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Ionic Modulation of Electrically Induced Fusion of Mammalian Cells

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Summary. During the last few years, a new technique has been developed for the "electrofusion" of mammalian cells. No previous treatment of the culture is needed, for the contact between cells is spontaneous. Short DC electric pulses are applied directly to a culture growing in monolayers on a culture dish. When the cell density is high enough, contacts occur between cells giving the so-called contact inhibition. In the present study, a systematic investigation of the modulation of the extent of the fusion by the ionic content of the bathing medium during the pulsation is described. An increase in the content in monovalent ions decreases the fusion yield. But this decrease is modulated by the nature of the ion; Li^+, a potent "water structure maker," is less effective than Na^+ or K^+. Ca^{2+}, when present in the millimolar range, leads to the lysis of the cells. Mg^{2+}, when present at concentrations smaller than 4 mM, promotes the fusion but prevents it at larger concentrations. Microelectrophoresis measurements show that the electric surface charge is not strongly affected by these changes in ionic content. Our observations are relevant of a modulation of the cell-cell interactions by the ionic content of the bathing medium.

Key Words electroporation · electrofusion · mammalian cells · cell contacts · ionic properties of cell membranes · organization of water by ions

Introduction

Cell fusion is a basic process in cell biology being responsible in the formation of myotubes for example. In biotechnology, it offers a way to obtain hybrid cells sharing the genetic characters of the parental cells. Different techniques were developed in the 60's and 70's where the fusion was achieved by the use of inactivated viruses [5, 6, 12, 19] of polyethyleneglycol (PEG) [7, 17] or lipid liposomes [18]. However, that the yield of viable hybrids was always low may be due to the presence of exogenous reagents, some of them now known to be lethal for the cells [11]. A new physical technique is now used: "electrofusion" (i.e. electrically induced fusion), where cell fusion is obtained by the application of a transient electric pulse on cells previously brought in close contact [3, 15, 22, 25, 26]. In our experimental procedure, cells grown in monolayers on a culture dish are directly submitted to repetitive microsecond square-wave electric pulses. The solution bathing the cells during the pulsations is isoosmotic to the culture medium, pH buffered and contains a small amount of magnesium ions. These experimental conditions appear as a great advantage as shown in the production of viable hybrids [9, 25]. Our previous results showed that a key step in this fusion was the electric field-induced pore opening in the cell membrane [13, 24]. Previous studies have shown that the ionic content of the medium was affecting the electric field-induced hemolysis of red blood cells, both by altering the size [14] and the sites [23] of the induced pores. No explanations were given for such observations but it should be taken into account that in lipid monolayers, the organization of the matrix is dependent on the ionic content of the subphase [20]. The internal energy of a lipid layer appears to be dependent on the ionic content of the bathing medium. Such a modulation may alter the pore-opening process linked to the growth of structural defects [1]. As far as electric field-induced fusion is concerned, the process was inhibited in slime molds when the medium contains millimolar concentrations of Mg^{2+} or Ca^{2+}; in our former work on mammalian cells [22], we showed that the fusion was strongly enhanced when the pulsing medium was 1 mM $MgCl_2$.

In the present work, electrofusion of mammalian cells is shown to be under the control of the ionic content of the pulsing medium. These conclusions are obtained by a systematic investigation of the yield of fusion observed by pulsing cells in medium containing different mono- and divalent ions.

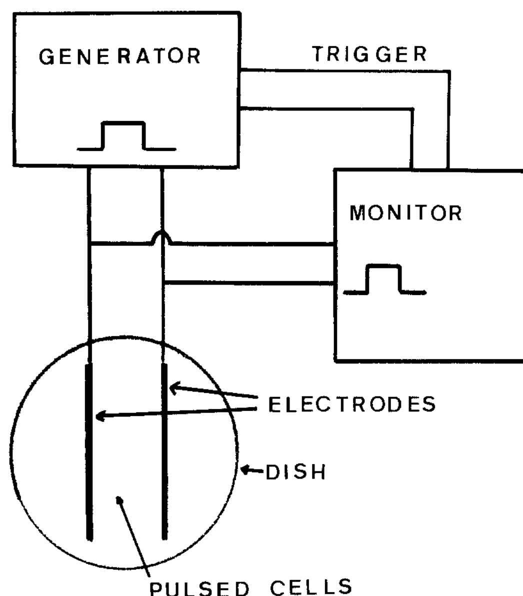

Fig. 1. Experimental set-up. The high voltage supplied by the generator is applied on the cell culture attached to the dish by the two electrodes. A fraction of the voltage is fed onto the data acquisition unit

Materials and Methods

Electric Field-Induced Fusion

The cell fusion protocol was described previously [4]. Briefly, CHO cells grown in Petri dishes in monolayers are submitted to square-wave electric pulses. These are generated by two thin stainless steel parallel electrodes connected to a voltage generator. The electrodes are seated on the bottom of the culture dish. Just before pulsation, the culture medium (MEM 0111) is substituted by 2 ml of a "pulsing buffer." Voltage pulses are then applied. Their shapes (magnitude and duration) are stored on a digital data acquisition unit; thus the actual field is monitored. After the electric treatment (5 pulses, 1.2 kV/cm, 100 μsec duration, 1 sec delay), the pulsing buffer is substituted by 5 ml of culture medium. The dishes are incubated at 37°C in a 5% CO_2/95% air atmosphere for 2 hr. The cells are then fixed and observed. All these experiments were performed under sterile conditions in a laminar flow hood (Fig. 1). The yield of fusion was the ratio between the number of nuclei belonging to polynucleated cells to the total number of nuclei [4]. In CHO, a background of polynucleation was always present which represents an apparent yield of fusion of 8 to 20%.

The standard "pulsing buffer" was 250 mM sucrose, 10 mM phosphate buffer, pH 7.7, 1 mM $MgCl_2$. Its ionic content was altered by adding small volumes of concentrated salt solutions.

Taking into account the power of the generator, the duration of the pulses and the volume between the electrodes, the temperature rise associated with the electric current during the five applied pulses was calculated to be less than 6°C. Furthermore, we recently described the interaction between electric pulses and cells in cell fusion to be vectorial [21].

Table 1. Effect of the external pH on the cell electrofusion[a]

pH	5.7	6.3	7.7	8	9
R	0.98 (±0.03)	1.02 (±0.02)	1	1.02 (±0.02)	0.87 (±0.03)

[a] R is the ratio of the fusion yield observed at the given pH to the one obtained at pH 7.7 (standard conditions). Fusion was triggered by 5 pulses (1.6 kV/cm, 100 μsec).

Fluorescence

CHO cells were grown in suspension in the MEM 0111 medium. They were washed and suspended in the "pulsing medium" at a density between 1.5 to 2.5 × 10^6 cells/ml. Fluorescence was measured by a microprocessed fluorimeter (ISA JY3D) with a thermostated cuvette holder (22°C). The magnesium salt of ANS was used in these experiments.

Microelectrophoresis

CHO cells were grown in suspension in the MEM 0111 medium. They were washed and suspended in the "pulsing medium" at a density of 0.2 × 10^6 cells/ml. Measurements were performed at 22°C with a Rank Mark II (U.K.) using a flat cell and platinum electrodes. The tension applied on the sample was adjusted between 50 and 100 V taking into account the ionic content of the sample.

Results

Effect of the Osmolarity

Keeping the ionic strength low and the same pH buffer (10 mM phosphate, pH 7.5), the osmolarity of the pulsing medium was changed by altering the sucrose concentration. The yield of fusion was the same in a hyperosmotic (400 mM sucrose), an isoosmotic (250 mM sucrose) and a hypoosmotic (200 mM sucrose) medium (*data not shown*). As it is going to be described, increasing the ionic strength decreases the yield of fusion. The same inhibitory effect is observed with a pulsing medium being either 0.1 M NaCl in 84 mM sucrose (isotonocity) or 0.1 M NaCl in 250 mM sucrose (hypertonicity) (*data not shown*).

Effect of pH

The pH of the pulsation medium was adjusted between pH 5.7 and 9 by adjusting the ratio between the base and the acid. The ionic strength was thus kept constant. As shown in Table 1, this change in pH of the pulsing conditions is not observed to alter

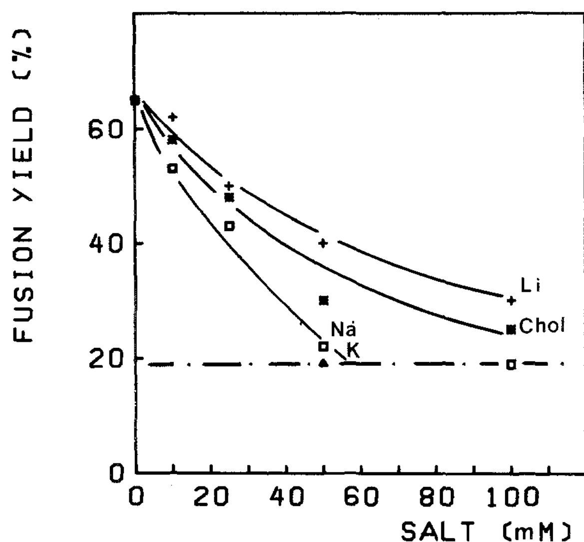

Fig. 2. Inhibition of electric field-induced cell fusion by monovalent ions. The pulsing medium was 10 mM phosphate, 250 mM sucrose, 1 mM $MgCl_2$ complemented by the indicated monovalent ion concentration. Fusion was triggered by 5 pulses (1.2 kV/cm, 100 μsec). The polynucleation background level is given by Δ

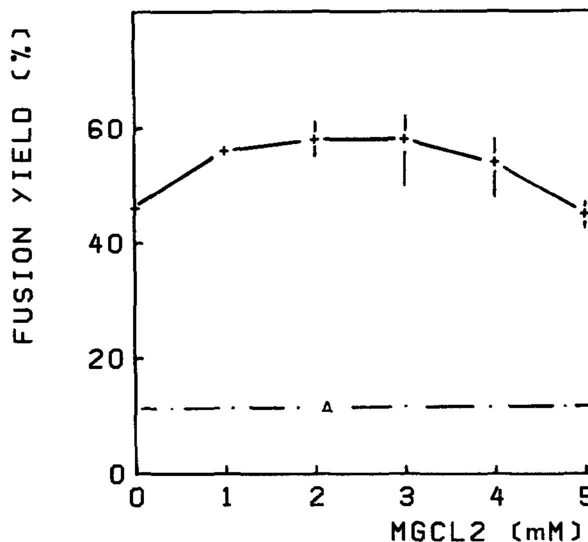

Fig. 3. Effect of magnesium ions on the electric-field induced cell fusion. The pulsing medium was 10 mM phosphate, 250 mM sucrose and increasing magnesium concentration. Fusion was triggered by 5 pulses (1.2 kV/cm, 100 μsec). The polynucleation background level is given by Δ

the yield of fusion between pH 5.7 and 8, a decrease appearing in an alkaline medium (pH 9).

Effects of Monovalent Cations

Keeping the pH buffer (10 mM phosphate) and the sucrose and the magnesium concentrations (respectively, 250 and 1 mM) unchanged, increasing concentrations of chloride salts were added in the pulsing buffer, the cations being Na^+, K^+, Li^+ and choline. The salt concentration was increased between 0 and 150 mM. The results are shown in Fig. 2. Increasing the ionic strength induces a decrease in the fusion yield. A complete inhibition of fusion is observed when 50 mM NaCl or KCl or 100 mM choline chloride was present. LiCl induces a weaker inhibitory effect. All these experiments were performed under pulsing conditions known to induce the largest fusion yield under the standard conditions. Changing the counter-ion Cl^- by SO_4^{2-} did not affect the observed inhibition. Nonpermeant dye penetration was always observed proving the occurrence of an electroperforation.

Effect of Magnesium Ions

Keeping the pH buffer (10 mM phosphate) and the sucrose concentration (250 mM) the same, the concentration in Mg^{2+} as a chloride salt was increased in the pulsing medium. Results are shown in Fig. 3. They were similar when the sulfate salt was used in place of the chloride. Pore opening was always detected. The inhibitory effect induced by monovalent ions (Na^+, K^+) is the same if the Mg^{2+} content is increased from 1 mM (as described above) to 4 mM. No competition for putative binding sites between Mg^{2+} and Na^+ exists.

Effect of Ca^{2+}

Ca^{2+} is known to form a complex in phosphate buffer. Taking into account this effect, the concentration in free Ca^{2+} was computed using a pK_D value of 2.7. In other experiments, a Tris-HCl 10 mM buffer was used. A control experiment showed that the fusion yield under standard conditions (250 mM sucrose, 1 mM $MgCl_2$) was unaffected by the buffer substitution. When using a pulsing medium containing a Ca^{2+}-free concentration (chloride salt) of more than 1 mM, the pulsed cells were all destroyed, the control samples on the same culture dish being unaffected. If lower free concentration was used, the fusion yield was observed to decrease strongly with increasing Ca^{2+} concentration (Fig. 4). Electroperforation which is detected by the penetration of nonpermeant dyes is always observed under our experimental condition.

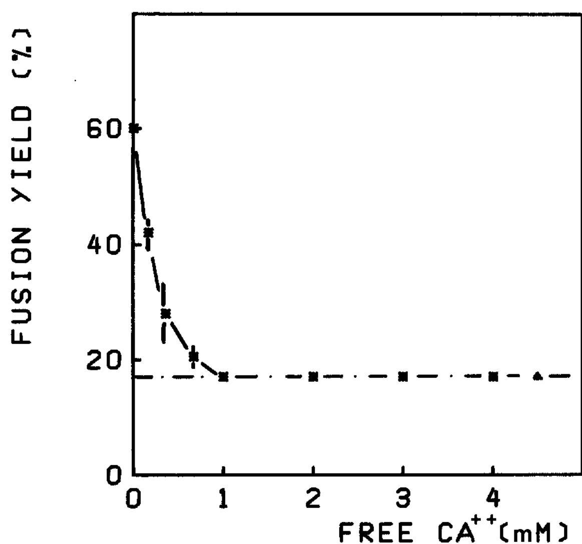

Fig. 4. Inhibition of electric field-induced cell fusion by free calcium ions. The pulsing medium was 10 mM phosphate or Tris, 250 mM sucrose, 1 mM $MgCl_2$ supplemented by $CaCl_2$. Fusion was triggered by 5 pulses (1.2 kV/cm, 100 μsec)

ANS Binding

Taking into account the above-described experiments, an attempt of the determination of surface charge changes was undertaken. The affinity of ANS, an amphiphilic fluorescent dye, for membranes, has been described as being under the control of the electric surface charges of the host matrix [8, 10]. The emission spectrum of the bound probe has a maximum around 470 nm and was not affected by the ionic content of the medium. As the fluorescence intensity of the free dye was negligible, the apparent binding constants were obtained from the double-reciprocal plot of fluorescence intensity versus total dye concentration. These plots being linear are evidences that binding sites are homogeneous. The extrapolate value of fluorescence at infinite ANS concentration is thus a good indicator of the density of binding sites. Results are shown in Table 2. Changes in dissociation constants K_D and in density of binding sites (using the above-described index) were very subtle.

Microelectrophoresis Measurements

Changes in the surface charges are known to affect the mobility of cells in an electric field. Microelectrophoresis measurements were performed with increasing ionic concentration in the pulsation medium. Results are shown in Table 3. Monovalent ions do not affect significantly the electrophoretic mobility. Divalent cations (Mg^{2+} and Ca^{2+}) induce a decrease in this mobility. This effect remains limited and may be associated to the absorption of these ions onto the membrane leading to a screening of the surface charge.

Discussion

This work provides the first experimental evidence that the electrically induced fusion of mammalian cells is under the control of the nature and the amount of the ions present in the medium. This effect is proved not to be linked to the associated change in osmotic pressure. Altering this parameter by changing the sucrose concentration between 200 and 300 mM (i.e., a range equivalent to the ones obtained by changing the ionic content), produced no change in the fusion yield. Furthermore, these changes in osmotic pressure were not observed under the microscope to induce morphological modifications in the cells.

The most striking observation with monovalent cations is the inhibition of fusion which is associated to an increase in their concentrations. This effect is valid whatever the nature of the counter-ions (Cl^- or SO_4^{2-}). The origin in the inhibition is not associated with the pore-opening process. Pores are induced by the electric pulse leading to the penetration of dyes such as Trypan Blue. Previous studies [14] showed that the size of the induced pores was a function of the ionic strength of the suspension medium. But in the case of electrofusion, the pore closing is supposed to be intercellular. In such a case, the contact between the pulsed cells is a critical factor in the fusion event. Increasing the ionic content would decrease the magnitude of the electrostatic forces. As described in a review paper [2], the adhesion of cells to cells is linked to the balance between electrostatic and electrodynamic forces. The change in ionic strength would then modulate the strength of the cellular adhesion and the nature of the cell-cell contact. This behavior of Li^+ provided some kind of experimental evidence that in fact cellular contacts are affected. As we showed, the inhibitory effect of Li^+ is weaker than with the other ions. Physical-chemical studies of aqueous solutions of monovalent ions have emphasized the point that Li^+ is a "water structure maker" and the other monovalent ions are "structure breakers." The water layer between cells is supposed to be organized due to the strong electric field arising from the membranes. Li^+ would thus be less perturbing for the cell-cell contact than the other ions, keeping nevertheless an inhibitory effect linked to the increase of ionic strength and of the associated decrease in electrostatic interactions. This pro-

Table 2. Parameters of ANS binding to CHO cells in different saline conditions[a]

mM	F_∞	K_d (μM)	F_∞	K_d (μM)
	KCl		Choline chloride	
0	8.89 ± 0.20	1.97 ± 0.25	7.06 ± 0.09	1.65 ± 0.36
25	8.80 ± 0.29	1.88 ± 0.33	6.45 ± 0.20	1.68 ± 0.43
50	7.62 ± 0.22	2.43 ± 0.33	6.45 ± 0.20	1.98 ± 0.20
75	8.25 ± 0.25	2.27 ± 0.33	7.14 ± 0.20	2.70 ± 0.30
100	8.85 ± 0.24	2.12 ± 0.29	6.82 ± 0.57	2.35 ± 0.35
	$MgCl_2$		$CaCl_2$	
0	6.12 ± 0.12	1.42 ± 0.17	6.90 ± 0.24	2.12 ± 0.45
1	7.27 ± 0.13	1.46 ± 0.15	7.54 ± 0.14	2.29 ± 0.44
2	7.27 ± 0.13	2.10 ± 0.15	7.57 ± 0.43	2.93 ± 0.61
3	7.27 ± 0.13	1.73 ± 0.37	6.69 ± 0.44	2.89 ± 0.50
4	7.41 ± 0.27	1.49 ± 0.18	6.45 ± 0.21	2.71 ± 0.44
5	6.26 ± 0.20	1.85 ± 0.30		

[a] Experiments were performed in the standard phosphate buffer, except for $CaCl_2$ where a 10 mM Tris buffer was used.

Table 3. Electrophoretic mobilities of CHO cells in different saline conditions

$MgCl_2$ (mM)	U($10^{-8}m^2sec^{-1}V^{-1}$)	$CaCl_2$ (mM)	U
0	1.63 ± 0.003	0	1.81 ± 0.1
1	1.36 ± 0.062	1	1.39 ± 0.05
3	1.12 ± 0.009	3	1.16 ± 0.019
5	1.04 ± 0.011	4	1.03 ± 0.031
NaCl (mM)	**U**	**Choline (mM)**	**U**
0	1.86 ± 0.089	0	1.91 ± 0.071
7.5	1.73 ± 0.012	7.5	1.79 ± 0.002
15	1.72 ± 0.055	15	1.61 ± 0.008
30	1.89 ± 0.064	30	1.76 ± 0.035
60	2.14 ± 0.025	60	1.72 ± 0.042

posed change in cellular adhesion is rather subtle and is in any case not due to a change in surface electric charge. This conclusion is derived for our three experimental evidences: a) no effect of pH between 5.7 and 8; b) no change in the characteristics of ANS binding; c) no critical change in the electrophoretic mobility. Binding of ANS to membranes is under the control of the surface negative charge because of the repulsion of the charged group of the dye [10]. The electrophoretic mobility is a direct function of the surface charge. It is well known that the thickness of the electric double layer, the surface potential and the associated zeta potential, are strongly affected by the ionic strength of the surrounding medium [8]. Nevertheless, as the buffer is 10 mM phosphate, the relative change in ionic strength is limited even with the high ionic content (0.1 M monovalent ions). As a conclusion, our observation of a minute change in surface charge is not amazing and such a subtle change cannot be considered as inducing the inhibition of fusion.

The effects of divalent cations (Mg^{2+}, Ca^{2+}) can be only partly explained by such a scheme. The inhibition by Ca^{2+} even at low concentration of free cation (0.7 mM) is clearly to associate to the penetration of this ion in the cytoplasm. The cytoplasmic Ca^{2+} concentration is then completely unbalanced leading to the death of the pulsed cells as we observed under the microscope. The action of Mg^{2+} can be explained by two antagonist effects. At low concentrations, Mg^{2+} is a promoter of fusion but at higher concentrations an inhibition is observed. This last effect can be correlated to the effects of

monovalent ions and linked to the increase in ionic strength. The rather unspecific character of the inhibition of fusion by high monovalent ion concentration is emphasized by the lack of competition between Mg^{2+} and Na^{+}. The same inhibition is observed with increasing Na^{+} concentration, the Mg^{2+} concentration being either 1 or 4 mM. Increasing the ionic strength abolishes the effect of Mg^{2+}. This increase may be self provided by an increase in the content in Mg^{2+} (as observed for concentrations larger than 5 mM). The promoting effect of Mg^{2+} on fusion may be explained in two different ways: a) organization of interfacial water; b) "cross-linking" of adjacent cells [16]. As described above for Li^{+}, Mg^{2+} is known to be a potent "water structure maker," its effect being much more efficient than Li^{+}. Thus here again, we may propose that a structured interfacial water layer would be a promoter of cell fusion. But one should keep in mind that Mg^{2+} is bearing two positive charges and can act as a bridge between cells, interacting with a negative charge on one cell and with another one on another cell. Such an effect would induce a very intimate contact between the cells. This role of Mg^{2+} in the aggregation of cells in suspension is well documented [16].

Our results are evidences that at least two different kinds of effects are associated to the action of ions on the electrofusion. At the present state of our knowledge, at least three different steps occur during electric field-induced mammalian cell: 1) intercellular contact, 2) electric field-induced pore opening, and 3) intercellular membrane mixing. Under our experimental conditions any step occurring before the modification of the ionic content is not affected. As impermeant dye penetration was always observed in our experiments, step 2 is always present whatever the ionic status of the pulsing medium. As a conclusion, step 3 appears as the critical one which is affected by the ions. Two different effects of ions on the fusion were observed in this study: 1) an unspecific inhibition clearly related to the increase in ionic strength and 2) a specific one with Li^{+} and Mg^{2+} which may be related to the structure of the water. In the case of the divalent cation, a link between adjacent cells may be present. As in step 3, where the close contact between cells is the key parameter, we should include that it is affected by the ionic content of the medium. The observed inhibition of electric field-induced cell fusion appears as a direct consequence of a modulation of the cellular contact. This conclusion is of great importance in cell biology. It proves that at a molecular level the contact between cells is strongly modulated by the ionic content of the medium. The effect is due to 1) a modification of the electrostatic interactions and 2) an organization of the interfacial water.

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