# Tension-voltage relationship in membrane fusion and its implication in exocytosis

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Received 3 November 1999

Edited by Maurice Montal

**Abstract In this study, new methods are used to control cellular membrane tension to evaluate the role it plays in electrofusion. The data show that membrane tension present during the application of an electric field facilitates electro-induced membrane fusion. No enhancement was detected if the strain was applied after the pulse. Analysis of the electromechanical process of fusion revealed a synergy between the two kinds of constraints in the membrane fusion. Both mechanical and electrical constraints apparently play a key role in membrane fusion between the granule membrane and the plasma membrane, i.e. the exocytosis process.**



*Key words:* Tight intermembrane contact; Electromechanical fusion; CHO cells

## 1. Introduction

Membrane fusion is a ubiquitous process occurring in every intracellular compartment. In order to maintain the compartmental identity and structural integrity of a eukaryotic cell, this process must be controlled. The specificity of exocytosis involves a number of different factors including small GTPases of the Rab family, $Ca^{2+}$ and various factors in combination with SNARE (soluble *N*-ethylmaleimide sensitive factor attachment protein receptor) proteins. The sequence of events leading to exocytotic secretion is still unclear [1]. In particular, whereas the steps preceding it, such as vesicle docking [2], seem well understood, the mechanism that fuses the vesicle and plasma membrane, leading to pore formation, remains to be explained. A bioelectrochemical modification of the membrane organisation must be considered. It is known that negatively charged lipids are preferentially distributed in the inner leaflet of the bilayer plasma membrane [3]. Furthermore, $Ca^{2+}$ interacts with negatively charged lipids and this may induce a lamellar to hexagonal phase transition with the generation of local micellisation foci and the breakdown of the bilayer [4]. One other potentially relevant factor is the intracellular electrostatic forces that could influence the interaction between the vesicle and plasma membranes leading to the fusion event [5–7].

It was previously shown that subjecting cells to short, strong electric pulses makes the membrane transiently permeable [8]. Such an electropulsation induced fusion of contacting cells [9]. The driving force is a change in the membrane potential difference that induces a membrane structural change. When the membrane potential difference attains a critical local value of 250–300 mV, the membrane becomes permeabilised and fusogenic in that part of the cell surface [10,11]. It is now known that voltage-induced permeabilisation is a precondition for the fusion between membranes [12]. Even for cells that are in close apposition, a time lag occurs between membrane permeabilisation and fusion [13]. The biological relevance of electrofusion is further supported by the magnitude of the electrostatic field present in the contact locus of exocytosis. It is also shown that when the two membranes approach each other during apposition [14], the electrostatic field reaches values known to cause electrical breakdown of biological membranes. It was recently suggested for the adrenal medullary chromaffin cell that the fusion pore could be ascribed to an electropermeabilisation of the granule membrane triggered by the strong endogenous electric field at the site of exocytosis [5].

The membrane tension in the contact area could make an important contribution in fusion. When the calcium signal reaches a secretory vesicle docked near an exocytotic site, there is a close approach and alignment of short segments of the granule and plasma membranes, possibly mediated by a scaffold of intermembrane proteins [15]. Electron micrographs show the two interacting regions lying flat against each for an interval that may extend several nanometers [16,17]. This supports the idea that proteins can induce a mechanical constraint provoking tension in the membrane of the contact zone. However, even when brought into close contact cell membranes were held apart by various repulsive forces. These repulsive forces can be reduced across the entire cell surface by single local membrane deformation, which limits the undulation forces. This constraint may act in synergy with the electrical forces described above. This idea is supported by work that showed that electropermeabilisation of liposomes occurs under lower field intensities when the membrane is mechanically stressed [18].

In the present work, the role of mechanical forces on mammalian cell electrofusion was evaluated in three different methods to create the cell-cell interaction: (1) the contact was ensured by protein interactions when plated cells were in a confluent state prior to the application of electric field [19], (2) cells in suspension after pulsation were subjected to mechanical forces by centrifugation [12], (3) tight membrane contacts were ensured by filtration providing strong cell deformations in the membrane contact area before application of the electric field. In the present paper, a role of membrane tension in the electropermeabilisation process and the subsequent fusion between cell membrane is reported.

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PII: S0014-5793(99)01739-1

## 2. Materials and methods

### 2.1. Materials

Chinese Hamster Ovary (CHO) cells (clone WTT) were grown in suspension under gentle agitation (100 rpm) at 37°C as previously described [12]. Cells were maintained in exponential growth phase ($5–10·10^5$ cells/ml) by daily dilution. CHO cells grown in suspension can be replated readily in Petri dishes (35 mm diameter, Nunc, Denmark) and kept at 37°C in a 5% $CO_2$ incubator (Jouan, France) to grow as monolayer. This was obtained only when cells were viable and was used as a selection tool.

Just before electropulsation (Fig. 1A), cells were washed in a pulsation medium with an iso-osmotic low ionic content (250 mM sucrose, 10 mM phosphate, 1 mM $MgCl_2$, pH 7.4).

For fusion with plated cells (Fig. 1B), monolayers of cells were obtained by plating cells in suspension on Petri dishes, and keeping them at 37°C in a 5% $CO_2$ atmosphere. The cell density was 600 cells/$mm^2$ for the fusion experiments. Numerous contacts were present.

For fusion and permeabilisation on filters, $2·10^6$ cells were carefully poured on a biocompatible filter (polycarbonate hydrophilic trans filter, 25 mm diameter, 1 μm pore diameter, Whatman, England) and the culture medium was pumped through. The polycarbonate membrane served as a matrix for forming a cell monolayer in less than 1 min (Fig. 1C). The pressure imposed to cell by the filtration was adjustable.

### 2.2. Methods

The cell fusion protocol for plated cells has been described elsewhere [20]. Briefly, cells in Petri dishes were subjected to square-wave electric pulses, generated by two thin stainless steel parallel electrodes in contact with the dish connected to a voltage generator which gave an uniform electric field (CNRS Cell Electropulser, Jouan, France). In this way, the pulse intensity and duration could be kept constant. The voltage pulse applied to the cell suspension was monitored with an oscilloscope incorporated in the cell pulsator. Just before pulsation, the culture medium was replaced by 2 ml pulsing buffer, and voltage pulses were applied.

The cell fusion protocol for cells in suspension was previously described [12]. Cells were washed in the pulsing buffer and resuspended at $10^7$ cells/ml. A volume (0.1 ml) of the cell suspension was placed between the thin stainless steel parallel electrodes in contact with a culture dish. Voltage pulses were then applied and the permeabilised cells were centrifuged immediately for 5 min (C500 Jouan centrifuge) in order to pellet the cells and create contacts for their fusion. The cell pellet was then incubated at 37°C for 10 min. After gentle deaggregation, the cell suspension was poured into a Petri dish of fresh culture medium. Viable cells spread on the dish surface.

The cell fusion protocol for cells on filter was as follows. The filter was kept wet and was placed on a clean microscope glass slide. Two steel parallel rod electrodes connected to the generator were brought into direct contact with the filter. The cell monolayer spread on the filter was then pulsed. After electric field delivery, the wet filter was incubated for 10 min at 37°C. Then cells were recovered by gentle flushing with culture medium. Viable cells spread on the dish surface. For permeabilisation, the protocol was similar except that the pulsing buffer contained 100 μM PI.

For all the methods, cells were incubated post pulsation during 2–4 h at 37°C in an air/$CO_2$ atmosphere. They were observed, when plated, under an inverted microscope (Leica, Wetzlar, Germany) by video monitoring (Sony, Aomori, Japan), and the percentage of polynucleated cells was evaluated. The fusion index was taken as the percentage of nuclei in polynucleated cells relative to the total number of nuclei. This approach where only plated cells were observed gave the advantage of selecting only viable cells.

## 3. Results

Cells in suspension (Fig. 1A) have a characteristic spherical shape that undergoes a deformation during filtration. A top view of the cells on filters (Fig. 1C) shows that cells are polygonal and tightly packed. Numerous large and very close intermembrane contacts are apparent. The cross section view monitored by electron microscopy (Fig. 1E) shows the

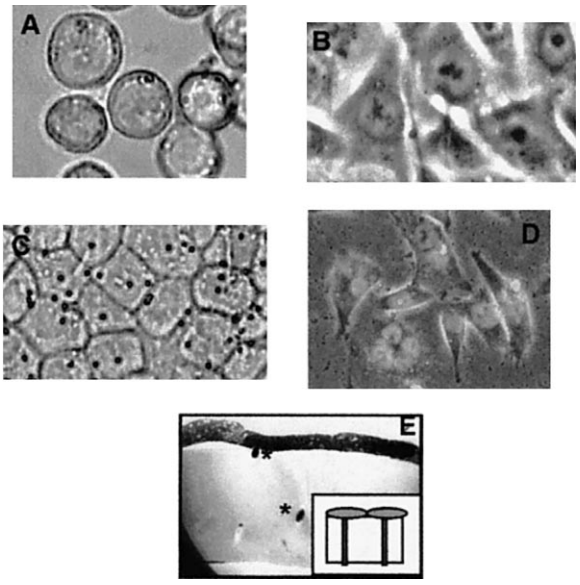


Fig. 1. Cell morphology. A: Picture of CHO cells in suspension with their spherical shape. B: Picture of adherent CHO cells. Numerous specific cell contacts were established on the culture dish when cells are in a confluence state. C: Picture of CHO cells on filter obtained just after filtration, cellular morphology was altered drastically on filter. D: Plated polynucleated cells obtained after 2–3 h incubation. E: Electron micrograph showing a low magnification view of the cells on the filter. The longitudinal section is cut on the bias. The monolayer organisation of flattened cells was clearly seen. Very close contacts were present between cells. Arrows indicate cell penetrations in filter pores. A model of filtered cells was also represented.

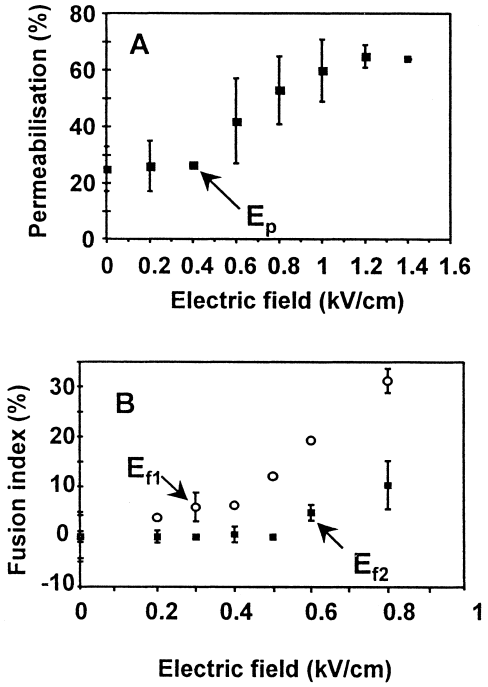


Fig. 2. Voltage dependence. A: The level of permeabilisation (i.e. the percentage of PI stained cells in the population) was plotted as a function of the electric field strength. B: The level of fusion index (i.e. the percentage of fused cells) was plotted as a function of the electric field strength. (○) Cells were electropulsed just after filtration and (■) cells were electropulsed 5 min after filtration. Cells were pulsed 10 times for 100 μs at 1 Hz frequency.

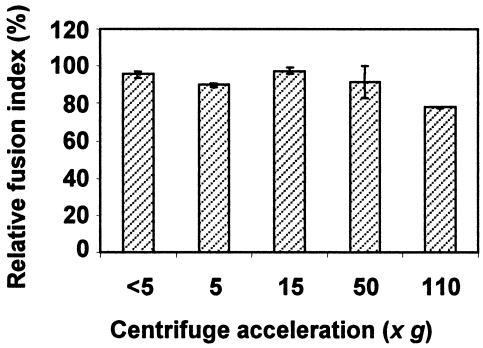


Fig. 3. Tension dependence. The relative level of fusion index was plotted as a function of the acceleration of the centrifuge. Pulsed cells in suspension were put in close contact by centrifugation at different acceleration. Cells were pulsed 10 times for 100 μs at 1 Hz frequency

monolayer organisation of flattened cells with tight contacts. Cells also penetrate into filter pores (represented by asterisks on photograph). Cell deformation and the associated local increase of membrane bending are present. Cells recover their initial spherical shape within 5 min after cessation of the pressure (data not shown). These penetrations are transient and slowly left the narrow pores. The flat contacts shown in Fig. 1C,E in contrast with random contact (Fig. 1A) or natural contacts (Fig. 1B), disappear with the post suction incubation.

Using CHO cells on filter within less than 1 min after suction, the effect of field strength on permeabilisation and fusion was evaluated when the mechanical constraint was present. Fig. 2 shows the dependence of the two processes on the electric field strength. Permeabilisation was influenced by field intensity (Fig. 2A). A field intensity about 0.4 kV/cm was required to observe electropermeabilisation. Fusion also required a threshold field intensity to noticeably augment the level of polynucleation. No fusion was observed for fields smaller than 0.4 kV/cm (Fig. 2B). The post filtration recovery step clearly affected the electrofusion process. If cells were pulsed 5 min after filtration, at a time when the constraint had disappeared (Fig. 2B, the field threshold value was higher. The loss of the membrane tension before pulsation was associated with an increase in the electrical field intensity required for fusion. A strong mechanical constraint before the pulse

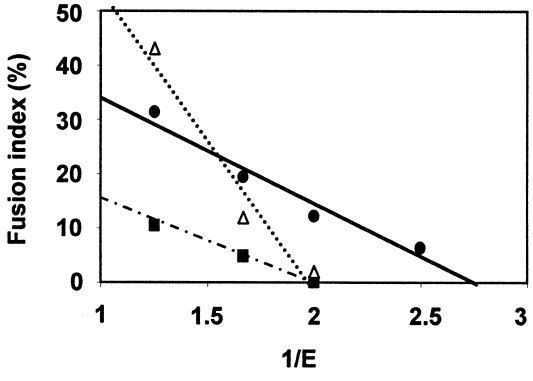


Fig. 4. Tension-voltage relationship. The percentage of fusion was plotted as a function of $1/E$. Adherent cells (△) or filtered cells were pulsed just after filtration (●) or 5 min later (■) 10 times at 100 μs.

did not affect the electrofusion. This tension dependence is not observed in cells in suspension that had been put in contact by centrifugation just after pulsation (Fig. 3). The tension on the membrane was determined by the centrifugation speed [21] but an increase of the centrifuge acceleration did not increase the fusion yield. Increase in membrane tension after pulsation had no effect on fusion. A loss of cell viability is observed only when the acceleration reached $110 \times g$, this imposed an upper limit to the range of our study. The mechanical contribution observed above must be associated with pulsation to influence the fusion yield.

Electrofusion yield depended on the electric field intensity (see Fig. 2B). This dependency is a function of the reciprocal of the electric field strength which reflects the membrane area electropermeabilised [10]. Results from plated and filtered CHO cells, pulsed immediately after filtration or 5 min later, showed a linear relationship between electrofusion index and the reciprocal of the field intensity (Fig. 4). Extrapolation to the zero value of fusion index gives the threshold value of electric field intensity where electrofusion could be detected. They were 0.5 kV/cm for plated cells and for cells pulsed 5 min after filtration and 0.37 kV/cm for cells pulsed just after filtration. The critical voltage $E_p$ was clearly lowered when a membrane tension was present during the pulse in comparison with the value for relaxed cells (i.e. 5 min after suction) or with adherent cells. The geometry of contacts and the size of cells were analogous in all cases.

## 4. Discussion

The aim of the present work was to evaluate the synergy between mechanical stress and electric field in the fusion process. In electrofusion, fusion starts from a perturbation in the structure of the lipid bilayer of adjacent membranes. This perturbation is triggered by the local alteration of the transmembrane potential by an external electric field. The electric field is presumed to locally 'disorganise' the lipid bilayer. It has been proposed that a large number of small defects due to structural mismatches are present in such circumstances [22]. This description is in agreement with the $^{31}P$ NMR studies, which showed that most lipids in the transiently permeabilised area were in an 'out-of-equilibrium' state and that the conformation of their headgroups was severely affected [23,24]. The data on the molecular changes of the cell membrane associated with electropermeabilisation suggest that a structural reorganisation of the membrane increases its permeability due to induction of random defects. This 'out-of-equilibrium' lipid state induced by electric field corresponds to a fusogenic state [12]. Fig. 2 confirms the correlation between the induction of permeabilisation and fusion.

The membrane tension during the application of an electric field influences the induction of the permeabilisation, but membrane mechanical constraint facilitates fusion only under well-defined conditions. When cells are pulsed just after filtration, permeabilisation occurs in low fields (Figs. 2B and 4). As the flat contacts disappear in the post suction incubation, the mechanical contribution vanishes. Its consequence on the electropulsation effects is a shift of the critical electropulsation field strength which returns to what was observed for plated cells (Figs. 2B and 4). If the mechanical constraint is imposed to cells after the pulses, no effect on fusion was observed. Nor did an increase of the centrifuge acceleration augment the

fusion yield (Fig. 3). A facilitating effect has been observed by Abidor et al. [25] with cells pulsed during centrifugation. The major conclusion was that as first reported on lipid liposomes [18], electropermeabilisation, i.e. electrofusion was triggered under milder conditions when membrane tension was present. Permeabilisation was triggered by the combination of mechanical and electrical stresses on the membrane [26]. Our conclusion is re-enforced by the work showing the contribution of pulse-induced dipole-dipole interaction to the total pressure acting normal to the membranes of closely positioned erythrocytes by dielectrophoresis during electrofusion [27]. A membrane tension is present in the flat contact zone. The electrofusion threshold was shown to be less when cell contacts induced by dielectrophoresis occur during the fusogenic pulses [28]. The electromechanical model developed to explain the tension-voltage relationship in lipid bilayer vesicles [18] appears to be equally valid for the present study of biological membranes.

These conclusions must be considered for exocytosis. It was proposed that the fusion pore formation starts as a perturbation in the structure of the lipid bilayer of the secretory vesicle membrane [5]. The perturbation at the site of exocytosis results from the forces exerted on charged dipolar constituents of the lipid bilayer by the high electrostatic field strength, due to the plasma membrane surface charges. Furthermore, patch-clamp studies of secretory granules from beige mouse mast cells have demonstrated that pipette-induced lateral tension in the granule membrane and high voltage transmembrane pulses can be used interchangeably to create fusion pore-like events [29]. However, the details of the process are still unknown due to the complexity of the interactions and the variety of the components involved in exocytosis. It is clear that SNARE proteins play an indispensable role in membrane fusion [30]. The role of *trans*-SNARE complexes seems to be confined to an intermediate stage of the reaction, probably docking [31]. SNARE complex formation could actively promote local deformation of the bilayer similar to that observed in our filtration system (Fig. 1D). Mechanical tensions in the lipid bilayer could develop during apposition, as a result of the interactions with the protein scaffold. Qualitatively, it would be expected that tension in the granule membrane would reduce the electric field strength required for electropermeabilisation (Figs. 3A and 4). This supports the observation that when the two membranes approach each other during the stage of apposition [14], the electrostatic field attains values known to cause electrical breakdown of biological membranes. Furthermore, the time course of single-vesicle amperometric transients of catecholamine secretion from isolated chromaffin cells [32] is similar to that of electric field pulse-induced membrane permeabilisation in chromaffin granules [33].

Our data show that the membrane tension present in the focal zones involved in exocytosis facilitates the electromechanical process of fusion. They support the biological relevance of this approach [5].

*Acknowledgements:* Thanks are due to Dr B. Gabriel for helpful discussion and Dr H. Krisch for rereading the manuscript. Financial support was given by Association pour la Recherche sur le Cancer and Region Midi Pyrénées.

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