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EVIDENCE FOR EARLY FLUIDITY CHANGES IN THE PLASMA MEMBRANES OF INTERFERON TREATED L CELLS, FROM FLUORESCENCE ANISOTROPY DATA

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SUMMARY : A significant increase of the plasma membrane fluidity is observed in L cells upon treatment with mouse β interferon, by the means of fluorescence anisotropy measurements of DPH. The effect is dose dependent and, at the difference with previously reported membrane effects of interferon, is an early one (maximum for a 30 min. treatment), and may be directly related to the initiation of antiviral activity.

When interferon interacts with responsive cells, it is known to bring about various effects (1,2), the most extensively studied among them being the development of antiviral activity. Evidence has been provided that the antiviral state is initiated by a high affinity binding of interferon to specific cell surface receptors (3,4). The subsequent metabolic response, that takes place within 30 minutes to several hours, consists of the synthesis of new "antiviral" proteins (5). Throughout the whole process interferon does not penetrate intracellularly (4,6). This raises the question of whether transmission of such a specific message from the cell surface to the nucleus involves signal amplification across the plasma membrane, possibly leading to detectable changes in the bilayer structure. Interferon has been reported to induce a number of modifications on the cell surface and in the plasma membrane (reviewed in 7), but until recently only a few of them (8,9) have been related from a kinetic point of view to the antiviral activity. Our purpose was to use fluorescence anisotropy measurements of a lipophilic probe, 1,6-diphenyl-1,3,5 hexatriene (DPH), as a sensitive tool (10) to

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monitor possible early plasma membrane fluidity changes that would accompany the establishment of antiviral activity. In the course of these experiments, two related papers appeared, mentioning membrane fluidity changes resulting from interferon treatment. One of these papers (11) describes chemical modifications in the membrane phospholipid composition of interferon treated mouse S 180 cells ; the other (12) reports interferon induced time dependent fluidity changes in the plasma membranes of He-La cells and of L 929 mouse fibroblasts, revealed by E.S.R. spectroscopy. At the difference with these papers concerned mainly with late effects of interferon we here present results showing that mouse β interferon induces *early* fluidity changes in plasma membranes of mouse L fibroblasts, which may match the kinetics of antiviral activity.

MATERIALS AND METHODS

Cell cultures : Mouse L fibroblasts were grown to confluency as monolayer cultures in Eagle's minimum essential medium (MEM) (BIOMERIEUX) supplemented with 10 % foetal calf serum (Flow Laboratories) in 175 cm^2 Nunclon (Nunc) flasks.

Interferon : Mouse fibroblast β-interferon with a specific activity of 2×10^7 units/mg of protein prepared from mouse C 243 cells (13), as well as the corresponding mock interferon (a preparation at the same purity level without inducing virus) were generous gifts from Dr. M. Tovey and Dr. I. Gresser (Institut de Recherche sur le Cancer, Villejuif France). The antiviral activity of the interferon was checked by plaque reduction assay on mouse L cells, challenged with vesicular stomatitis virus.

Interferon treatment : At confluency the cell medium was quickly replaced by fresh medium containing interferon at concentrations of 10^3 or 10^2 units/ml. For kinetic measurements it is important that the temperature does not drop markedly below 37°C during the procedure (14). Control cells were processed the same way with the appropriate concentration of mock interferon. In both batches the number of treated cells was about 8×10^7. The cells were then incubated at 37°C under sterile conditions for varying periods from 15 to 120 minutes. At the end of the incubation time the cell layers were washed thoroughly 3 times with 0.05 M Tris HCl pH 7.8 at 4°. This treatment is not expected to dissociate the bound interferon from its receptors (4) but is believed to stop any further metabolism.

Plasma membrane preparation : In intact living cells the fluorescent probe DPH presents no specificity for plasma membranes, but has been shown to partition into all the lipid regions (15). Then studying changes

in plasma membranes, it was thus necessary to isolate these membranes and further incorporate DPH into the purified material. For this purpose fractionation by an aqueous two phases polymer system was used, according to the procedure described by Brunette and Till (16). One of the features of this method consists of a pretreatment of the cells with a 3 mM $ZnCl_2$ solution in order to stabilize the membranes ; this step has been suppressed to avoid additional unwanted cell surface modifications. Experimental conditions were also changed. The cell layers were gently scraped (Costar disposable scrapers) off the container wall. The cell pellet from a 500 g (10 min.) centrifugation was washed with 0.05 M Tris HCl pH 7.8 and subjected to cell rupture in a tightly fitting Dounce homogenizer until about 97 % of the cells were broken (60 up and down strokes). After a 200 g (10 min) centrifugation to remove whole cells and nuclei the supernatant was centrifuged at 4000 g (10 min) and the resulting pellet suspended at the top of the two phases system (Polyethylene-glycol 6000 Merck, Dextran 500 Pharmacia). The two phases were mixed and centrifuged at 3000 g (10 min) in a Sorvall SS 34 angle rotor. Plasma membranes assemble at the interface. This step was repeated twice. After collection of the material at the interface, the membranes were sedimented at 12 000 g (10 min.) in 0.05 M Tris HCl pH 7.8 and washed twice with the same buffer. The method yields 1 % (in proteins) of plasma membranes with an acceptable level of purity. The average enrichment in the ouabain-sensitive Na^+/K^+ ATPase is seven-fold, whereas the mitochondrial contamination as determined by the cytochrome C oxydase specific activity, represents 20 % of the initial specific activity. Enzymes were assayed as in (16) and the protein determinations were done by the method of Bradford (17), using bovine serum albumine as standard. The advantage of the two phases method is its rapidity which allowed the fluorescence anisotropy measurements to be performed as soon as 4 hours after the interferon treatment.

DPH incorporation and sample preparations : After Potter homogeneization the membrane suspensions were incubated at 4°C with gentle stirring for one hour with DPH (Kochlight) in tetrahydrofuran solution, at a final concentration of 10^{-6}M (1 mole DPH for 10^3 moles of membrane phospholipids). Aliquots of each sample were brought to the same level of turbidity by addition of 0.05 M Tris HCl buffer, in order to equalize the contribution of depolarization due to light scattering (18). The turbidity of the samples was routinely adjusted to 0.200 $\pm$ 0.005 absorbance units at 340 nm (excitation wavelength of DPH). It has been checked that a variation of turbidity corresponding to values as high as 0.05 OD units did not result in any detectable variation of the measured anisotropy of DPH. It has been checked also, by measurements at different concentrations of the membrane suspensions, that the factor turbidity has a negligible contribution to the uncertainty of the results. Interferon treated samples only differed from the mock samples by interferon molecules bound to their receptors : the related contribution of this phenomenon to light diffusion is quite negligible since the protein concentration is less than 10^{-6} that of the membrane preparation, i.e. less than 10^{-7} mg/ml in the measure cell.

Fluorescence anisotropy and DPH fluorescence lifetime measurements : The steady state fluorescence anisotropy is defined as

$rs = \frac{I_{//} - I_{\perp}}{I_{//} + 2I_{\perp}}$, where $I_{//}$ and $I_{\perp}$ are the polarized emission intensities (425 nm) respectively parallel and perpendicular to the polarization direction of the excitation light (340 nm), of the DPH probe, embedded in the membrane samples. rs was measured at 20°C under

continuous illumination using a SLM 8000 SC spectrofluoropolarimeter. Interpretation of rs values imposes the knowledge of the fluorescence lifetime τ (see below). The τ measurements were performed by the single-photon counting technique (19). A computer non-linear regression least square analysis was used for deconvolution and parameter calculation.

It is particularly important that in all the experimental procedure the interferon-treated and the control samples were handled in strictly the same manner.

RESULTS AND DISCUSSION

rs variations : The relative differences $\frac{\Delta rs}{rs\ average}$ % between the values of rs in the mock-interferon-control samples and in the interferon-treated samples, have been plotted against the duration of incubation (figure 1). The results significantly show that interferon induces an early and transitory decrease of the fluorescence anisotropy of DPH, peaking at 4 % after 30 minutes incubation. The intensity of the effect appears to depend upon interferon concentration. Error bars represent

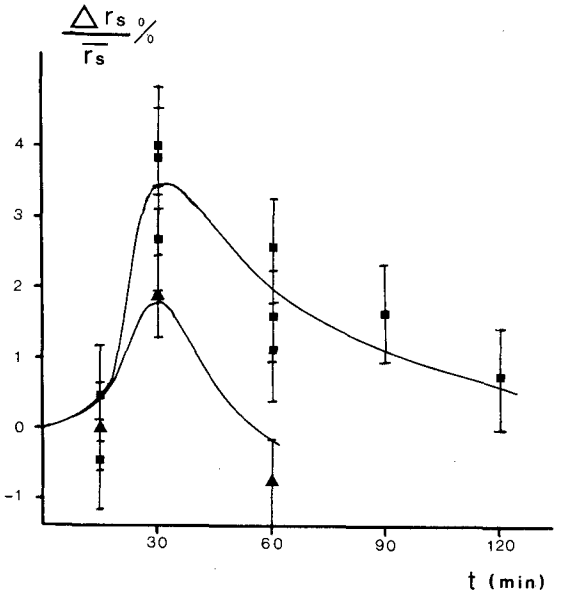

Figure 1 :

Interferon induced variations of the fluorescence stationary anisotropy rs of DPH in plasma membranes of L cells.

The relative difference $\frac{rs\ mock - rs\ interferon}{rs\ average}$ $\frac{(\Delta rs\ \%)}{rs}$ between the values of rs for control cells and for interferon treated cells, has been plotted against the duration of interferon treatment, t.

■ 1000 units/ml

▲ 100 units/ml

the standard deviation from 20 successive determinations. Normal rs values of DPH in the plasma membranes of untreated cells were about 0.23. The use of mock interferon for control cells has proved to be appropriate since the mock preparation itself induces a slight but non negligible, dose dependent, increase of rs in comparison with untreated cells.

DPH fluorescence lifetime :

As previous authors (20), we found for DPH a bi-exponential decay. The long lived component τ_1 (typically 8 ns in biomembranes (21)) always represents the main part ($\alpha_1 \sim 0.8$-0.9) of the emission. Interferon treatment had practically no effect on it : for a 30 min. incubation (corresponding to the maximum rs change), values of τ_1 were 8.4 $\pm$ 0.2 ns for the interferon treated samples and 8.2 $\pm$ 0.2 ns for the control. Such a variation accounts for less than 10 % of the observed effect on rs. The fast component τ_2 was too short and of too low intensity to be measured with accuracy (τ_2 = 0.7 - 1.3 ns ; α_2 = 0.10 - 0.20). This however was of minor importance in the present case, since for a bi-exponential decay rs in biomenbranes can be shown to be given by the expression $rs = \frac{(r_o - r_\infty)}{\alpha_1\tau_1 + \alpha_2\tau_2} \phi \left[\frac{\alpha 2 \tau 2}{\phi + \tau_1} + \frac{\alpha 2 \tau 2}{\phi + \tau 2} \right] + r_\infty$ (22,23) where the constant r_o is the limiting anisotropy when the probe is immobilized, r_∞ is the time resolved anisotropy at very long times after excitation and ϕ is the fluorescence correlation time (ϕ and r_∞ are the parameters which account for the dynamic and static restrictions of the oscillatory motion of the probe, imposed by the bilayer environment i.e. those which give informations about the fluidity and the lipidic order around the probe). $\alpha_2\tau_2$ is always much lower than $\alpha_1\tau_1$ (by a factor of about 50) and the expression reduces to $rs = \frac{r_o - r_\infty}{1 + \tau_1/\phi} + r_\infty$.

Discussion :

With respect to the very low concentration of interferon needed to induce the effect : 10^{-6}mg/ml (10^3 U/ml), which almost corresponds to the

saturation of the receptors (10^3 per cell (4)), the observed decrease of rs (4 % maximum) should be considered as rather important. In comparison prolactin, a polypeptidic hormone induces a dramatic rs decrease (18 % maximum for DPH in membranes of rat hepatic cells (24), but it acts at concentrations 10^3 times higher (25). Since practically no DPH lifetime variation following the interferon treatment is observed, the resulting rs decrease is explained by an overall increase of membrane fluidity of the treated cells, i.e. the constraints imposed by the bilayer structure on the oscillatory motion of the DPH molecule are reduced. DPH may also interact with hydrophobic regions of membrane proteins giving rise to high rs irrespective of microviscosity (26). However we assume such a mechanism to be of secondary importance : experiments performed in the Laboratory (27), have shown that the quantum yield of DPH is about 10 times higher in purified hepatocytes plasma membranes than in pure proteins (bovine serum albumin) ; on the other hand interferon has been reported (8) to increase the concentration of membrane glycoproteins and this would tend to increase rs, at the opposite of the observed effect. From a kinetic point of view the early (20 min.) appearance of the effect could correlate with the initiation of antiviral activity. The fact that the curve passes through a maximum suggests that the overall effect arises from the sum of two or more counterbalancing effects. In this respect our results are to be compared with those obtained by Pfeffer et al. (12) by E.S.R. spectroscopy. These authors were mainly concerned with *late* effects of interferon ; however they also report an early and transitory membrane fluidity change in interferon-treated L 929 cells, reaching a maximum at about 30 minutes, but their observed effect is of opposite sign (increase in rigidity). At the difference with our condition they used suspension cell cultures in calcium deprived spinner medium, instead of monolayer cultures. Calcium is probably involved in the mechanisms of action of interferon (28, 29), although the exact role is not yet clear.

It should be pointed out that a possible uptake of membrane bound Ca^{2+} (29) concomittant with the development of interferon activity could account for a membrane fluidity increase (30) as well as for a previous reported increase of the cell surface negative charge (9). Complementary interferon effects responsible for an increase in membrane rigidity would then be associated with modifications in the membrane lipid metabolism (11, 31). Further investigations of these phenomena are planned.

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