and isotonic and hypotonic buffers are within the standard deviation of the experiments.

Electrofusion

In the second part of the study we investigated the electrofusion of B16-F1 and CHO cells in isotonic and hypotonic buffers at different electric field amplitudes. Based on the difference in cell size, we chose such electric field amplitudes that the ITV values were the same in isotonic and hypotonic buffers. In Fig. 2 micrographs of control and electrofused B16-F1 and CHO cells in hypotonic and isotonic buffer are presented. In Figs. 3 and 4 the percentage of fusion yields in isotonic and hypotonic buffers for B16-F1 and CHO cells are presented. The highest fusion

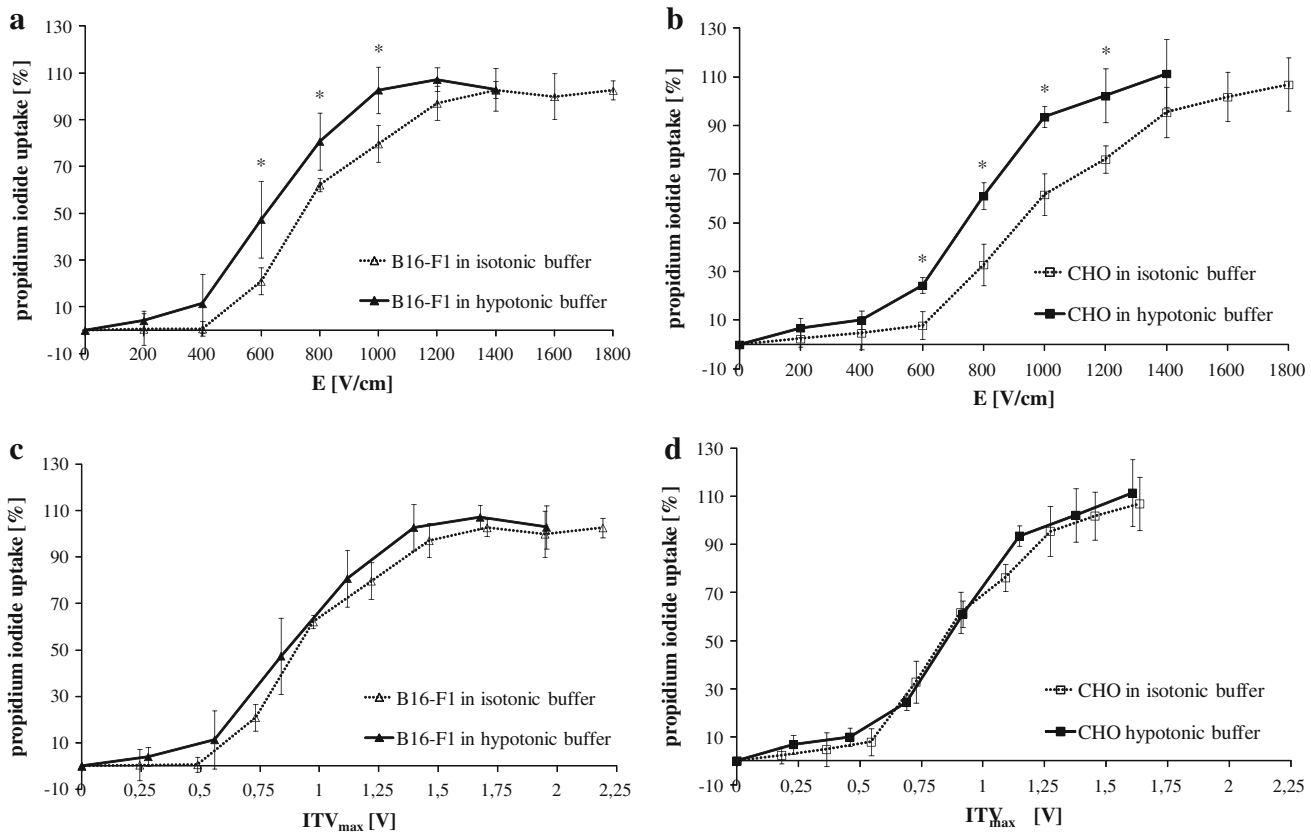

Fig. 1 Electroporation efficiency determined by propidium iodide uptake. Cells were electroporated 2 min after hypotonic or isotonic treatment with a train of pulses (8 × 100 µs, 1 Hz) at different electric field amplitudes. The percentage of propidium iodide uptake versus electric field amplitudes is presented for **a** B16-F1 and **b** CHO cells. Values of ITV$_{max}$ were then calculated based on cell radii and applied electric field amplitudes using Eq. 1 for **c** B16-F1 and **d** CHO cells. *Asterisks* represent statistically significant differences ($P < 0.05$). Each data point represents the average ± SD of at least four independent experiments

yield was obtained in hypotonic buffer with B16-F1 cells (up to 41 ± 9 %, Fig. 3). Nevertheless, a reasonably good fusion yield (32 ± 11 %) of B16-F1 cells was also found in isotonic buffer (Fig. 3). In contrast to B16-F1, when fusion was performed with CHO cells, <1 % of the fused cells were observed in isotonic buffer (Fig. 4). The hypotonic treatment increased the fusion of CHO cells up to 10 % (Fig. 4), suggesting that the hypotonic treatment plays a critical role in electrofusion of CHO cells.

Discussion

In this article a systematic comparison of cell electroporation and electrofusion in isotonic and hypotonic buffers using two cell lines (CHO and B16-F1) is presented. The main question was how hypotonic treatment affects cell fusion. Does it affect cell membrane permeabilization or the contact between cells? In our recent study (Usaj et al. 2010) we found that electrofusion efficiency in hypotonic buffer was considerably affected by the cell line used, suggesting that the biological characteristics of cells have a significant impact on cell electrofusion. Here, we extended our previous study (Usaj et al. 2010) and compared the effect of isotonic and hypotonic treatments on electroporation and electrofusion efficiency using two cell lines with different fusogenic abilities. In both isotonic and hypotonic buffers an increase in electric field amplitude led to an increase in electroporation efficiency. However, lower electric field amplitudes were required for electroporation in hypotonic buffer, while the shape of the curve was not affected (Fig. 1a, b). This is in accordance with the published literature, where a similar effect of hypotonic treatment has been reported (Barrau et al. 2004; Rols and Teissie 1990; Wang and Lu 2006). In order to exclude the effect of hypotonic treatment on cell size, which affects the ITV and therefore cell electroporation (Kinosita and Tsong 1979; Weaver and Chizmadzhev 1996), we calculated ITV$_{max}$ values (Fig. 1c, d). The results show that electroporation is not affected by buffer osmolarity and biological characteristics of the cells. This is in accordance with the study of Golzio et al. (1998), who did not find any significant effect of the hypotonic buffer on cell electroporation. We have to mention here that our experimental

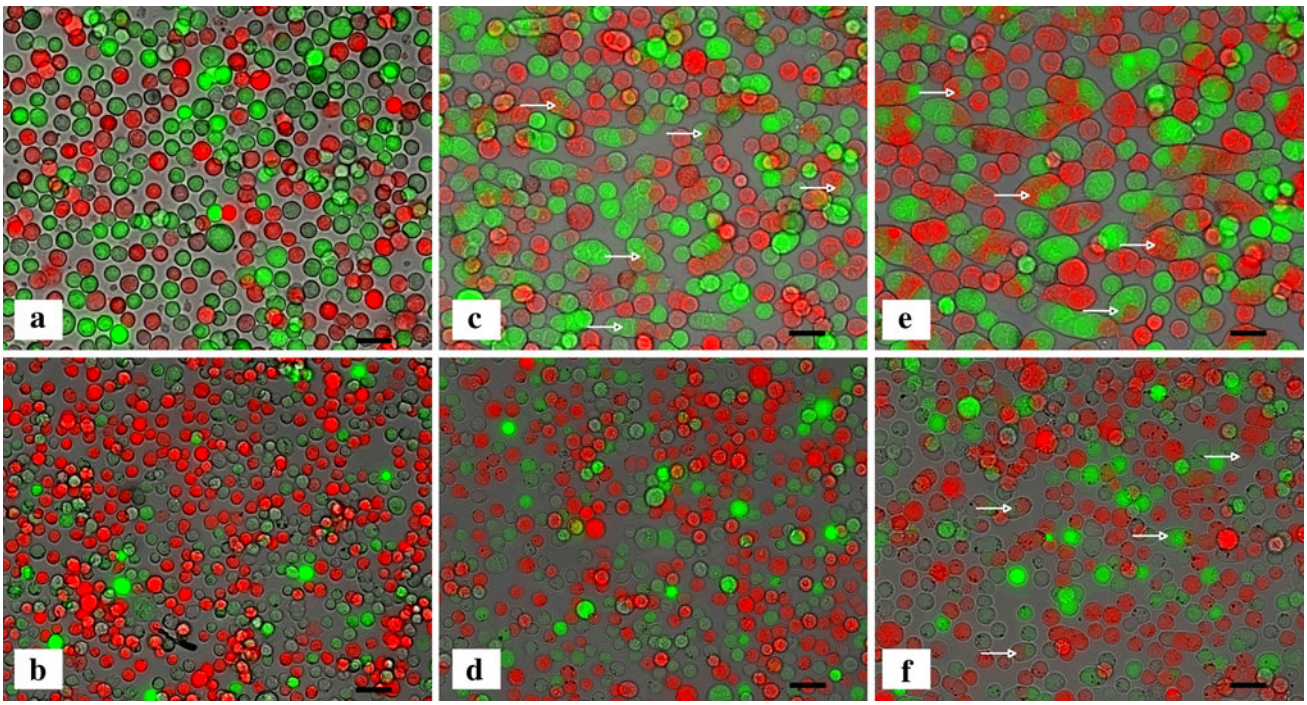

Fig. 2 Three-channel microscopic images of cell electrofusion: B16-F1 control cells (**a**) and fused cells at $ITV_{max} = 1.68$ V in isotonic (**c**) and hypotonic (**e**) buffer as well as CHO control (**b**) and fused at $ITV_{max} = 1.84$ V in isotonic (**d**) and hypotonic (**f**) buffer. Images of cell electrofusion were captured 10 min after electric pulse treatment under ×20 objective magnification. In order to keep images clearer, only a few fused cells are marked with *arrows*. <1 % of fused CHO cells were obtained in isotonic buffer. *Bars* = 30 μm

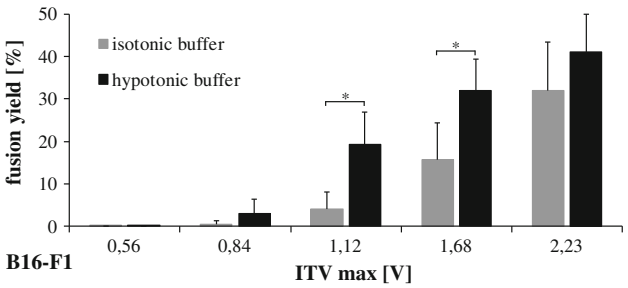

Fig. 3 Electrofusion efficiency in isotonic and hypotonic buffer of B16-F1 cells for different ITVs. The increase in ITV improves the fusion yield in hypotonic and isotonic buffer; however, higher fusion yields are observed in hypotonic buffer. *Asterisks* represent statistically significant differences ($P < 0.01$). Columns represent the average ± SD of at least three independent experiments

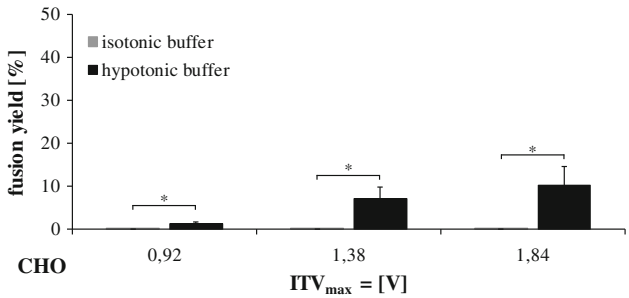

Fig. 4 Electrofusion efficiency in isotonic and hypotonic buffer of CHO cells for different ITVs. The increase in ITV improves the fusion yield only in hypotonic buffer, whereas in isotonic buffer <1 % of fused cells were obtained. *Asterisks* represent statistically significant differences ($P < 0.01$). Columns represent the average ± SD of four independent experiments

protocol was slightly different. In the study of Golzio et al. (1998) the electroporation was performed on cells after the regulatory volume decrease (RVD) induced in hypotonic buffer took place and the cells were back to their initial size. In our study electroporation was performed when cells reached their maximal size before RVD was activated. Therefore, the only effect observed in hypotonic buffer was cell electroporation at lower electric field amplitudes caused by cell swelling itself (Fig. 1a, b). Another effect of the hypotonic buffer on cell electroporation was proposed by Barrau et al. (2004), who suggested that cell swelling causes an increase in cell surface area, which requires the unfolding of undulations and envaginations of the cell membrane and increases membrane lateral tension. It was calculated that ≈100 mV lower ITV is needed to trigger cell membrane electroporation in hypotonic buffer (Barrau et al. 2004). However in our study (Fig. 1c, d) this was not observed due to low data resolution around the electroporation threshold.

In contrast to electroporation, cell physiology and the cell response to stress induced by hypotonic treatment seem to play crucial roles in electrofusion. It is interesting

to note that different degrees of electroporation are needed for electrofusion of B16-F1 and CHO cells. For example, in hypotonic buffer considerably higher electroporation efficiency (70 ± 11 %, at $ITV_{max} = 0.92$ V) is needed for CHO cells to start to fuse (fusion yield = 1.2 ± 0.4 %) than for B16-F1 cells (electroporation efficiency = 44 ± 8 %, at $ITV_{max} = 0.84$, fusion yield = 3.1 ± 3.2 %).

The hypotonic treatment improves cell fusion in both cell lines. The fusion yield of the more fusogenic cell line B16-F1 in hypotonic buffer was up to 41 ± 9 %. As far as we know this is one of the highest reported in situ electrofusion efficiencies determined by fluorescence microscopy (Gabrijel et al. 2004). We should not forget that not all of the fused cells can be detected by dual-color fluorescence microscopy. If we take into account (Scott-Taylor et al. 2000) that only one-half of fused cells can be detected by this method, then our total fusion yield exceeds 80 %. Here, we have to mention that our approach for fusion yield determination does not distinguish between binucleated cells as a result of the fusion between two cells and polynucleated cells as a result of multiple fusion events. From this point of view our fusion yields are underevaluated since polynucleated cells were often obtained. Such high fusion yields were obtained with a simple but efficient modification, described in "Materials and Methods", of our adherence method published previously (Usaj et al. 2010).

However, even with the highly efficient method for cell electrofusion, we did not obtain high electrofusion of CHO cells. The difference between the highly fusogenic B16-F1 and the poorly fusogenic CHO cells still exists. This different electrofusion ability of the two lines was described in our previous work (Usaj et al. 2010). The improved electrofusion method presented here results in a good fusion yield (32 ± 11 %) of B16-F1 cells even in isotonic buffer at the highest ITV_{max}. In contrast to B16-F1 cells, we obtained <1 % of fused CHO cells in isotonic buffer even at the highest electric field amplitude used (2,000 V/cm, $ITV_{max} = 1.84$ V). The use of hypotonic buffer improved the fusion yield of both cell lines. In our experimental conditions the hypotonic treatment seems to be crucial for CHO cells, where up to 10 ± 4 % of fused cells were observed. Compared with our previous study (Usaj et al. 2010), we improved the electrofusion of CHO cells on average by 67 %. Our results are in agreement with previous studies, where it was reported that the use of hypotonic treatment improves electrofusion efficiency (Perkins et al. 1991; Rols and Teissie 1990; Schmitt and Zimmermann 1989; Sukhorukov et al. 2006; Zimmermann et al. 1990). Several explanations were proposed, as discussed in our previous report (Usaj et al. 2010). Also, cell membrane fluidity alternation caused by a hypotonic environment should be considered as a possible explanation for the effect of the hypotonic treatment on cell electrofusion (Toplak et al. 1990).

From our results we can confirm that the better fusion yield in hypotonic buffer is not caused by the effect of the hypotonic treatment on cell electroporation since the same degree of cell permeabilization was achieved in both isotonic and hypotonic buffers for a given ITV. These results suggest that the beneficial effect of hypotonic treatment is indeed caused by membrane–membrane interactions due to improved physical cell contacts or due to enhanced fusogenic state of the cell membrane itself. Further studies and analyses are, however, needed to specify and evaluate one or both hypotheses, especially to determine the role of the cell cytoskeleton and membrane fluidity in cell electrofusion.

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