EFFECTS OF THE NDV-GLYCOPROTEINS ON THE ELECTROPERMEABILIZATION OF LIPID BILAYERS

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ABSTRACT

Newcastle disease virus (NDV) is an enveloped RNA virus. Certain biological activities of the NDV are determined by two surface glycoproteins –haemaglutinin-neuraminidase (HN) and fusion-protein (F). These proteins play an active role in the virus-cell interaction altering the lipid order in the virus membrane. Liposomes from L-α-phosphatidylcholine with embedded viral components, HN and F, were exposed to an alternating electric field of 120 V peak to peak square electric field waves at three different frequencies of 0.01, 1 and 100 kHz to perturb the liposome permeation. Two independent sets of experiments were conducted: liposomes without viral components, and liposomes with embedded viral components. To monitor the changes in permeation of liposome particles, the leakage of calcium ions from liposomes was measured by recording of the fluorescence emission due to the complex formation reaction with fluorescence dye in the medium solution. The increase of temperature of the solution in the experimental chamber for electropermeabilization was also registered. The frequency dependence of both the leakage of Ca ions and temperature increments was obtained. It was observed that the efficiency of the Ca ions leakage in the both cases has a minimum at 1 kHz. The percentage of leakage in the second experiment increased by a factor of 25% in comparison to the same data valid in the first experiment. The values of temperature increment followed similar frequency dependencies as those for Ca ions leakage with some varieties. There is accumulating evidence to show that higher values of temperature increment correspond to higher values in Ca ions leakage in the presence of HN and F. Experimental similarity between both, as a function of frequency, suggests that the energy dissipation of electric field by the sample is the main reason for these phenomena.

Introduction

Recently numerous studies have been devoted to the effects of electric field (EF) on cell suspensions and artificial lipid membranes (1, 2, 3, 4). It is well known that a bilayer lipid membrane is a good barrier for ions and hydrophilic molecules. Such a permeation barrier is readily modified by imposing a transmembrane electric potential exceeding the dielectric strength of the membrane. In this case the membrane specific conductance increased dramatically, sometimes to values as high as 1 S cm $^{-1}$ in microseconds. This effect is reversible: repetitive voltage scans did not alter the V-I characteristics of the membrane. Nevertheless the information which has already been obtained in these studies concerning the effects of EF on membrane permeability, the process of electroporation of cell membranes as well as related phenomena remain unclear.

Of all such phenomena, by far the most important is the membrane contact which may activate and regulate the membrane "docking" process. It is an essential prerequisite to fusion , but it does not necessarily lead to fusion. How ever, the role of EF

in such phenomena is still obscure. Here we review some data which demonstrate the role of virus components of NDV on the electropermabilization of assembled lipid bilayers to Ca ions. The data show the existence of frequency dependence window at 1 kHz. Furthermore, the data were interpreted according to the theory of interactions of electromagnetic fields with highly polarized and deformed materials such as lipid vesicles (5). The frequency dependence in the presence of embedded virus components in the lipid bilayer, could be also explained as a decrease of the membrane binding rigidity and disordering effect of these proteins (6).

Materials and Methods

Virus. Newcastle disease virus, vaccinal strain La Sota, was grown in 10- day old embryonated chicken eggs. The eggs were incubated at 37 °C for 72 h and the allantoic fluid was harvested. After sedimentation of the cellular debris at 1000 x g for 20 min at 4 °C, the virus in the supernatant was pelleted at 28 000 rpm/h for 30 min. The viral pellet was dispersed in PBS (pH 7.2) and applied to a 5-60% (w/w) sucrose lineal gradient in TNE buffer (pH 7.2) at 28 000 rpm/h for 3 h at 4 °C in Beckman SW 28 rotor. The virus band was collected, dialyzed against PBS and pelleted at 28 000 rpm/h for 90 min at 4 °C.

Purification of the NDV-glycoproteins. The purified virions were suspended in PBS, pH 7.4 containing 2% octyl-glucoside (OG) at room temperature (RT) during 1 h to solubilize glycoproteins. The solubilized glycoproteins were separated from membrane (M)-protein by centrifugation at 30 000 rpm/min for 45 min.

Preparation of samples. For preparation of lipid vesicles liposomes, commercial product of Sigma L-α-phosphatidylcholine from frozen egg yolk was used. Fluorescence marker Quin-2 from Dijondo (Japan) with molecular weight of 693.8 was purchased product. Lipid, 100 mg/ml in 9:1 solution of chloroform/methanol was dried under nitrogen gas flow in a round-bottomed tube. Then required amounts of $CaCl_2$ were added to the dried lipid in 10 ml dH_2O and the mixture was vortexed for 60 min at room temperature. These liposomes were used as controls. The samples of reconstituted lipid assemblies with embedded virus HN and F proteins were formed by solubilization of lipid dried film in 10 ml. 2% octylglycoside solution containing the required amounts of mixture HN and F (total average molecular weight of 132 kD) at molar ratio lipid/virus components 100:1. The samples were then dialyzed against 2 mM $CaCl_2$ for several days to remove the detergent. The residue was resolved in 10 ml destilled water and the mixture was vortexed as above. Control liposomes and such containing virus proteins were characterized for particle size distribution using a dynamic light scattering size meter (Photal Otsuka Electronics LPA 3000m, Japan).

Instruments and electric field treatment. The apparatus for application of EF was described in details in previous paper (6).The voltage applied between the electrodes had an effective value of 80 V peak to peak. The chamber was from a round glass tube with diameter 1 cm and two wire Pt electrodes at a distance of 1 mm. The enhancement of temperature due to the EF was measured before and after EF application by a sensitive miniature thermocouple. EF of 30 seconds duration was applied to the sample in the chamber. The electropermeabilization protocol was as follows: to a part (1.5 ml) of liposomes suspension or reconstituted assemblies respectively, a solution of 1.5ml. of fluorescence dye Quin 2 with concentration 1.10^{-3} M in water was added. The mixture was incubated for several minutes before, just prior to the EF application.

Fluorescence intensities and increments of temperature were then measured for different frequencies of EF. The excitation

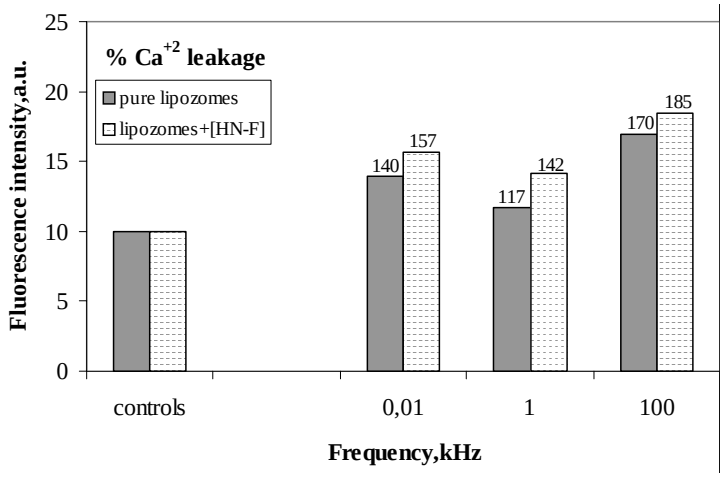

Fig. 1.

wavelength was at 339 nm and detection at 492 nm.

Results and Discussion

Ca ions leakage and effect of frequency

All date related to the leakage of Ca ions were obtained as an increase of fluorescence dye intensity after EF treatment of controls and samples in the chamber at three different frequencies. The histograms at **Fig. 1** showed permanent increase of Ca ions leakage in comparison to controls without embedded virus proteins. The maximal Ca ions release in the individual samples was achieved within 2-3 minutes after the EF application. The frequency dependence of Ca ions leakage at Fig. 1 demonstrates a gradual decrease at frequency of 0.01 and 1 kHz, and then an increase was at 100 kHz. This tendency was obvious in both cases, pure liposomes and liposomes with embedded virus proteins. The data above confirm that the applied EF may affect the process of liposome leakage under the conditions of our experiment. The presence of virus components in the liposomes undoubtedely play an additional role in this phenomenon. Two conclusions can be drawn in this case - the effect of frequency is connected with polarization of liposome particle and this effect is more pronounced in the presence of HN and F.

Effects of HN and F virus components on Ca ions leakage

The maximum changes in the leakage and temperature increment of Ca ions were obtained with reconstituted lipid assemblies containing embedded virus proteins, HN and F, and perturbed with EF at 100 kHz. The significance of the differences between these particles and free liposomes, and electric field frequency may be evaluated as reported firstly in the theory of lipid-protein interactions (7,8) and secondly in the theory of interactions of electromagnetic fields with high polarized and deformed materials as liposome particles. (5). In this paper we shall restrict only to the first, since our chief conclusion is that the leakage of Ca ions from liposomes is a result of the EF- induced transmembrane potential. The heating of the solution in the chamber is due to the high electroconductance of the medium.The leakage of Ca ions through the membrane into chamber increased the ionic strength and osmolarity of the medium. Experimental similarity between the fluorescence measurements characterizing the Ca ions leakage and those of increment of temperature as a function of frequency, suggests that the energy of dissipation of EF by the sample

is the main reason for those phenomenon. Then, we will explain the differences in the data in the presence of HN and F in the liposomes. It is quite conceivable that the organization of lipid assemblies may be affected by the presence of HN and F in the bilayer to a significant extent. A number of theoretical models and explanations have put forward to provide a consistent interpretation of similar results. An adapted explanation for the case when external EF is applied concerns the appearance of fusion pores and channels during the application of EF. They open instantaneously and have strictly defined levels of conductance. Further, it is well known that lipid bilayer fusion is very sensitive to the spontaneous curvature and deformation of lipid membrane. Proteins can interact with lipids to change membrane spontaneous curvature and some membrane functions as membrane permeability. In the course of EF application these processes are to the highest degree. Assimilating the body of knowledge on possible role of HN and F in this phenomenon, we may add that membrane surface proteins increase significantly the pore stability so that they can be stable for seconds and longer. Taking in account this fact we kept the time of EF application within seconds. The proteins in one membrane can also promote a topological rearrangement of all membrane components and structures, influencing the membrane functions. Interpretation of such phenomena in terms of protein-membrane interactions leads naturally to certain implications and consequences. Thus the role of HN and F on the electropermeabilization of liposome particles probably contribute potentially to an increase of new research interests. At molecular level these dependences involve the changes in membrane curvature and deformation of lipid bilayer due to the EF. The last may be significantly influenced in the presence of HN and F in the bilayer. The well known hypothesis regarding the possible role of frequency in membrane electropermeabilization is that at lower values the EF dependent processes on the lipid membrane are not so intensified. In our experiment it is valid for frequencies of 0.01 and 1 kHz as the electroporation has not yet reached its maximum value. At values above 1 kHz the EF dependent processes on the membrane surface and inside in the particle will perturb the membrane either through interaction with surface charges, dielectric forces or local heating due to the highly promoted membrane currents. In the case of local heating a breakdown of the membrane may be observed which leads to a significant increase in liposome leakage.

Joule-heating of the chamber solution

A rapid thermal heating from 5-7 °C of the solution in the chamber was recorded after the EF application. In **Fig. 2** dependence of temperature increment $\Delta T = T_2 - T_1$ are given as a function of frequency in two separate experiments 1 and 2, where T_2 is the measured temperature after EF application and T_1 is the initial temperature in the chamber. As can be seen there is a correlation with the results found for Ca ions leakage at different frequencies. In Fig. 2 the values of temperature increment for the sample (experiment 2) are higher than those for control (experiment 1) in all cases, but the shape of both dependencies is similar. The values of ΔT decreased gradually with increasing the frequency from 0.01 to 1 kHz. The changes are negligible (about 1-1.5 °C or less). More obvious changes were registered at a frequency of 100 kHz. The values of increment in experiment 2 are about 4 °C higher than those in experiment 1. These results show a similarity with the results for Ca ions leakage measured by fluorescence intensity changes as a function of EF frequency. There is accumulating evidence to show that the higher values of temperature increment correspond to higher values of Ca ions leakage, as can be seen from Figs 1 and 2., but could not found a correlation

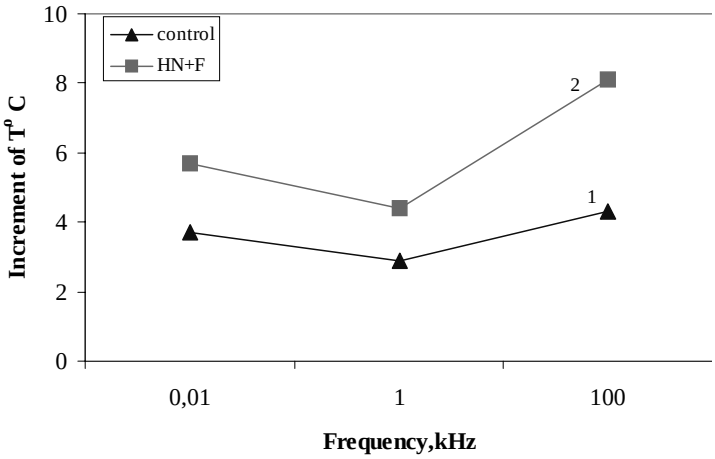

Fig. 2

between the percentage of Ca ions leakage and temperature increment.

REFERENCES

1. **Hubiniec R.T., Liang H., Hui S.W.** (1990) Biotechniques, **B.**, 16-21.

2. **Hibino M., Shigemori M., Itoh H., Nagayama K., Kinosita K.** (1991) Biophys. J., **59,** 209-220.

3. **Golzio M., Mora M.P., Raynaud C., Delteil Ch., Teissie J., Rols M.P.** (1998) Biophys. J., **74,** 3015-3022.

4. **Rols M.P., Teissie J.** (1990) Biochemistry, **29,** 4561-4567.

5. **Dumey C., Christensen D.** (1999) In: Basic introduction to bioelectromagnetics. Ch. 1,7 Adsorption energy. (C. Dumey, Ed.), CRC Press. Boca Raton London, New York, Washington, D.C., 10-13.

6. **Neitchev V., Terezova N., Matsumura H., Tomov T.** (2002) Histol. and Histopathol., **17,** 649-656.

7. **Mouritsen O., Biltonen R.** (1992) In: Comprehensive Biochemistry. (A. Watts, Ed.), Protein-lipid Interactions, Elsevier Scientific Publishers, 12-28.

8. **Zimmerberg J., Vogel St., Chemomordik L.** (1993) Ann. Rev. Biophys. Biomol. Struct., **22,** 433-466.