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Electrostimulated uptake of DNA by liposomes

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High molecular mass DNA was efficiently taken up by large unilamellar vesicles exposed to a short pulse of electric field (0.1–1 ms) with an intensity as high as 12.5 kV/cm. The efficiency of uptake increased significantly in presence of Mg^{2+} ions and was approximately 0.6 and 1.5 μg of DNA per μmol of lipid for T7 DNA and plasmid pBR 322, respectively. The results presented indicate that DNA was taken up as a result of the electrostimulated formation of endosome-like vesicles rather than via field-induced membrane pores.

1. Introduction

The introduction of DNA into procaryotic and eukaryotic cells by electroporation is widely used, both for obtaining stable genetic transformants and for the investigation of transient gene expression [1–3]. Yet, the mechanism of the voltage induced uptake of DNA by cells remains unclear. It is commonly suggested that DNA crosses cell membranes through pores induced by the electrical treatment of the cells [4,5]. However, this model has been challenged by the data indicating that the characteristic diameter of the pores does not usually exceed 5–7.5 nm [4–7].

In the present study the unilamellar vesicles were emloyed as a simple model system to study electrostimulated DNA uptake. It was shown that large unilamellar vesicles (LUVs) efficiently take up high molecular mass DNA when exposed to a short, high intensity electric pulse. Evidence is presented that DNA molecules are taken up into endosome-like vesicles and this process is related neither to the heating of LUVs suspension by electric pulse nor to fusion of the liposomes.

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; EtB, ethidium bromide; LUV, large unilamellar vesicle; DNA_s, sonicated T7 DNA; DNA_n, native T7 DNA; DNA_p, plasmid pBR 322; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NBD, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl); Rh, Rhodamine.

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2. Materials and Methods

We used dipalmitoylphosphatidylcholine (DPPC) from Calbiochem, cholesterol, fructose and EDTA-Na from Serva, ethidium bromide (EtB) and Kieselgel 60 for TLC from Merck, Sepharose 4B-CL and Sephadex G-75 (fine) from Pharmacia, Tris and agarose from Sigma, [α-^{32}P]dNTP (specific activity 1000 Ci/mmol) from Isotope (U.S.S.R.). [^{14}C]Fructose was purchased from Amersham. *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE) were purchased from Avanti Polar Lipids. Sucrose and inorganic chemicals were of analytical grade. Bidistilled water was used to prepared all solutions.

Phage T7 DNA was isolated and sonicated as described in Ref. 8. Native (DNA_n) and sonicated (DNA_s, mean molecular mass 25 kDa) T7 DNA were ^{32}P-labeled using consecutively 3-exonuclease and polymerase activities of DNA-polymerase 1 (Klenow fragment) [9]. Plasmid pBR 322 (DNA_p) was isolated, linearized and ^{32}P-labeled using standard techniques [9]. [^{32}P]DNA_n and DNA_p were analyzed by electrophoresis in a 0.7% agarose gel and were shown to migrate as a single band.

LUVs were prepared following the reverse-phase evaporation technique described in Ref. 10 with few modifications. The starting emulsion contained 34.3 mg of a DPPC/cholesterol mixture (molar ratio, 7 : 3), 3.1 ml of buffer 1 (130 mM sucrose, 10 mM Tris-HCl, pH 7.5) and 6.2 ml of diethyl ether. No additional aqueous phase was added during the preparation of LUVs. The resulting suspension of LUVs (3 ml) was diluted 4 fold with buffer 2 (65 mM NaCl, 1 mM EDTA, 10 mM

Tris-HCl, pH 7.5), the LUVs were sedimented by centrifugation (14000 × *g*, 6 min) and the pellet was resuspended in 1 ml of buffer 1. The mean diameter of the liposomes was approx. 0.5 μm, assessed by electron microscopic evaluation [13].

A high-voltage generator producing single quasirectangular impulses was designed for applying the electric field. A sample: 60 ml of buffer 1 containing LUVs (1.5 μmol of DPPC); labeled and unlabeled DNA (22 μg DNA_s, 6 μg DNA_p or 3,5 μg DNA_s) or fructose (0.5 mM); $MgCl_2$ (where indicated), was placed at room temperature in a cylindrical Teflon chamber with a 4 mm gap between two stainless electrodes (4 mm diameter). To control the temperature increase of the suspension of LUVs in buffer 1 inside the chamber resulting from the applied electric field, we used the measurement of the resistance decrease of the chamber from the initial value of 5.7 kOhm after application of voltage pulse and the corresponding calibration curve (the dependence of sample resistance on the temperature). The value of the electrically induced temperature jump by this procedure was similar to the results of the simple estimation of sample heating.

After field application, a 55-μg aliquot was withdrawn from the sample, diluted 15-fold with buffer 2 and LUVs were sedimented by centrifugation (14000 × *g*; 2 min). The pellet was resuspended in buffer 2 (1 ml), the LUVs were resedimented and the radioactivity of the pellet was measured.

In another variant the resulting pellets were resuspended in buffer 1 (2 ml) and EtB was added to the samples to a final concentration of 2.5 μM. The fluorescence of the DNA-EtB complex prior to and after sonication of the samples was measured for wavelengths from 540 to 640 nm (λ_{em} = 520 nm) using a spectrofluorimeter MPF-4 (Hitachi). Sonication of LUVs was carried out in an ice/water bath for 1 min using an ultrasonicator UZDN-2T (U.S.S.R.).

In some experiments liposomes were electrically treated in presence of DNA and washed as usual. Then DNase 1 (Sigma) was added to the samples (final concentration 50 mg/ml). The liposome were incubated with DNase I (30 min at 20 °C) in 10 mM Tris-HCl buffer (pH 7.5) containing 130 mM sucrose and 0.5 mM $MgCl_2$. Then EDTA (final concentration 5 mM) and SDS (final concentration 2%) were added to the incubation mixture. After protein extraction by phenol, gel filtration on Sephadex G-75 was carried out in 10 mM Tris-HCl buffer containing 1 mM EDTA.

To establish whether the electric field could induce the fusion of LUVs under the experimental conditions used, we used a NBD/Rh fluorescence assay for monitoring the mixing of membrane lipids [11]. Two populations of LUVs, containing, respectively, 0.5 mol% *N*-NBD-phosphatidylethanolamine or 0.5 mol% *N*-Rh-phosphatidylethanolamine, were mixed in a 1 : 1 ratio. Then the fluorescence of *N*-Rh-label was measured at 590 nm after excitation of the *N*-NBD label at 450 nm.

3. Results and Discussion

Fig. 1 shows the relationship between the irreversible binding of DNA_s (curve 1) or DNA_n (curve 2) to the LUVs and the intensity of the electric field applied. The strong treatment resulted in binding of a considerable portion of the DNA to the LUVs. Electrophoresis of the bound DNA_n in a 0.7% agarose gel and examination of the DNA for the presence of single-stranded regions with the use of Tb^{3+} as a probe [12], demonstrated that the binding of DNA to the LUVs was not accompanied by its destruction and/or denaturation.

The electrical treatment of the suspension resulted in an increase of the sample temperature (4 °C for a 0.3 ms pulse of 12.5 kV/cm). To determine whether the heating of the suspension can contributed to the DNA binding stimulated by electric field, we measured the binding at different temperatures. No irreversible binding of DNA by LUVs was observed after a 10 min incubation of the suspension at the temperatures as high as 60 °C, without electrical treatment.

To localize the bound DNA, fluorescent dye, EtB, which does not significantly penetrate liposome membranes, was used as a DNA-specific probe [13]. Fig. 2 shows that the addition of EtB to the field treated LUVs after separation of the LUVs from free DNA, was not followed by the appearance of the specific DNA-EtB complex fluorescence (curves 1 and 2). The fluorescence was developed only after sonication of the LUVs in the EtB solution (curve 3). Therefore the DNA bound by electrically treated liposomes can not interact with EtB present in the external solution. In addition bound DNA was not accessible to DNase 1 activity (Fig. 3). After the electrical treatment of LUVs in

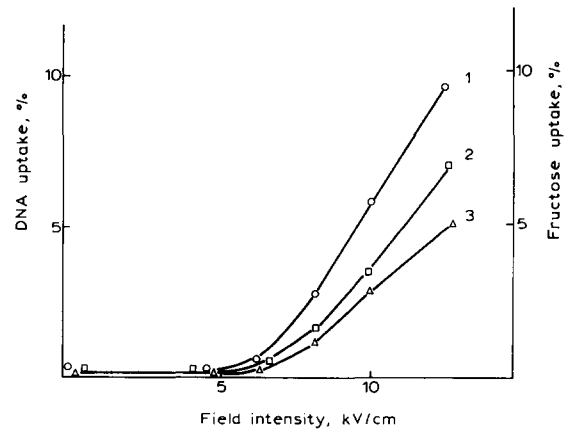

Fig. 1. The DNA_s (curve 1), DNA_n (curve 2) and fructose (curve 3) uptake by electric field-exposed LUVs. The electric pulse of 0.3 ms duration was applied to samples containing [^{32}P]DNA or [^{14}C]-fructose.

presence of DNA_n, the LUVs were washed by the standard procedure to remove the unbound DNA and then incubated with the enzyme. The gel filtration profile of DNA was determined (curve 1) and compared to that obtained in absence of DNase 1 in the medium (curve 2). Another control curve (curve 3) shows the profile of products of DNA digested by the enzyme when DNA was introduced into the suspension of electrically treated liposomes just before DNase 1. The similarity of gel filtration profiles 1 and 2 indicates that irreversibly bound DNA is insensitive to DNase 1. It was shown in Ref. 13 that the adsorption of DNA onto liposomes induced by divalent cations does not prevent the interaction of DNA molecules with EtB and DNase 1. Thus the most probable interpretation of the inaccessibility of the DNA bound by liposomes after electrostimulation to EtB or to DNase 1 is that the location of the bound DNA is inside the LUVs.

The special experiments have shown a sharp electroinduced increase of LUVs membranes permeability for water-soluble tracer molecules ([^{14}C]fructose) in the same range of field intensities as that of DNA uptake (Fig. 1, curve 3). A simple estimation based on the Maxwell equation [4] gives for the maximal voltage V, applied to the membrane of liposome (radius, r, 0.25 μm) for the field intensity, E, 12.5 kV/cm the value $V = 1.5 \cdot E_r \approx 0.45$ V. At similar voltages the electroporation of LUVs was observed by others [14,15]. However, the value obtained is significantly lower than the membrane voltage at breakdown ≈ 1.5 V obtained in Ref. 16. The difference can be related to the shorter duration of pulses (60 ms) used by these authors. There is also the possibility that in Ref. 16 the later stage of the electropermeabilization process was monitored since the direct visual control of vesicle shape was used instead of permeability measurements.

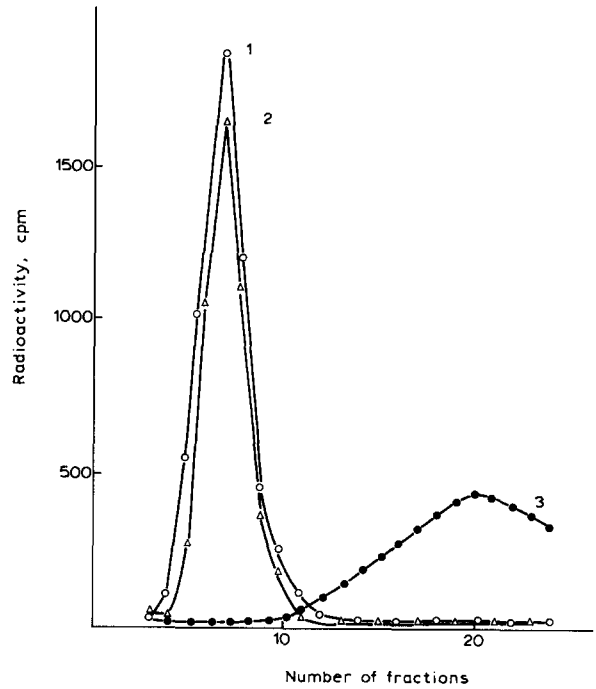

Fig. 3. Insensitivity of bound [^{32}P]DNA_n to DNase 1. Curve 1, the gel filtration profile of products of bound DNA digest by DNase 1. The control incubation mixture contained no DNase 1 (curve 2). In the other control (curve 3) 0.3 μg of [^{32}P]DNA_n was introduced into LUVs suspension 5 min after electric treatment and just before DNase 1.

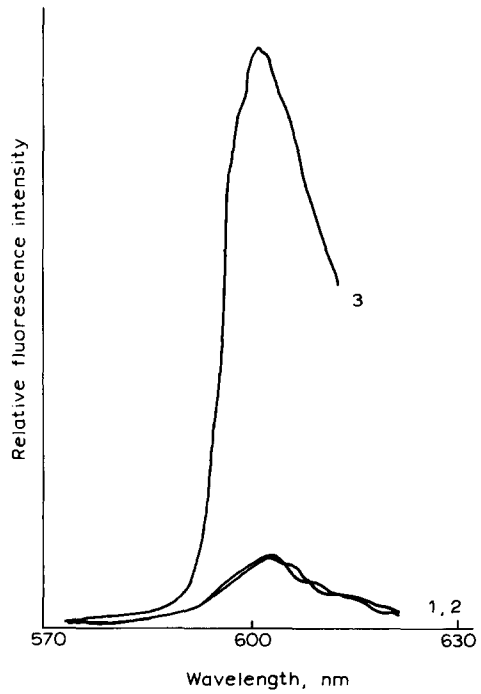

Fig. 2. Inaccesibility of irreversibly LUVs-bound DNA to EtB. Field-exposed LUVs (12.5 kV/cm; 0.3 ms) were separated from unbound DNA. Fluorescence spectra were registered immediately after addition of EtB to the samples to the concentration of 2.5 μM (1), after 10 min incubation with EtB (2), after sonication of the samples in the presence of EtB (3).

Thus there are electroinduced pores in membranes of LUVs as well as of cells when the DNA molecules cross the membranes. According to the model of pore-mediated DNA uptake, DNA, on its route into LUVs, should be accessible to water-soluble agents if they are present inside the LUVs prior to field application. To verify this hypothesis, we used LUVs prepared in buffer 1 containing EtB. The special calibration experiments showed that field application (12.5 kV/cm; 0.3 ms) to the LUVs (in the absence of DNA) resulted in the release of about 50% of the internal EtB to the medium. Then, the voltage was applied to the EtB-containing LUVs in the presence of DNA (Fig. 4). The treated LUVs were separated from external DNA and EtB by the standard washing procedure and the fluorescence spectrum of EtB was monitored. The specific fluorescence of the DNA-EtB complex appeared only after sonication of the LUVs. If DNA had entered the liposomes through

the pores which did not release EtB resulting from electrostimulation, then the specific fluorescence of the EtB-DNA complex would have been detected before the membranes were disrupted by ultrasound. It is possible that the DNA molecules enter through the pores into the liposomes releasing EtB simultaneously. However, this interpretation is not supported by the data since DNA would have to interact with EtB while passing through the pores and according the calibration experiments the fluoresence of the DNA-EtB complex would be detected before sonication of the liposomes. Therefore the results presented in Fig. 4 point out that the electrostimulated DNA uptake is accompanied by spatial isolation of the DNA both from the interliposomal medium and from the intraliposomal space.

The experiments involving of the NBD/Rh fluorescence assay showed the absence of the mixing of membrane lipids between two populations of liposomes after electrical treatment of LUVs suspension in presence of DNA (data not shown). This result suggests that electroinduced DNA uptake is not accompanied by fusion of liposomes [11].

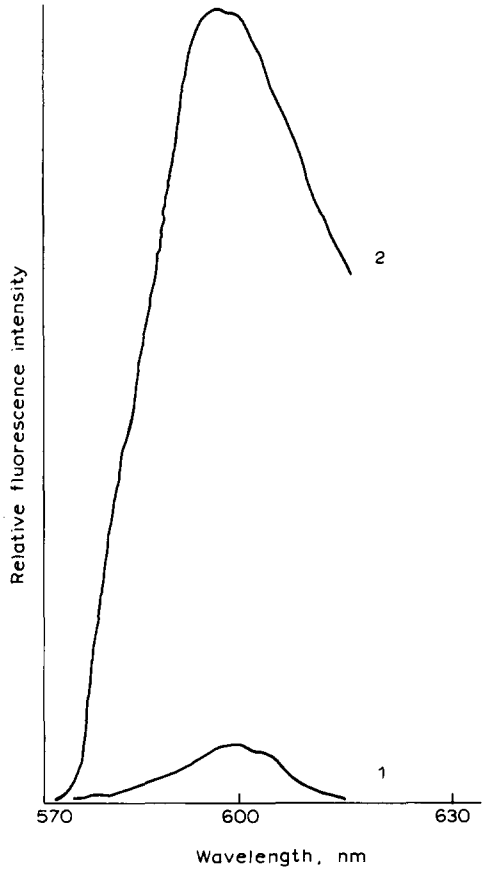

Fig. 4. Field-induced irreversible binding of DNA to LUVs containing 0.2 mM EtB. The external EtB was removed by precipitating the LUVs twice in buffer 2. The resulting pellet was resuspended in buffer 1. The LUVs were treated electrically (12.5 kV/cm; 0.3 ms) in the presence of DNA_s and the fluorescence spectra were registered prior (1) and after LUVs sonication (2).

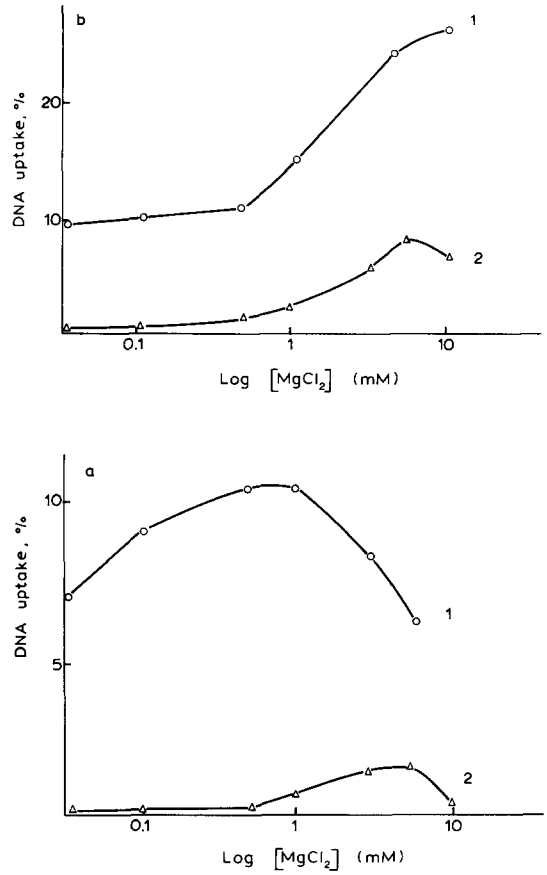

Fig. 5. The electrostimulated DNA_n or DNA_s (b) uptake by LUVs in the presence of Mg^{2+} ions. Curve 1, field-exposed LUVs (12.5 kV/cm; 0.3 ms); curve 2, electrically untreated LUVs.

Evidence was presented earlier that the divalent-cation-mediated adsorption of DNA onto LUVs results in the translocation of a part of the adsorbed DNA into the interior of the LUVs via the formation of DNA-containing endosome-like vesicles which bud off towards the interior of the LUVs [13,17]. This model was substantiated by experiments using the DNA-specific fluorescence probe (EtB) as well as membrane markers and electron microscopy. The DNA adsorption onto LUVs proved to be a necessary step in the uptake process [13,17]. Electrostimulated DNA uptake may occur by a similar mechanism consisting of two steps: field-induced adsorption of DNA onto LUVs and internalization of DNA binding membrane regions.

It is important that electric treatment can promote the uptake of preadsorbed DNA as well. For LUVs treated by an electric field in the presence of $MgCl_2$, the uptake efficiency was much higher (especially for DNA_n) than that without field application (Fig. 5). It should be noted that 95% of the DNA in the samples was adsorbed onto the LUVs at $MgCl_2$ concentration > 1 mM (data not shown). The divalent cation media-

ted DNA uptake observed in LUVs even without the electrical treatment (curves 2) was investigated in [13,17].

The DNA uptake efficiencies obtained by electrical treatment (3.7 μg; 1.5 μg of 0.6 μg of DNA per μmol of lipid or DNA_s, DNA_p and DNA_n) are similar or even higher than those achieved by the methods published previously [18,19]. Thus the electroinduced loading of LUVs seems to be a promising approach for obtaining stable LUVs containing high molecular mass DNA.

Estimations based on the applied field strengths in the medium [4] show that the electrostimulated DNA uptake by LUVs occurs at nearly the same membrane voltages as the electrostimulated DNA translocation across cell membranes [2,3]. For LUVs, the DNA molecules are apparently taken up within endosome-like vesicles which budd off into the liposomes. The applicability of relative mechanism to the case of cell membranes is still in question.

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References

1 Neumann, E., Schaeber-Ridder, M., Wang, J. and Hobschneider, P.H. (1982) EMBO J. 1, 841–845.
2 Chu, G., Hayakawa, H. and Berg, P. (1987) Nucleic Acids Res. 15, 1311–1326.
3 Cymbalyuk, E.S., Chernomordik, L.V., Broude, N.E. and Chizmadzhev, Yu.A. (1988) FEBS Lett. 234, 203–207.
4 Zimmermann, U. (1982) Biochim. Biophys. Acta 694, 227–277.
5 Sowers, A.E. and Lieber, M.R. (1986) FEBS Lett. 205, 179–184.
6 Chernomordik, L.V., Sukharev, S.I., Popov, S.V., Sokirko, A.V. and Abidor, I.G. (1987) Biochim. Biophys. Acta 902, 360–373.
7 Escande-Geraud, H.L., Rols, H.P., Dupont, M.A., Gas, N. and Teissié, J. (1988) Biochim. Biophys. Acta 939, 247–259.
8 Budker, V.G., Godovikov, A.A., Naumova, L.P. and Slepneva, I.A. (1980) Nucleic Acids Res. 8, 2499–2515.
9 Maniatis, T., Fritsch, I.E. and Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
10 Düzgünes, N., Wilschut, J., Hong, K., Fraley, R., Perry, C., Friend, D.S., James, T.L. and Papahadjopoulos, D. (1983) Biochim. Biophys. Acta 732, 289–299.
11 Topal, M.D. and Fresco, J.R. (1980) Biochemistry 19. 5531–5537.
12 Hoekstra, D. (1982) Biochemistry 21, 2833–2840.
13 Budker, V.G., Sokolov, A.V., Weiner, L.M. and Krainev, A.G. (1987) Biolog. Membr. 4, 55–66.
14 Harbich, W. and Helfrich, W. (1979) Z. Naturforsch. 34A, 1063–1065.
15 Teissié, J. and Tsong, T.Y. (1981) Biochemistry 20, 1548–1554.
16 Needham, D. and Hochmuth, R.M. (1989) Biophys. J. 55, 1001–1009.
17 Budker, V.G., Kiseleva, L.V. and Sokolov, A.V. (1987) Biolog. Membr. 4, 1201–1208.
18 Itany, T., Ariga, H., Yamaguchi, N., Tadakuma, T. and Yasuda, T. (1987) Gene 56, 267–276.
19 Szelei, J. and Duda, E. (1989) Biochem. J. 259, 549–553.