Control by electrical parameters of short- and long-term cell death resulting from electropermeabilization of Chinese hamster ovary cells

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Abstract

Chinese hamster ovary (CHO) cells were pulsed by using brief intense square-wave electric field pulses. The electrical treatment induced a transient local permeabilization of the cell membrane. The growth of CHO cells after electropulsation in an iso-osmotic pulsing buffer with low ionic content was measured. Parallel experiments evaluated cell death which took place in the minute range after electropulsation (short-term death) and the cell death upon 24 h (long-term death). Short-term cell death was defined as the case of cells with membrane still permeable to Direct-blue at 15 min after electropulsation. It was observed only under stringent pulsing conditions where electropermeabilization of the two sides of the cell was triggered. The long-term cell death, i.e., the inability of some pulsed cells to grow was observed as soon as permeabilization had been triggered. The higher the permeabilization level of the cell population was, the higher the long-term cell death level was. The cell death was linearly related to the reciprocal of the electric field intensity, i.e., to the fraction of the membrane area electrically brought to the permeable state. From this work, it appeared that for high levels of permeabilization of a cell suspension, best cell survivals were obtained if limited alterations were triggered over a large area of the plasma membrane (single pulse with high intensity) than if a small area of the membrane was strongly altered (repetitive pulses with small intensity). The highest yield of viable permeabilized cells was achieved when using one single pulse of duration up to 1 ms.

Keywords: Electropermeabilization; Cell survival; Short-term cell death; Long-term cell death; Chinese hamster ovary cell

1. Introduction

Application of brief and intense external electric field pulses to cells (electropulsation) is known to affect the membrane of the cell. This is due to an associated modulation of the membrane potential difference. A transmembrane electric potential difference ($\Delta V_i$) is created associated with the dielectric properties of the plasma membrane, under electropulsation. It is directly related to the external electric field intensity $E$. Theory predicts and experiments confirm that $\Delta V_i$, oriented from external medium to intracellular compartment, is dependent on cell parameters and on the position on the cell surface [1,2]:

$$\Delta V_i(M, E, t) = f(g(\lambda) \cdot r \cdot E \cdot \cos[\theta(M)] \cdot [1 - e^{-(t/\tau_p)}]$$

(1)

in which $M$ is the point on the cell surface we are considering, $t$ is the time lag after electropulsation is turned on, $f$ is a factor depending on the cell geometry, $g$ is a parameter controlled by the electric permeability $\lambda$ of the membrane, $r$ is the radius of the pulsed cell which is assumed to be a sphere, $\theta(M)$ is the angle between the direction of the electric field and the normal to the cell surface at $M$, and $\tau_p$ is the characteristic time constant of the membrane charge (in the microsecond time range). The electroinduced transmembrane potential difference is added to the native $\Delta V_n$. When the new transmembrane potential difference ($\Delta V_n + \Delta V_i$) reaches a critical value locally, an alteration of the membrane structure is observed which led to the occurrence of reversible membrane permeabilization (electropermeabilization). This is a local effect controlled by field strength. The threshold potential difference which triggers membrane electropermeabilization was experimentally evaluated to be about 200 mV for many cell systems [3]. Several models have been proposed in an attempt to describe the physical basis of the membrane electroperme-
2. Materials and methods

2.1. Materials

Direct-blue (Catalog no. D2535) was purchased from Sigma. Methyl violet (CI 42555) was obtained from United States Biochemical Corporation (Catalog no. 19210). Standard iso-osmotic pulsing buffer (PB) was 250 mM sucrose, 1 mM MgCl$_2$, and 10 mM potassium phosphate buffer (pH 7.4). Phosphate buffer saline (PBS) was 138 mM NaCl, 3 mM KCl, 1.5 mM KH$_2$PO$_4$, and 8 mM Na$_2$HPO$_4$ (pH 7.4). Solutions were prepared with ultrapure water obtained using a Milli-Q system and sterilized on Sterivex filters 0.2 μm (Millipore, USA). Salts were analytical grade.

2.2. Cell culture

Chinese hamster ovary (CHO) cells (WT
cell type but such cell death is observed as soon as cell electropermeabilization is triggered [32]. It was shown that the cytoskeleton was involved in mammalian cell electropermeabilization resealing [26]. Even if electropermeabilization is reversible, death of some pulsed cells is generally observed [27-30]. This death of cells after electropulsation is associated firstly with a short-term effect, i.e., a high membrane permeability present more than 15 min after pulsation (short-term death). Secondly, a long-term effect is induced by the electric field pulse as shown by the partial loss of the ability of resealed cells to grow (long-term death) [28,31]. Sensitivity to electric field depends strongly on cell type but such cell death is observed as soon as cell electropermeabilization is triggered [32]. It was shown with mammalian cells [33] and vegetal protoplasts [34] that, in many cases, short-term and long-term cell deaths are not related.

In this work, we analyzed the control of the losses of cell viability by the field intensity and by the cumulated pulse duration.

2.4. Kinetics of the recovery of electropermeabilized cell membrane integrity

Reversibility of the permeable state of the cell membrane to Direct-blue was assayed by measuring the ability of cells to be stained by delayed addition of Direct-blue after being electropermeabilized in the absence of the dye [21]. After different time lags following the electrical treatment, Direct-blue containing PB was added to obtain a final concentration of 10 mg per ml. Cell suspensions were then incubated for 5 min at room temperature before counting the percentage of blue-stained cells. Direct-blue
was then used as a reporter for cell membrane integrity. Again this method reflects population statistical effects.

2.5. Electropulsed cell viability

Cells ($2 \cdot 10^5$ cells in 100 $\mu l$ of PB) were pulsed on a petri dish and kept for 5 min at room temperature. 2 ml of culture medium were then added. Viability was measured by quantifying the growth of plated cells over more than one generation (about 24 h) by Crystal violet staining [36]. Briefly, the cell monolayer was washed with PBS to remove all non-adherent cells, and was incubated for 30 min with PB containing Methyl violet (0.1% w/v), under gentle agitation. The cell monolayer was washed five times with PBS, and the cells were lysed with 500 $\mu l$ of 10% acetic acid solution. The absorption of the cell extract at 595 nm was measured, and was linearly related to the cell number [37]. Kinetic analysis of CHO cell growths showed that the electrical treatment did not induce any delay in the onset of cell growth after electropulsation (data not shown).

2.6. Statistical analysis of the data

‘Linefit’ and ‘Expofit’ computer software (Elsevier-Biosoft, Cambridge, UK) were run on an Apple IIe computer to calculate data fittings using respectively straight lines and exponential decays. Each point was mean $\pm$ S.D. from three independent experiments.

3. Results

Electropermeabilization of CHO cell suspension induced death of some cells according to two criteria (scheme 1): Short-term death, i.e., an electrically induced membrane permeability still present 15 min after electropulsation of the cell population at 21°C. This took into account that complete resealing is fast at 21°C if the cells are not damaged [21].

Long-term death, i.e., the inability of electropulsed cells to grow over one generation time (24 h) when spread on culture dishes.

3.1. Short-term death of electropulsed CHO cells

Electric field-mediated membrane permeability is well known to depend on the electric field strength. Fig. 1A shows an electropermeabilization plot obtained with CHO cells in suspension. Electrical conditions were 10 pulses of 100 $\mu s$ duration applied with a frequency of 1 Hz and with variable intensity. Direct-blue incorporation into cells was only detected for electric field intensities higher than an apparent threshold value (about 0.6 kV per cm). The relative number of electropermeabilized cells increased when the electric intensity was increased. This reflected the heterogeneity of the cell size (variation of $r$ in Eq. (1)) and of the membrane properties (variation of $g(\lambda)$ in Eq. (1)) of the cell population. Permeabilization of the complete cell population was achieved for intensities higher than 1.2 kV per cm. CHO cell electropermeabilization is partially a reversible process but nevertheless high membrane permeability can be maintained in the minute time range after the electrical treatment. Fig. 1B shows the kinetics of the recovery of electropermeabilized cell membrane integrity obtained for three electric field intensities. The kinetics depended on the field intensity. Recovery curves obtained with intensities of 0.9 and 1 kV per cm showed that 15 min after electropulsation, all the cells regained their initial membrane impermeability to the dye. Under these two electrical conditions, no short-term cell death was observed. But when the cell suspension was electropulsed using 1.4 kV per cm intensity, the percentage of stained cells decreased down to 40%, 15 min after pulsation. A short-term death was present. The recovery kinetics for a given intensity E, could be described as in [38] by the equation:

$$P(E,t) = L(E) + \left[ (P(E,0) - L(E))e^{-Kt} \right]$$

in which $P(E,t)$ was the permeabilization level at time $t$, $L(E)$ was the percentage of short-term cell death, and $K$
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Fig. 2. Short-term cell death in relation to the level of permeabilization. CHO cell suspension was pulsed in PB, at 21°C, 10 times, 100 μs duration (1 Hz frequency). A: short-term cell death was measured 15 min after electric field application, using the Direct-blue exclusion assay (see Fig. 1). B: short-term cell death in relation to the reciprocal of the electric field intensity. The percentage of cell death is reported in relation to \((1 - E_b/E)\) with \(E_b = 1.06\) per kV cm.

the apparent rate of recovery observed at the level of the cell population.

Short-term cell death was only observed for electric field intensity higher than an apparent threshold value (about 1–1.1 kV per cm) (Fig. 2A). From Figs. 1A and 2A, we observed that using 10 pulses of 100 μs duration (1 Hz frequency), if \(P(E,0) < 90\%\) then \(L(E) = 0\) and if \(P(E,0) > 90\%\) then \(L(E)\) increased strongly with \(E\). If \(L(E)\) was plotted as a function of the reciprocal of the field intensity \((1/E)\), a linear fit was obtained when \(E\) was larger than a critical value, \(E_b (1.06 \pm 0.03\) kV per cm) (Fig. 2B).

3.2. Long-term death of electropulsed CHO cells

Long-term death of the electropermeabilized CHO cells was observed as less cells were counted 24 h after cells had been pulsed.

Effect of electric intensity

Cells were electropulsed in PB and electrical conditions were as above. Long-term cell death \((D(E)\) in Scheme 1) was detected as soon as cell permeabilization had been triggered, i.e., when the electric field intensity was higher than 0.6 kV per cm (Figs. 1A and 3A). The higher the field intensity (i.e., the initial electropermeabilization level, Fig. 1A) was, the higher the long-term cell death was.

Fig. 3A shows results obtained with 6 different cell populations spaced out over three weeks culture in suspen-

Fig. 3. Dependence of the long-term cell death on the electric field intensity and on the cell ageing (A) and relation of the long-term cell death to the induced cell permeabilization level (B). Electrical treatment was as in Fig. 1. A: CHO cell death level 24 h after electropermeabilization. Data were obtained from six different cell cultures spaced out over three weeks, CHO cell cultures were 2 days (△); 4 days (▲); 9 days (○); 11 days (●); 16 days (◆) and 18 days (■) old respectively. The curves shift onto the right upon ageing. B: death/permeabilization plot. The grey area represents the statistical variation of long-term cell death in relation to the cell ageing over three weeks.
Fig. 4. Effects of number of pulses (A) and of duration of the pulse (B) on suspended CHO cell permeabilization and death. CHO cells were pulsed in PB. A: electrical conditions were 100 μs duration and 1.2 kV per cm. When multiple, the pulses were applied with a frequency of 1 Hz. ●, permeabilization curve; ○ long-term cell death. B: electrical conditions were a single pulse of 1.2 kV per cm with variable duration. ●, permeabilization curve; ○ long-term cell death.

Effect of number of pulses

CHO cell populations were electropulsed in PB with constant pulse duration (100 μs) and field intensity (1.2 kV per cm) but with variable number of pulses. Long-term cell death was observed to increase when the number of pulses increased (Fig. 4A). Electropermeabilization of cells increased with the number of pulses and electropermeabilization of the quasi-complete cell population was achieved when five pulses were applied, the long-term cell death being about 60%.

Effect of duration of the pulse

At given electric field intensity (1.2 kV per cm) and for one single pulse, increasing the pulse duration resulted in an increase of electropermeabilization up to 100%, reached with one pulse of 6 ms duration (Fig. 4B). Concomitantly, long-term cell death increased.

Effects of electric field parameters are summarized in Table 1. Smaller long-term cell death levels were obtained when one single pulse of 500 μs (30%) and 1 ms (50%) were applied rather than respectively 5 pulses of 100 μs (60%) and 10 pulses of 100 μs (70%). The pulsing conditions gave the same permeabilization levels (about 90%).

Control by extent of permeabilization

The dependency of the long-term cell death on the electric field intensity (when 10 100-μs pulses were applied, see Fig. 3A) was plotted as a function of \((1 - E_c/E)\) (Fig. 5), \(E_c\) (0.58 kV per cm) being the critical threshold.
intensity value from which electropermeabilization of CHO cells in suspension in PB was detected [39,40]. A linear relationship between long-term cell death and the initial electropermeabilized membrane area is then present. Assuming that long-term death was an exclusive consequence of the electric field mediated transient membrane permeability, the level of electropermeabilized CHO cells remaining alive 24 h after the electrical treatment (relative survival, $S_r(E)$) can be described by the equation (Scheme 1) [38]:

$$S_r(E) = P(E,0) - D(E)$$  \hspace{1cm} (3)

in which $D(E)$ was the percentage of long-term cell death and $P(E,0)$ the initial permeabilization level. When 10 pulses of 100 $\mu$s duration and with variable strength were applied, for $P(E,0) < 40\%$, $S_r(E) = 0$ (Fig. 6). For $P(E,0) > 40\%$, $S_r(E)$ increased up to a maximum of 20$\%$ where 80$\%$ of the cell population had previously been electropermeabilized. The permeabilization level of the cell population being constant (about 80$\%$), this maximum value was increased up to 60$\%$ when one single short pulse with a higher intensity was applied (Fig. 6).

4. Discussion

Electropermeabilization of CHO cells suspended in PB, while being a transient event, induced cell death which depended on electrical conditions. A short-term effect, i.e., in the minute time range after electropulsation, was associated with membrane permeability to Direct-blue. A long-term effect was observed through the incapacity of some electropermeabilized cells to grow beyond a generation time (about 18 ± 1 h for plated CHO cells).

The short-term cell death was observed only when the initial permeabilization level of the population was higher than 90$\%$ (Fig. 2), whereas long-term cell death was detected as soon as cells were electropermeabilized (Fig. 3A). Results from Figs. 2B and 6 demonstrated that there was no direct correlation between the short-term and the long-term electroinduced cell deaths, as previously reported for plant protoplasts [34] and mouse carcinoma cells [33]. The recovery of native specific membrane impermeability to Direct-blue, just after electropermeabilization, is therefore not a reliable index for long-term cell survival.

Short-term cell death was triggered for electric field intensities higher than a threshold value $E_b$ (1.06 kV per cm) which was 1.8 times higher than $E_c$ (Fig. 2). Such observation can be explained by the fact that cell electropermeabilization is a position-dependent phenomenon, i.e., it would occur for lower field intensities on one side of the cell than on the other [3,41,42]. Assuming that $\Delta V$, the resting transmembrane potential difference, was 70 ± 10 mV for CHO cells in suspension, and that $\Delta U_c$, the critical potential difference inducing membrane electropermeabilization, was 200 ± 20 mV, the critical intensity $E_c$ which triggered electropermeabilization of the two cell sides was estimated as described in Ref. [3] from the equation:

$$(f \cdot g(\lambda,r,E_f)) - \Delta V = \Delta V + (f \cdot g(\lambda,r,E_c)) = \Delta U_c$$

We obtained $E_f = 1 ± 0.1$ kV per cm. This result shows that $E_f$ and $E_c$ are very similar. This strongly suggests that short-term death is related to the electropermeabilization on the two sides of the cell. The linear dependency of the short-term death on $(1 - E_b/E)$ (Fig. 2B) is then indicative that it is the appearance and then the extent of the permeabilization on the cell surface facing the anode which control this negative consequence of electropulsation.

The long-term cell death/permeabilization profile (Fig. 3B) indicated the contribution of a cellular parameter in the modulation of long-term cell death. A shift was observed upon ageing of the cell culture (Fig. 3A). Such physiological dependencies had already been observed with leucocytes [43] and tobacco protoplasts [44]. Furthermore, cell physiology appeared as a limiting factor for electrotransformation of mammalian cells [45]. However, it was possible to minimize long-term cell death while keeping the permeabilization level high and constant if the electrical conditions were optimized (Table 1). For practical use, it was more efficient, for a given electric field strength, to use one single long duration pulse than a set of repetitive pulses with the same cumulated duration.

Electropermeabilization induced a local flow of molecules $S$ across the cell membrane at a time $t$ after electropulsation ($\phi_S(t)$) as described by the equation [16]:

$$\phi_S(t) = G(S).X(N,T).(1 - E_f/E).e^{-k_t}$$  \hspace{1cm} (4)

in which $N$ is the number and $T$ the duration of the pulses, $G(S)$ is a function which depends on the reporter $S$.
concentration gradient and on the cell membrane properties, $X(N,T)$ a function of $N$ and $T$ which controls the permeabilization extent on the potentially electropermeable membrane surface defined by $E$. For given electrical conditions and cell ageing, the flow of molecules associated with electropermeabilization is directly correlated to the term $(1 - E_c/E)$. A linear correlation is present between long-term cell death and this geometrical parameter representing electroinduced membrane restructuration (Fig. 5).

Dependency of long-term cell death on the number $N$ (Fig. 4A) and on the duration $T$ (Fig. 4B) of the pulses is analogous with that previously reported for the electroinduced flow [11]. Long-term cell death is directly associated with the molecule flow induced through the electropermeabilized membrane area. Using electrical conditions which did not cause short-term cell death, when comparing the relative effect of field intensity and cumulated pulse duration on the percentage of permeabilized cells which remain viable (Fig. 6), it is clear that a less damaging effect is obtained with a short cumulated pulse duration. Local alteration of the membrane organization is then small [16]. To obtain a large number of permeabilized cells, one should then use a high intensity field. This has two consequences (1) smaller cells are affected, and (2) a larger part of the cell surface is affected for a given cell [16]. According to the hypothesis that only the electropermeabilized cells might die after the electrical treatment, no electropermeabilized cell survival was observed when permeabilization levels of the population were small (Fig. 6). From Fig. 6 and Eq. (1), cells which are permeabilized with the lower field strengths are very sensitive to field effect. As larger cells are permeabilized at lower field intensities than the smaller ones [46], one may conclude that in a given population, the larger cells are the most sensitive to the lethal effect of the field.

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