Spontaneous lipid vesicle fusion with electropermeabilized cells

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Abstract Fusion is obtained between electropermeabilized mammalian cells and intact large unilamellar lipid vesicles. This is monitored by a fluorescence assay. Prepulse contact is obtained by Ca^{2+} when negatively charged lipids are present in the liposomes. The mixing of the liposome content in the cell cytoplasm is observed under conditions preserving cell viability. Electric conditions are such that free liposomes are not affected by the external field. Therefore destabilization of only one of the two membranes of the partners is sufficient for fusion. The comparison between the efficiency of dye delivery for different liposome preparations (multilamellar vesicles, large unilamellar vesicles, small unilamellar vesicles) is indicative that more metastable liposomes are more fusable with electropulsated cells. This observation is discussed within the framework of the recent hypothesis that occurrence of a contact induced electrostatic destabilization of the plasma membrane is a key step in the exocytosis process.

Key words: Liposomes; Mammalian cells; Electrofusion; Exocytosis

1. Introduction

Membrane fusion must be a well-controlled process in order to maintain the compartmental identity and structural integrity of a eukaryotic cell. The specificity of exocytosis involves a number of different soluble factors in combination with SNARE (soluble *N*-ethylmaleimide sensitive factor attachment protein receptor) proteins [1]. But whereas the steps involved in vesicle docking [2] seem well understood, the physical mechanism that fuses the vesicle and plasma membrane, leading to pore formation and vesicle content release, remains to be explained. A bioelectrochemical modification of the membrane organization must be considered. Negatively charged lipids and amino acids are distributed on the outer leaflet of the bilayer plasma membrane and of the vesicles they fuse with [3]. Local Ca^{2+} release through Ca^{2+} channels [4,5] may induce a change in membrane organization by charge neutralization inducing breakdown of the bilayer [6]. Intracellular electrostatic forces could also influence membrane integrity at the site of contact between the vesicle and the plasma membrane [7–9]. In this way, it was computed that when the two membranes approached each other [10] the electrostatic field reached values known to cause electrical breakdown of biological membranes. It was also recently suggested for adrenal medullary chromaffin cells that the fusion pore formation could be ascribed to a granule membrane electropermeabilization triggered by the strong endogenous electric field at the site of exocytosis [7].

Artificial electropermeabilization is obtained by subjecting cells to short, strong electric pulses, which make the membrane transiently permeable [11]. This membrane-permeabilized state is a precondition for cells to fuse [12]. Cell fusion can only be obtained between electropermeabilized surfaces. Within Rosenheck's model [7], only one partner was permeabilized and this was proposed to be sufficient to induce membrane fusion. This implies that when two membranes are in close contact, electropermeabilization of only one of the partners induces a destabilization of the other resulting in their fusion. A theoretical approach was given in the so-called coaxial-pore mechanism of cell membrane electrofusion [13].

This part of Rosenheck's hypothesis was experimentally checked in the present study. Taking advantage of the size dependence of electropermeabilization [14], cells in contact with smaller size liposomes were electropermeabilized under conditions preserving their viability. Liposomes were too small to be directly affected by the field. Fusion was assayed by content mixing. In our experiments, fusion was observed, proving as predicted that the electric destabilization of one partner brings the coalescence of its membrane with that of the other.

2. Materials and methods

2.1. Materials

8-Hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS) was obtained from Molecular Probes (Eugene, OR, USA). Lipids were obtained either from Avanti Biochemicals (Alabaster, AL, USA) (egg yolk phosphatidylcholine (PC), bovine brain phosphatidylserine (PS), cholesterol) or from Sigma (St. Louis, MO, USA) (hexadecyltrimethyl ammonium bromide, tetradecyltrimethyl ammonium bromide, stearylamine). Phosphate buffered saline (PBS) was purchased from Gibco (Grand Island, NY, USA). Salines were all analytical grade. Ultrapure water was obtained from a MilliQ system (Millipore, France). Chinese hamster ovary cells (clone WTT) were grown in suspension under gentle agitation (100 rpm) at 37°C. Cells were maintained in exponential growth phase ($5–10\times10^5$ cells/ml) by daily dilution.

2.2. Liposome preparations

Small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs) were prepared in a 5 mM HPTS solution dissolved in a 5 mM HEPES buffer. The lipid mixture (8.5 mg) (PC/PS/cholesterol: 6/1/3 molar ratio) was dissolved in chloroform/methanol (2/1). The solvent was removed under a stream of nitrogen and then under vacuum for 30 min. The resulting lipid film can be stored under argon at −20°C for several months. 1 ml of the HPTS solution was added, the mixture was stirred with a vortex to obtain dispersion.

SUVs were obtained by treating the dispersion in a bath sonicator

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for 30 min. The free dye was partly removed by centrifugation and washing the pellet with a dye free solution. A mixture of SUVs and multilamellar vesicles (MLVs) was present.

LUVs were obtained by thawing and freezing the dispersion five times. The lipid suspension was then forced five times through two successive 0.1 μm pore polycarbonate membranes with an extruder (Lipex Biomembranes, Canada). The free HPTS was separated from the liposomes by a Sephadex G 75 gel filtration equilibrated with a NaCl (0.145 M) HEPES 10 mM buffer (pH 7.4).

MLVs were prepared as in [15]. The different lipid mixtures (PC/PS/cholesterol: 6/1/3 molar ratio, PC/cholesterol/cationic lipid: 1/1/1 molar ratio) in HPTS, HEPES 10 mM solution were prepared as described above. The dispersion was obtained by hand shaking and the free dye was washed out by centrifugation.

2.3. Electropulsation protocol

Just before electropulsation, cells were washed in a pulsation medium with an iso-osmotic low ionic content. When cationic lipids were present, the pulsing buffer was a 10 mM phosphate buffer containing 250 mM sucrose and 1 mM $MgCl_2$. In the experiments where PS was present, cells were resuspended in a different buffer (PBCa) (HEPES 10 mM, sucrose 250 mM, $CaCl_2$ 5 mM).

Cells washed in the appropriate pulsing buffer are finally resuspended at 4×10^7 cells/ml. 25 μl of the cell suspension was mixed with 150 μl of the liposome solution. 5 min incubation at 4°C was observed. The volume was placed between thin stainless steel parallel electrodes in contact with a culture dish. Voltage pulses were performed then by a voltage generator that gave a uniform electric field (CNRS Cell Electropulser, Jouan, France). In this way, the pulse intensity (1.2 kV/cm) and duration (100 μs) could be kept constant. The voltage pulse applied to the cell suspension was monitored with an oscilloscope incorporated in the cell pulsator. Repetitive pulses (5, 10 or 20) were applied with a 1 s delay. The suspension was then incubated for 10 min at room temperature, washed and then resuspended in 1 ml PBS. Ca^{2+}, when present, was therefore chelated and almost no free ions were left. This procedure was chosen (i) to preserve cell viability [12,24] and (ii) to avoid a long contact between cells and liposomes, where a significant endocytotic uptake may occur.

2.4. Detection of fusion

Single cell fluorescence emission was evaluated by flow cytofluorometry on a Facscan (Becton Dickinson, Cockeysville, MD, USA) using the FL1 channel.

Cells were also observed under an inverted microscope (Leica, Wetzlar, Germany) by video monitoring (Princeton, NJ, USA) and emission was evaluated with Winview software (CSPI, USA). Images were obtained by using NIHimage software.

3. Results

3.1. HPTS fluorescence emission

The intensity of the emission in solution was observed to be a function of the dye concentration. At low concentrations (up to 1 mM), the emission was a linear function of the concentration. At higher concentrations, the intensity was observed to decrease down to a plateau value obtained above 10 mM (Fig. 1). The spectral analysis of the emission showed that a slight shift in the emission (from 510 nm to 520 nm) was present when high concentrations were used. The practical consequence is that the dilution of a concentrated HPTS solution will induce an increase in the fluorescence.

Due to the surface charge associated with cationic and anionic lipids and the putative p*K* shift if HPTS was inserted in the lipid matrix of liposomes, the fluorescence characteristics of the dye (5 mM) when present in the internal layers of MLVs were analyzed. No effect on the emission intensity and spectra was detected for liposome concentration up to 1 mg/ml. HPTS was not affected by the presence of the liposomes whatever their electrical charge. Its transfer from the liposome internal volume to the cell would bring an increase in its emission due to the dilution in the cytoplasm, not to the change in environment. Fusion gave an increase in the mean fluorescence of the population. Of course, fusion resulted in a fluorescent labeling of the cell volume which was detected at the single cell level.

In all further experiments, liposomes were prepared with HPTS at a self-quenching concentration (5 mM).

3.2. MLVs

Different mixtures of lipids were used to prepare the liposomes. A direct electrostatic contact to the negatively charged cell surface was obtained when cationic surfactants were present. When PS was added, the contact was obtained by adding Ca^{2+} in the pulsing buffer (0.7 mM final concentration).

As described in Section 2, four different lipid mixtures were investigated using the three different numbers of applied pulses. Fusion (i.e. enhanced fluorescence in the cells) was never detected.

3.3. SUVs

Liposomes were prepared only with the addition of PS in the neutral matrix (PC/cholesterol). Ca^{2+} (0.7 mM) was present in the pulsing buffer to bring the electrostatic bridge between the partners. This condition was closer to what was present during exocytosis.

Fluorescence intensities were observed to first increase with the number of pulses, but a sharp decrease was obtained when

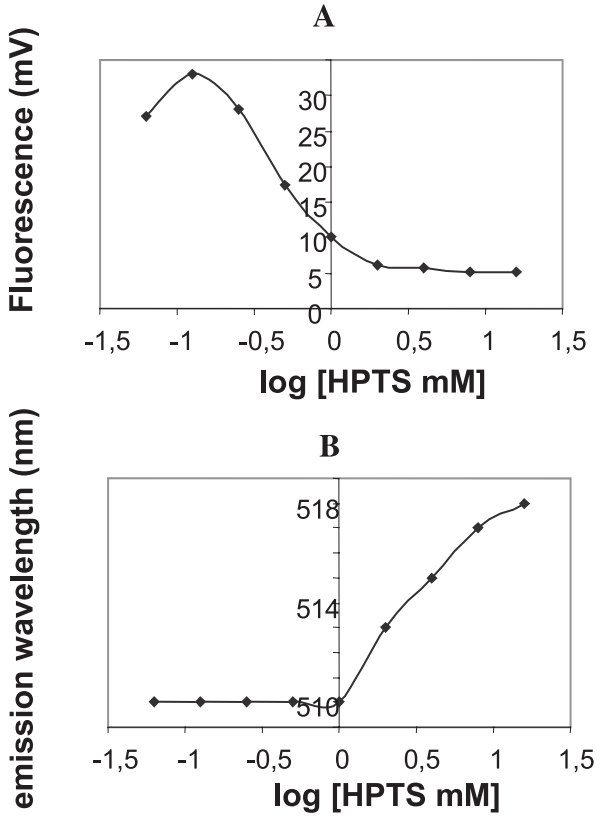

Fig. 1. HPTS emission. A: Dependence of the emission intensity at 510 nm as a function of the dye concentration. B: Dependence of the wavelength of the emission peak as a function of the dye concentration. Emission was detected with a Jobin Yvon JY3 fluorimeter (λ_{exc} was 413 nm). The buffer was 5 mM HEPES.

20 repetitive pulses were applied (Fig. 2A). A background of fluorescence was detected when liposomes were mixed with unpulsed cells. This confirmed that HPTS was still present in the solution of liposomes after centrifugation and contributed to the fluorescence signal on a population. Cells were observed under the microscope. A high background of fluorescence was indeed detected outside of the cell. A peripheral labeling was detected on the unpulsed cells (Fig. 2B), but the cytoplasm of pulsed cells was more fluorescent. Two patterns of labeling were observed with pulsed cells: strongly emittive patches among a uniform one in the cytoplasm, indicative of the liposome to cell content mixing (Fig. 2C).

3.4. LUVs

As for SUVs, liposomes were prepared with PS as the charged partner and Ca^{2+} was added in the pulsing buffer to mediate the electrostatic interaction between liposomes and cells (0.7 mM final concentration).

Fluorescence intensities were observed to increase with the number of pulses and the amount of liposomes that were added (Fig. 3A,B). No background of fluorescence was detected, showing that the exclusion chromatography was effective in removing the non-internalized dye. The unpulsed cells displayed a faint peripheral labeling (Fig. 3D). The direct observation of the pulsed cells under the microscope showed that a uniform cytoplasm labeling was obtained in a significant number of cells (Fig. 3F). Image analysis gave a transversal profile of emission with a 'ball shape', proving the homogeneous volume distribution of the dye in the cytoplasm (Fig. 3G). This is indicative of the liposome to cell content mixing.

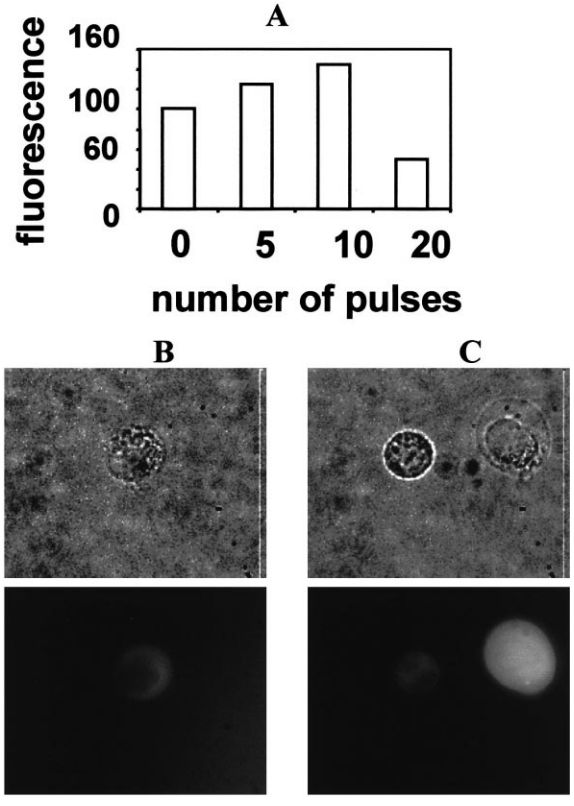

Fig. 2. Fusion with SUVs. Cells were mixed with PC/PS/cholesterol SUVs and pulses of 1.2 kV/cm were applied. HPTS emission was measured on a cytofluorometer in A or observed under a microscope in C. A: Mean fluorescence intensity of cells for different number of pulses. B: Unpulsed cells (top: contrast; bottom: fluorescence). C: Pulsed cells (top: contrast; bottom: fluorescence).

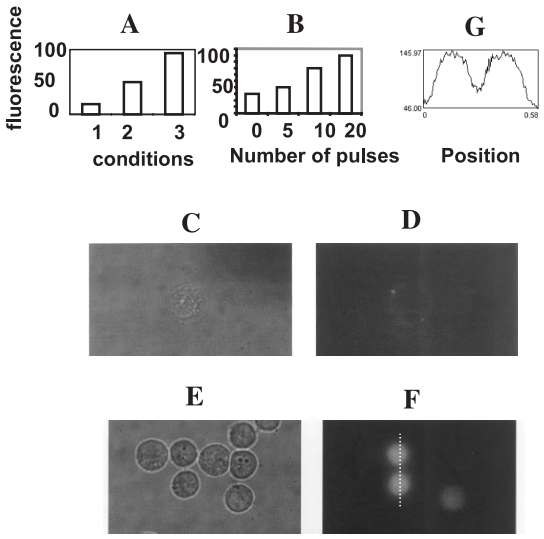

Fig. 3. Fusion with LUVs. Cells were mixed with PC/PS/cholesterol LUVs and pulses of 1.2 kV/cm were applied. HPTS emission was measured on a cytofluorometer in A and B or observed under a digitized microscope in D. A: Mean intensity of cells with increasing amounts of LUVs. Twenty pulses were applied. Different volumes of liposomes were added to 25 μl of cells, the final volume being kept constant. 1: no liposome present; 2: 75 μl of the liposome preparation was added with 75 μl of buffer; 3: 150 μl was added. B: Mean fluorescence intensity of cells for different numbers of successive pulses. 25 μl of cells was mixed with 100 μl of liposomes. C: Phase contrast micrographs of unpulsed cells. D: Fluorescence picture of the cells viewed in C. E: Phase contrast micrographs of pulsed cells. Twenty pulses were applied. F: Fluorescence picture of the cells viewed in E. G: Fluorescence emission plot along the dotted line shown in F.

4. Discussion

The present observations with LUVs bring the conclusion that electropermeabilized cells can fuse with small lipid vesicles as shown by the content mixing assay detected in Fig. 3F. A similar conclusion is obtained with SUVs (Fig. 2C). Metastability of the lipid assemblies is apparently required to obtain the mixing of the vesicle content. No fusion was obtained with MLVs whatever the composition of the lipid matrix or the nature of the electrostatic binding to the cell surface was. The experiments with MLVs were in line with the experiment at the single cell level already published [16], where it was observed that the cell–MLV fusion yield was always very low. The authors suggested that this could be due to the multilamellar nature of the vesicles and that the internal layers may inhibit fusion. Our previous study of the packing of the outer layer in different kinds of vesicles showed that a tightly packed assembly was present in MLVs while a lot of defects were detected in SUVs [17].

A moderate field intensity was used to prevent extensive cell lysis. This makes our approach very different from the enhanced reversible binding previously described [18]. As a result, the field pulse (1.2 kV/cm, 100 μs) was not enough to trigger permeabilization of the lipid vesicle even with the large

MLVs [19–21]. No leakage of HPTS was induced by electropulsation under the present conditions. Our observations cannot be explained by a reentry in the cells of leaked dye. A direct content mixing was indeed present.

These observations provide experimental support to Rosenheck's hypothesis that the electrostatic field due to the surface charge present on the interacting membranes may trigger fusion in exocytosis [7]. In his calculation only one partner was supposed to be electropermeabilized and this was enough to trigger the fusion. This description was clearly valid in the present experiments. Only the plasma membrane was electrically affected, and fusion was observed when the lipid vesicle was metastable. This last condition brought us to the conclusion that this model for exocytosis was valid only if it was assumed that the exocytosis vesicle was metastable, a prediction supported by its high curvature (30 nm for synaptic vesicles, 250 nm for chromaffin granules).

Fusion was detected only when electropermeabilization was induced. As a constant electric field intensity was used in all experiments and as the delay between the pulses was too short for the cell to rotate during the application of the train of pulses, the part of the cell surface that was brought to the permeabilized state was constant in all experiments [12]. An increase in the number of pulses is known to bring an increase in the number of defects in the permeabilized part of the cell [12]. Such an increase in defects may affect the cell viability by making the membrane permeabilization irreversible. Cell fusion was shown to be controlled by the number of pulses [12]. It was proposed that this was linked to the density of defects as long as the cell viability was not affected. The conclusion was that a high density of defects brings a high level of permeabilization and a membrane state highly competent for fusion. The present study is in full agreement with this description in the case of SUVs and LUVs. Vesicle spontaneous fusion with an electropermeabilized cell was facilitated when the membrane state was strongly competent for fusion for a given number of vesicles. This suggests that, within Rosenheck's model, the magnitude of the electrostatic destabilization, by affecting a larger part of the cell surface, will control the probability of fusion in exocytosis. An increase in fusion was obtained when more vesicles were present. This suggests that as the probability of contact between vesicles and cells was increased at high vesicle concentration, there was either not a saturation of the cell surface with vesicles or only transient contacts were present from which only a limited fraction brought about fusion as suggested by the reversibility of docking [22].

As the vesicles were purely lipidic, there was no contribution of a protein scaffold in the present study. We previously showed that mechanical constraints play a positive role in electrofusion [23]. This strongly suggests that, within the present description, exocytosis will be facilitated by the mechanical strain in the contact area due to the SNARE proteins [24].

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