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Rapid Report

Electric field-mediated glycophorin insertion in cell membrane is a localized event

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Purified soluble glycophorin, an intrinsic protein, can be back 'electroinserted' in the membrane of Chinese hamster ovary cells by submitting the cell/protein mixture to short electric field pulses. Previous studies showed that this complex between pulsed cells and proteins, which is detected only when the cell membrane is electropermeabilized, was very stable. This strongly suggested that the protein was indeed inserted in the membrane. The basic processes involved in this phenomena are studied in the present work. The association is observed at the single cell level by means of videoimmunofluorescence. Electric field-mediated insertion occurs firstly in a limited patch of the cell surface, which size is in agreement with the prediction of Electropermeabilization theory. A free diffusion of the inserted proteins then follows on the cell surface. The diffusion coefficient is computed to be less than 10^{-10} cm^2/s as observed for transmembranous proteins. This slow process gives an homogeneous distribution of the inserted protein.

The physics of intrinsic protein membrane insertion remains one of the challenging problems in cell biology. It has been proposed that it is mediated by specific translocators where the transfer of the hydrophilic part of the protein occurs but where the transmembranous hydrophobic segments are stopped. They are then transferred laterally to the lipid matrix as a consequence of a conformational change of the translocator [1]. The inserted protein is then supposed to diffuse freely in the membrane where it remains stably inserted. Two steps are clearly present: a very localized one at the level of the translocator and a second one where the protein diffuses freely in the membrane. This second step supports the homogeneous distribution of the inserted protein. Direct experimental evidences of such a protein insertion process are still missing.

Therefore, artificial implantation of xeno-proteins in cell membrane has been used to give such an experimental approach to this problem. In the most successful approaches, i.e., virus or liposomes fusion to the recipient cell, insertion is due to the local event of fusion but again a free diffusion of the newly inserted protein should occur to give an homogeneous distribution [2,3]. One should mention that nevertheless, up to now, this second step has never been detected and an homogeneous protein labeling has always been observed on the cell surface. Ability of 'inserted' proteins to diffuse was supported by the induction of patching when antibodies were added.

Very recently, a new physical approach for integral protein insertion was described. When a glycophorin A (or CD4)/red blood cells mixture was submitted to an electric pulse, a stable association (insertion) was observed [4–7]. Cell integrity was not affected by the treatment as shown by the very long life-time of the re-injected modified erythrocytes in animals. Glycophorin was indeed shown to be present with, in most cases, its extracellular domain remaining accessible to specific monoclonal antibodies [6]. The long lived stability of the glycophorin/cell complex was suggested to be one strong evidence that indeed insertion was taking place, not a strong adsorption. This consequence of cell electropulsation was called 'electroinsertion' [4–7]. An homogeneous distribution of the electroinserted proteins was always observed on the cell surface but glycophorin was able to be patched when treated with antibodies [6]. No analysis of the diffusion of electroinserted proteins was given.

In a previous study (El Ouagari et al., data not shown), we induced the 'electroinsertion' of glycophorin A in the membrane of nucleated mammalian

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cells, the Chinese Hamster Ovary (CHO) cells. 'Electroinserted' proteins were detected by immunofluorescence. 'Electroinsertion' was only observed when the field strength was larger than a critical value larger than the one needed to trigger Electropermeabilization. This observation was in conflict with the results published on erythrocytes [4]. Viability of cells was not affected as shown by their ability to grow. In the present study, 'insertion' is observed to be a fast process which occurs only locally on the cell surface. Inserted proteins then diffuse freely in the membrane to give an homogeneous distribution on the cell surface. This is the first experimental observation of the two step hypothesis on the protein insertion in the membrane.

Chinese hamster ovary cells (CHO cells) are used in many somatic cell genetics laboratories. The WTT clone, which was given to us by Prof. Zalta (this institute), was selected for the present study due to its ability to grow in suspension under gentle stirring. Cells were grown in Eagle's minimum medium (MEM 0111, Eurobio, France) supplemented with 8% newborn calf serum (Boehringer, Germany), antibiotics, glutamine and tryptose phosphate broth. They were maintained in exponentional growth phase by daily dilution.

Pulsing buffer (PB) was 10 mM phosphate buffer (pH 7.4), 1 mM $MgCl_2$, 250 mM sucrose. PBS was 42 mM K_2HPO_4, 8.3 mM NaH_2PO_4, 125 mM NaCl (pH 7.4). Salts were analytical grade. Solutions were prepared in Milli Q water and sterilized on Sterivex filters 0.2 μm (Millipore, USA).

Cells were carefully washed with the PB in order to work in a low ionic content medium where Joule heating is reduced. Electroinsertion of glycophorin A type MN (Sigma, USA) was obtained by pulsing cells at room temperature, in PB containing 50 μg glycophorin as follows. 18 μl of CHO cells (10^7 cells) in glycophorin containing PB were pulsed with five successive square wave pulses of 0.9 kV/cm and 7 ms applied at a frequency of 1 Hz, by using a CNRS Electropulser (Jouan, France). Cell viability was only slightly affected by the electric treatment as shown by their ability to grow during the days following the pulsation.

Electroinsertion was observed by the following immunoassay. After pulsing, cells were washed three times in PBS. They were then fixed at different delays (0, 1 and 8 h) by paraformaldehyde (1% w/v in PBS). For the long delays, due to their spontaneous plating, cells were bathed in a Chelex-100 treated PBS (Ca^{2+} free) to be brought in suspension before fixation. Cells were then incubated at 4°C during 30 min with 2 μg of antihuman glycophorin A monoclonal antibody (Immunotech, France). Cells were then washed three times with PBS and incubated at 4°C during 30 min with 12.5 μg of a secondary antibody ($F(ab')_2$) goat anti-mouse fluoresceinated IgG (Immunotech, France). The cells were washed three times in PBS and fluorescence labeling was analyzed under a videomicroscope.

Cells were observed under a Leitz Fluovert fluores-

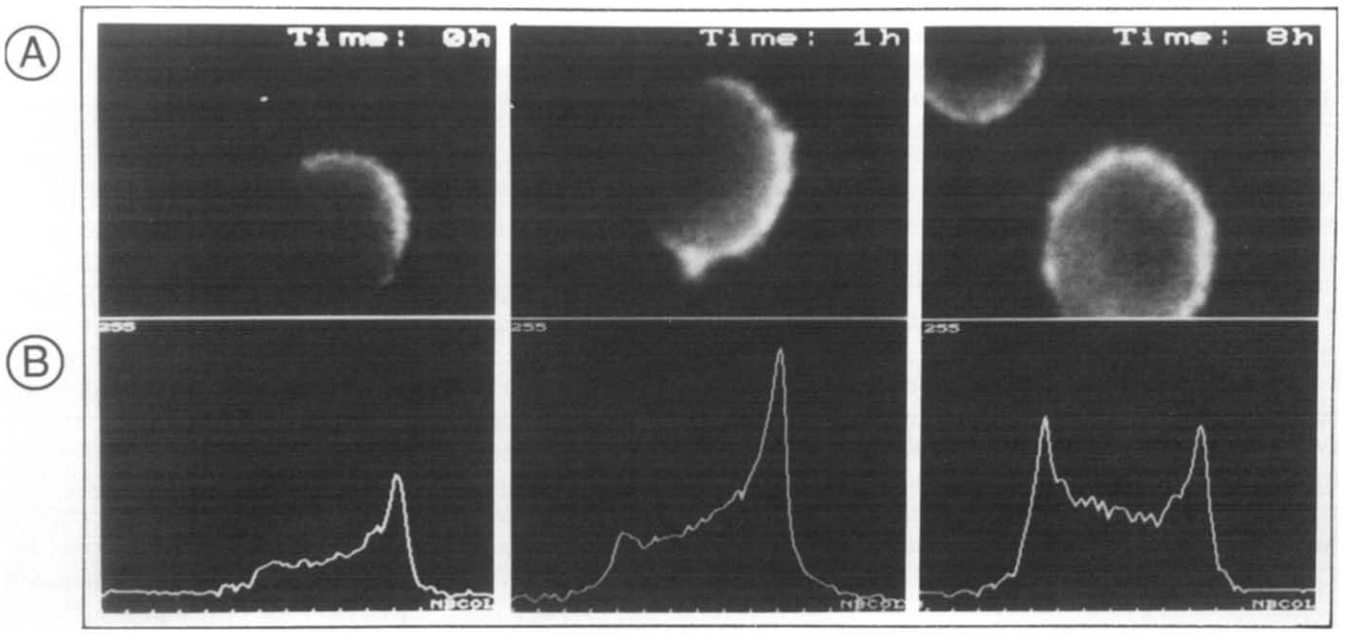

Fig. 1. Video microscopy observation (A) and 1-D light level distribution profiles (B) of the CHO immunolabeling. (A) After electropulsing with glycophorin, CHO cells are labeled as described in Methods. The immunoassay is run just after the pulse, 1 h, or 8 h after it. The contrast of each picture is adjusted by the computer to be the same for all incubation periods. (B) Video pictures shown in part A are digitized and analyzed. Light levels are recorded along the horizontal cell diameter. The emission is high only at the membrane level.

cence inverted microscope. A 63 × oil-immersion objective was used to observe the cells. The light wavelengths were selected by a Leitz H3 filter block. The fluorescence emission was detected by a light-intensifying camera (Lhesa, Pontoise, France) associated with a black-and-white monitor. The video setup was connected a digitizer (Info'Rop, Toulouse, France) driven by a computer (CPU 68010, Motorola, Tempe, AZ). In this way, the video signal was converted to a matrix with 8-bit gray scale, namely, 256 different light levels. The size of these matrix was selected to be 256 × 256. The software library (Trimago, Ifremer, Paris, France) contains the major routines for digital image processing [8]. All digitized pictures were thus corrected from the background signal and from the illumination heterogeneity, as previously described [9].

No immunolabeling is detected with control unpulsed cells with the light amplification setting we use on the camera (data not shown). Under the experimental conditions we use, no morphological damage is detected and cell viability is only decreased by 25% while all pulsed cells are immunolabeled. The observation of the immunofluorescence labeling pattern of the pulsed cells shows that, just after the pulse, glycophorin is located in a small patch on the cell surface (Fig. 1A). This localization can be quantified by use of the 1-D light level distribution profile of the picture (Fig. 1B). The emission intensity of the antibody is strong only on one side of the cell. This is in conflict with a previous report on erythrocytes where a uniform distribution was observed [6]. Electric field mediated insertion is then a localized phenomenon in cultured cells as previously observed with the electropermeabilization process [10]. This is the direct experimental evidence of that was mathematically suggested by the dependence of the electroinsertion on the field strength (El Ouagari et al., data not shown). It was proposed that electroinsertion occurred only in a limited part of the cell surface which organization has been altered by the electropermeabilization process [10].

Observation of the pulsed cells at different delays following the pulse, shows that the immunolabeled part of the cell surface increases with the delay before observation, i.e., sample fixation (Fig. 1A). The asymetry of the labeling which was clearly observed just after the pulse is lost progressively as shown by the emission patterns we computed through the digitizing of the video micrographs (Fig. 1B). An homogeneous labeling of the cell membrane is in fact observed 24 h after the pulse (data not shown).

The annulus shape of the immunofluorescence labeling, observed under the microscope, is characteristic of a membrane localization of the protein. The diffusion is then occurring on the cell surface. The position of the diffusion front can be easily defined and the diffusion process can be quantified as described in

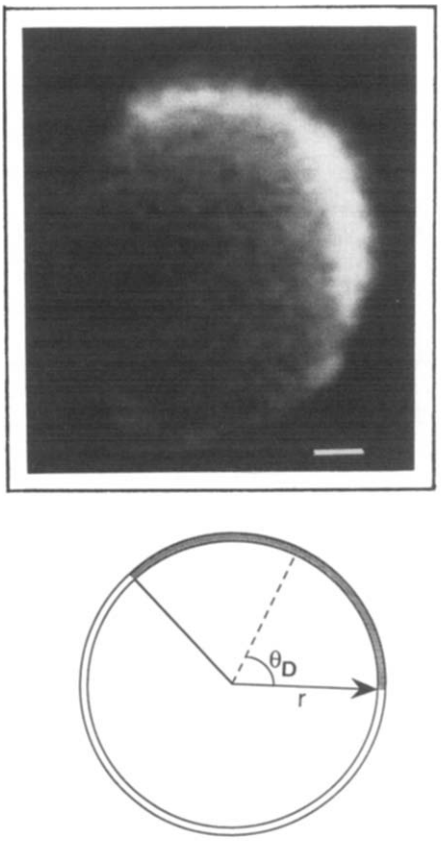

Fig. 2. Definition of $\theta_D(t)$. The diffusion front on the cell membrane is referred in polar coordinates. The dashed area in the cartoon is figuring the labeled part of the cell surface shown in the upper micrograph. A symetric diffusion is assumed to take place.

experiments on the electrofusion of erythrocytes ghosts [11]:

(i) by assuming that the diffusion process is occurring symetrically,
(ii) by taking advantage of the spherical geometry of CHO cells in suspension
(iii) and by measuring the polar angle on the labeled part of the cell surface (Fig. 2). We obtained a time dependence of the characteristic angle $\theta_D(t)$ (the half of the polar angle) by comparing the pattern in many cells for different times before fixation.

As the diffusion is occurring on the cell surface, if the radius of the cell is r ($r = 6.5 \pm 0.2$ μm for CHO cells, sample $n = 300$; this narrow distribution is obtained by the direct videoobservation of the cells under the microscope), we obtain the square of the diffusion length as:

$$l^2 = [r \cdot \theta_D(t)]^2$$

When plotting l^2 as a function of t, we obtain a linear fit with a good correlation coefficient when

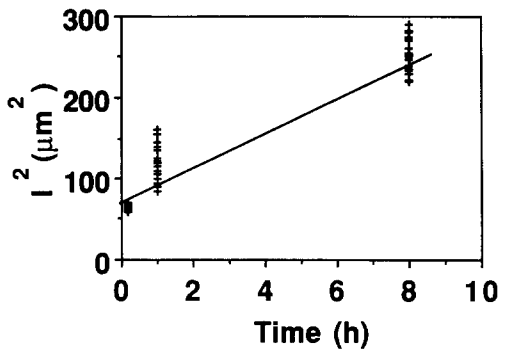

Fig. 3. Dependence of the square of the diffusion length on the post-pulse incubation time. Diffusion lengths are determined for different post-pulse incubation times by a random observation of pulsed cells (sample, $n = 20$ at each time) in the population. The linear fit is computed by using 'linefit'software (Biosoft, UK). A correlation coefficient of 0.98 is obtained by treating the data obtained from the 60 cells observed at the three different post-pulse times.

working with a population of pulsed cells ($n = 3 \times 20$) (Fig. 3). This was predicted by the lateral diffusion theory [16]:

$$l^2 = 4D \cdot t$$

The slope of the linear fit gives a rough access to D, the lateral diffusion coefficient of the electroinserted glycophorin [11]. From our experiments, D can be evaluated as $(0.6 \pm 0.13) \cdot 10^{-10}$ cm^2/s. This is in agreement with what one can expect for the free movement of an intrinsic protein which is not connected to the cytoskeleton [16].

The present study provides the first experimental evidence that electric pulses mediate the membrane insertion of glycophorin and not an increased adsorption. Two sequential steps are clearly present:

(i) an initial fast very localized insertion,

(ii) a slow lateral diffusion on the cell surface from this local patch.

A key phenomenon in this sequence of events consists in the local insertion of the protein. Absorbed glycophorin molecules give a low immunofluorescence background (detected only with a high light amplification) (data not shown) showing that absorbed glycophorin epitopes remain sensible to antibodies (data not shown). But under our experimental conditions (low gain of the video camera), this low background is not detected and the signal, we observe, is specific of 'electroinserted' proteins.

Due to the vectorial character of the electric field, many of its effects are position dependent on the cell surface. Previous results showed that only a very small part of the cell surface is affected by the electropermeabilization [10,12,13]. This patch is proposed to remain immobile on the cell surface [13,14]. The life-time of the permeabilized state is very short under our experimental conditions (post pulse incubation temperature of 37°C) [14]. Such a perturbed part of the cell surface is a good candidate to support spontaneous protein insertion.

We can evaluate the extent of the electrically affected part of the cell surface associated to glycophorin insertion [14]:

$$A = (A_{tot}/2)(1 - E_s/E)$$

where E_s is the threshold field we detect to be needed to induce electroinsertion and E the field intensity we use [15].

This gives us a predicted value (16.5% of A_{tot}) ($E_s = 0.6$ kV/cm; $E = 0.9$ kV/cm) in good agreement with the part, where insertion is detected just after the pulse, that is observed in Fig. 1A ($A = (A_{tot}/2)(1 - \cos \theta_D)$) (about 15.5% from extrapolation to $t = 0$ where θ_D has an average value of 46.2° ($n = 20$)) (One should notice that this value of θ_D is highly reproducible for the different cells in the population as shown by the small S.D. of l^2 in Fig. 3).

From this localized 'reservoir' of inserted glycophorin, we observe that a slow lateral free diffusion process is then occurring, which gives a uniform cell membrane labeling 24 h after pulsing (Fig. 1).

Our observation supports the hypothesis that the localized reorganization of the cell membrane associated to electropermeabilization [14] is responsable for the insertion. Insertion cannot occur later because (i) non-inserted glycophorin molecules are washed out and (ii) no absorbed glycophorin remains bound to the cell surface after washing as shown by the very localized immunofluorescence pattern detected just after the pulse (Fig. 1A). This slow expansion of the glycophorin distribution is the evidence that electric field mediates the insertion of glycophorin, not simply an increased adsorption. If this alternative explanation was true, glycophorin would then diffuse in the bulk phase where its diffusion coefficient is larger than 10^{-6} cm^2/s [16] and this implicates that high-affinity absorption sites, which are present in the electropermeabilized part as shown in Fig. 1, would be still generated on the cell surface hours after the pulses, when the membrane organization is back to normal. This is in conflict with our observations that no immunolabeling is detected if glycophorin is added a long delay after the pulses (data not shown). The lateral diffusion we observe by videomicroscopy is characteristic of the movement of an intrinsic protein. The diffusion coefficient we calculate ($0.6 \cdot 10^{-10}$ cm^2/s) is smaller than the one observed by fluorescence recovery after photobleaching of glycophorin inserted in lipid multilayers above their phase transition [17]. But such a low value is in fair agreement with what can be expected for an intrinsic protein embedded in a viable cell membrane,

where diffusion is hindered by the protein and glycocalix organization [16]. Nevertheless the diffusion is fast enough to suggest that no connection exits between the 'inserted' xenoprotein and the cytoskeleton.

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