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Biochimica et Biophysica Acta 1189 (1994) 175–180

BBA Biochimica et Biophysica Acta

Real-time measurements of chemically-induced membrane fusion in cell monolayers, using a resonance energy transfer method

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(Received 25 March 1993)
(Revised manuscript received 9 July 1993)

Abstract

Fusion of mouse melanoma cells grown in monolayers has been directly monitored by fluorescence resonance energy transfer between fluorescein and rhodamine probes attached to octadecanoic acid. Various poly(ethylene glycol)s (PEG), either alone or in combination with amphipathic molecules, have been used as fusogens. Fusion starts at a maximum rate as soon as PEG is removed from the medium and reaches a plateau after 20–30 min. Both the initial rate and extent of fusion have been recorded for each experiment. The extent of fusion shows in general a positive correlation with the initial rate, although PEGs with different molar masses appear to induce fusion at different rates, but to a similar extent. A good correlation has been found between the extent of fusion, as measured by fluorescence, and the 'fusion index' computed from cell and nucleus counting; a calibration curve is provided for the interconversion of both parameters. Optimum fusion values are obtained with 50% (w/v) PEG 1500. The effect of pre-treatments with surfactants (Triton X-100, sodium dodecylsulphate) on PEG-induced fusion has also been tested. Sodium dodecylsulphate, but not Triton, enhances considerably both the rate and extent of cell fusion. The in situ generation of the amphipathic molecule diacylglycerol, through the catalytic activity of a phospholipase C, also enhances significantly the fusion parameters. These results are in good agreement with previous studies based on syncitia counting.

Key words: Membrane fusion; Poly(ethylene glycol); Surfactant; Phospholipase C; Resonance energy transfer; (B16 melanoma cell)

1. Introduction

Membrane fusion is an important event in many physiological and pathological processes, from egg fertilisation to neurotransmitter secretion and viral infection. In addition, cell membrane fusion has important biotechnological applications, e.g., in obtaining hybridomas for monoclonal antibody production. Poly(ethylene glycol) (PEG) has long been established as a fusogenic agent, and it is widely used in in vitro cell fusion experiments [1,2].

Previous work from this laboratory [3,4] has been directed to the improvement of PEG-induced cell fusion, mainly by combining PEG and surfactant treatments. We have also studied the effect of combining PEG with diacylglycerides, produced in situ via a phospholipase C. However, in those studies, as in most cell fusion studies, fusion was only measured under equilibrium conditions, i.e., a 'fusion index' was calculated by estimating nuclei/cell ratios under a light microscope once syncitia formation had taken place. In the present paper, we report on the application of a fluorescence method enabling us to perform kinetic studies of the cell fusion process. The procedure, suggested years ago by Keller et al. [5], is based on resonance energy transfer (RET) between two fluorescent molecules. When applied to our system, it provides a convenient method for the quantification of both rates and extents of cell fusion. The RET measurements have also been calibrated against the nucleus counting procedure.

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Abbreviations: CFS, calf foetal serum; F18, 5-(*N*-octadecanoyl) aminofluorescein; PEG, poly(ethylene glycol); R18, octadecylrhodamine B chloride salt; RET, resonance energy transfer; SDS, sodium dodecylsulphate.

SSDI 0005-2736(93)E0300-Y

2. Materials and methods

Triton X-100 and sodium dodecylsulphate (SDS) were purchased from Sigma (St. Louis, MO). PEG 1500 was 'for synthesis' quality, from Merck (Darmstadt). Phospholipase C (EC 3.1.4.1) from *Bacillus cereus* was purchased from Boehringer-Mannheim and used without further purification. SDS-PAGE revealed that our enzyme preparation was ≈ 90% pure.

The B16F10 cell line from C57-B1/6 mouse melanoma used in this work was kindly provided by Dr. M.F. Poupon (Villejuif, France). The cells were grown in monolayer 150 cm^2 Falcon flasks on a defined RPMI 1640 medium supplemented with 10% calf foetal serum, 10^6 I.U./l penicillin and 2.5 mg/l streptomycin. When required, RPMI 1640 without Phenol red (Sigma) was used. Final pH was adjusted to 7.4. Culture flasks were kept at 37°C under 5% CO_2 and relative humidity near saturation.

2.1. Fluorescent labeling

The probes used, 5-(*N*-octadecanoyl)aminofluorescein (F18) and octadecylrhodamine B chloride salt (R18), were purchased from Molecular Probes (Eugene, OR). Fluorescent labeling of the cells was carried out according to Keller et al. [5] with some modifications. The probes were dissolved in ethanol and added to 20 ml of growth medium (RPMI 1640) to a final concentration of 10 μM for R18 and 5 μM for F18. The ratio of ethanol to medium was 0.1% (v/v).

Cells in late exponential phase of growth were harvested and seeded in 150 cm^2 Falcon flasks, $20 \cdot 10^6$ cells per flask with RPMI 1640 and calf foetal serum (CFS). After incubation for 6 h at 37°C, the medium was decanted and the cells washed twice with RPMI without CFS. Then, 20 ml of medium containing either R18 or F18 were added. 16 h later, the labeling medium was decanted and the cells were washed with fresh medium. The cells were collected with trypsin and washed twice with RPMI 1640 in order to remove non-incorporated probe. Subsequently, the cells were resuspended in medium with CFS and counted in a hemocytometer. The viability, which was evaluated with Trypan blue, was found to be always higher than 85%. Cells labeled with R18 and those labeled with F18 were mixed in a 1:1 ratio, and allowed to grow to a monolayer on glass coverslips (32 × 13.5 mm) within Petri dishes with growth medium. After 3 h, the coverslips were diagonally placed in fluorometer cuvettes [6] and rinsed twice with RPMI medium without Phenol red, to remove unattached cells. RPMI without Phenol red is used in order to avoid dye interference in the fluorescence scans.

Quantification of fluorescence in the supernatants indicated that about 90% of the added fluorophores was incorporated after the 16 h incubation. Similar levels of incorporation were found for both probes. Examination of these cells under a confocal microscope revealed that the probe was basically incorporated to the plasma membrane, although minor amounts could also be detected in the internal membranes. A similar situation was found by Keller et al. [5].

Control experiments were performed in which multilamellar liposomes composed of egg phosphatidylcholine were labeled with the probes and treated with PEG and phospholipase C or detergents; spectral changes or other signs of probe degradation were not observed. Lipids were extracted with chloroform/methanol/HCl (2:1:0.01) and absorbances in both chloroformic and aqueous phases were measured. 99% of absorbance was recovered in the organic phase.

2.2. Fusogenic treatments

The coverslips with an attached monolayer of cells were pre-treated with either phospholipase C (10 min) or detergents (5 min) as detailed below. After this pre-treatment, a 50% PEG solution in RPMI without Phenol red was added to the cuvette (1 min at 37°C), and the monolayer was subsequently washed with different solutions of PEG (33%, 25%, 17%, 0%). Then the coverslip was rinsed twice with pure medium and immediately fluorescence measurements were started.

2.3. Fluorescence measurements

Fluorescence was measured in a Perkin-Elmer MPF-66 spectrofluorometer with a cuvette holder thermostated at 37°C. The coverslips were placed diagonally in the cuvettes [6]. The excitation wavelength was 460 nm (excitation wavelength of F18). An interference filter was used at 470 nm. Emission scans from 500 nm to 700 nm were recorded and the variation of the fluorescence intensities at 524 (emission wavelength of F18) and at 592 nm (emission wavelength of R18) were measured. ΔR at a given time was computed as follows:

$$\Delta R = \left(\frac{I_{592}}{I_{524} + I_{592}} \right)_T - \left(\frac{I_{592}}{I_{524} + I_{592}} \right)_C$$

where I_{524} and I_{592} are the fluorescence intensities at 524 and 592 nm, respectively. The subscripts C and T represent the control and treated cells, respectively. I_{592} was corrected by subtracting the F18 contribution to the emission at this wavelength; for this purpose, the ratio of F18 fluorescence emissions at 592 and 524 nm was measured when F18 was the only dye present, and assumed to be similar in the presence of R18. This assumption was confirmed by band decomposition and curve fitting of some RET spectra. At zero time, just after the treatment, a value of ΔR denoted ΔR_0 was

obtained which was subtracted from the values obtained at later times in order to normalize the results. Normalized ΔR values were denoted as $\Delta R'$ ($\Delta R' \equiv \Delta R - \Delta R_0$). Initial fusion rates were estimated from the initial (maximum) slopes of the '$\Delta R'$ vs. time' curves, and expressed as $\Delta R' \times 100 \times \text{min}^{-1}$.

The 'fusion index' in the monolayer was also determined for every system studied here after the fluorescence measurements. This was carried out by cell and nucleus counting under the light microscope by the method described in Ref. 3.

3. Results

When the monolayer consisting of both cell populations is excited at 460 nm, an emission spectrum is obtained as shown in Fig. 1 (continuous line). The corresponding spectrum after treating the same monolayer with the fusogenic agent (50% PEG) for 15 min is also shown in Fig. 1 (dashed line). The observed increase in the R18 emission peak after the treatment is the result of energy transfer between the two fluorescent molecules which occurs when both probes are close to each other due to the lipid mixing between adjacent cells, lipid mixing being in turn an indicative of cell fusion.

From these scans, recorded at different times, the $\Delta R'$ values were calculated as described above. When these values are plotted versus time, an asymptotic curve is obtained (Fig. 2). Initial rates, maximum amplitudes, and times required to reach saturation of the lipid mixing (fusion) process may be estimated from these curves. In general, the fusion rate is maximal

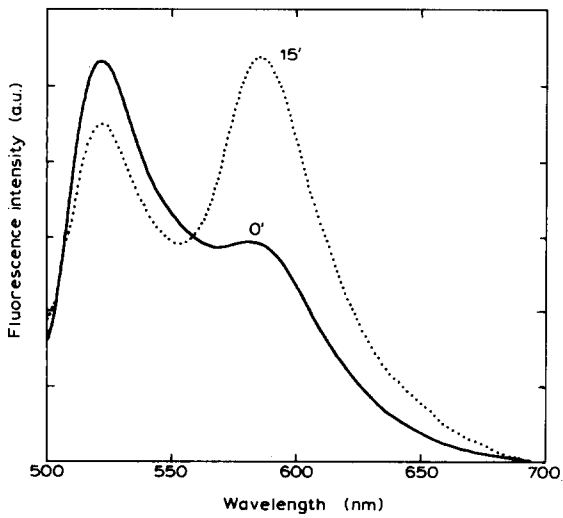

Fig. 1. Fluorescence emission spectra ($\lambda_{ex} = 460$ nm) of a mixed population of cells labeled with either F18 or R18. Continuous line: 0 min after PEG treatment. Dashed line: 15 min after PEG treatment.

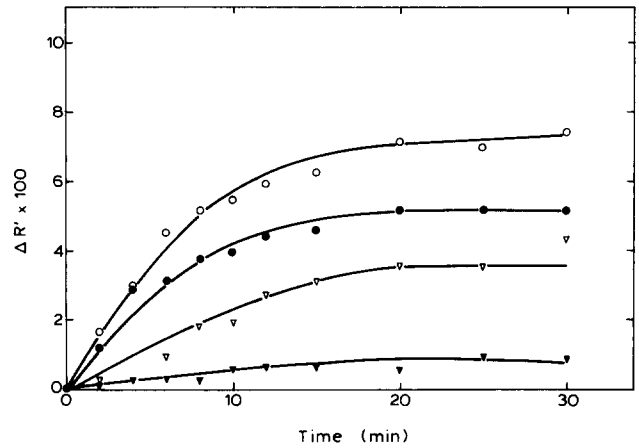

Fig. 2. Time-course of cell fusion measured by the resonance energy transfer method. Effect of PEG 1500 concentration: ▼, 20% (w/v); ▽, 30% (w/v); ●, 40% (w/v); ○, 50% (w/v).

immediately after the onset of measurements, and becomes virtually zero after 20–30 min.

The relationship between $\Delta R'$ values attained in the asymptotic region of the plots (as shown in Fig. 2) and the fusion index evaluated by optical microscopy for each coverslip is shown in Fig. 3. (This figure contains data from measurements carried out under each of the experimental conditions described along the paper.) It is apparent that a linear relationship exists; consequently, a fusion index can be estimated by determining $\Delta R'$ values through fluorescence experiments.

PEG is known to promote lipid transfer (including R18 exchange) even at concentrations below those producing fusion [16]; the good correlation observed between the spectroscopic and microscopy measurements, even at low levels of fusion (Fig. 3) suggests that, under our conditions, either lipid transfer is negli-

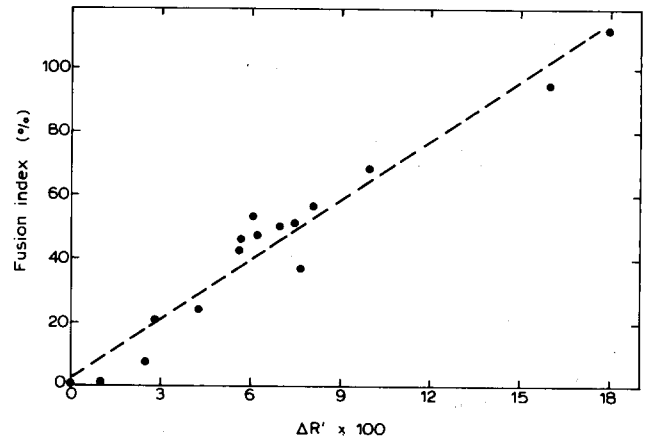

Fig. 3. Linear correlation between fusion index and $\Delta R'$. Fusion index was estimated from syncitia counting; $\Delta R'$ is a parameter of resonance energy transfer (see Materials and methods). The correlation coefficient is $r = 0.974$.

gible, or else it occurs at a very early stage, so that subtraction of ΔR_0 accounts also for this phenomenon. An experiment aimed at directly observing spontaneous probe transfer was performed as follows: two coverslips were used, each with an attached monolayer of cells. One of the coverslips was treated with both R18 and F18, while the other one was not stained. Both coverslips were immersed in RPMI (without Phenol red) at 37°C, with the cell monolayers facing eachother, at a distance of ≈ 1 mm; after treating with 50% PEG 1500, the slides were kept in RPMI medium under these conditions for up to 1 h. When appropriate, one of the slides was transferred to a cuvette in the fluorometer, and the corresponding spectrum recorded. As a control, the evolution of RET spectra from a double stained (R18 + F18) third coverslip, kept alone in RPMI medium (after PEG treatment) was followed. The results in Fig. 4 show that no RET signal is detected in the unstained monolayer, and that the time-course of the spectra of the double stained monolayers is the same irrespective of the absence or presence of unstained cells. More important, all recorded RET spectra show the same R value ($R \equiv I_{592}/(I_{524} + I_{592})$) of about 0.72. These experiments demonstrate that, under our conditions, probe transfer does not lead to artefactual measurements of cell fusion.

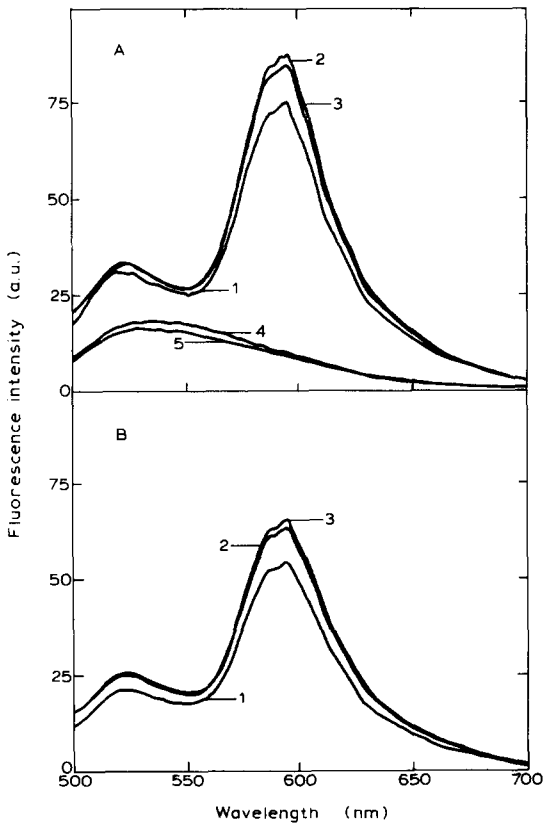

Fig. 4. Changes in resonance energy transfer spectra as a function of time. (A) Two cell monolayers, one unstained, the other stained with both R18 and F18. RET spectra: 1, stained monolayer, time zero after treatment with PEG ($r = 0.716$); 2, after 30 min ($r = 0.732$); 3, after 60 min ($r = 0.727$); 4, unstained monolayer before PEG treatment; 5, unstained monolayer 60 min after PEG treatment. (B) Only one cell monolayer, double stained. RET spectra: 1, time zero after treatment with PEG ($r = 0.720$); 2, after 30 min ($r = 0.720$); 3, after 60 min ($r = 0.720$).

Table 1
Fusion parameters calculated from resonance energy transfer measurements

PEG 1500 concentration (%w/w)	Initial fusion rates ($\Delta R' \times 100 \times \text{min}^{-1}$)	Fusion end-point ($\Delta R \times 100$)	Fusion index (%)
20	0.048	0.8	7±5
30	0.154	3.5	24±8
40	0.557	5.1	34±9
50	0.748	7.3	48±5

The results represent average values of three independent preparations (±S.D. for fusion index measurements). $\Delta R'$ is calculated as defined in the text. Fusion index is measured as in Ref. 1. Measurements were carried out 30 min after PEG addition.

Fig. 2 also shows the variation of $\Delta R'$ with time for cell monolayers treated with different concentrations of PEG 1500. The initial rates of the fusion process are observed to decrease with decreasing PEG concentrations. As mentioned above, $\Delta R'$ values and the fusion index are linked through a linear relationship. Accordingly, the fusion index for each PEG concentration can be calculated for the corresponding $\Delta R'$ values at saturation. The fusion indexes calculated in this way are shown in Table 1; the extent of the fusion process also decreases with decreasing PEG concentration. Since the most effective concentration tested is 50%, it was used in the remaining experiments.

The influence of PEG molar mass on the fusion process was also investigated. PEG of molar mass 1500, 4000 and 6000 g mol^{-1} were used. PEG of lower molar mass were not used because they are reported as cytotoxic for this cellular line, that is, they produce a drastic reduction of celullar viability [7]. The variation of $\Delta R'$ with time for cells treated with PEG of different molar masses has been represented in Fig. 5. The initial slopes of these graphs for PEG 4000 ($m = 1.125$) and 6000 ($m = 1.213$) are larger than the slope for PEG 1500 ($m = 0.748$), indicating a faster initial rate of lipid mixing for molar masses higher than 1500 g mol^{-1}. The influence of PEG molar mass is only relevant at short times; at or near saturation $\Delta R'$ are the same for all molar masses studied, suggesting that the fusion index is practically the same for them all.

As mentioned above, the effect of two synthetic amphiphiles (SDS and Triton X-100) on PEG-induced cell fusion was studied. Previous experiments revealed that the detergents were not able to produce any fusion by themselves [7]. Non-cytotoxic detergent concentrations were also selected according to our previous studies [1,2,7,8]. Fig. 6 shows the influence that the

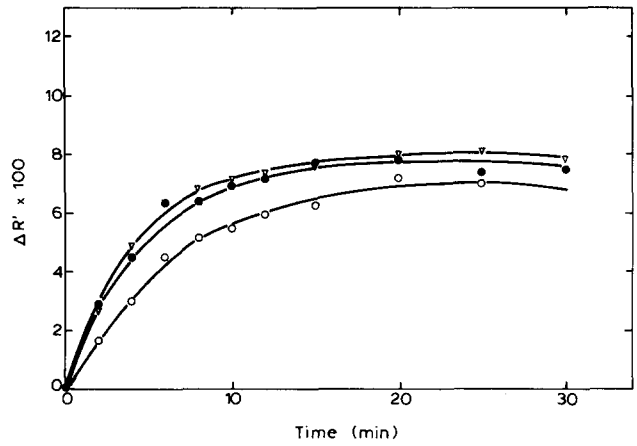

Fig. 5. Time-course of cell fusion measured by the resonance energy transfer method. Effect of PEG molar mass: (○) 1500; (●) 4000; (▽) 6000.

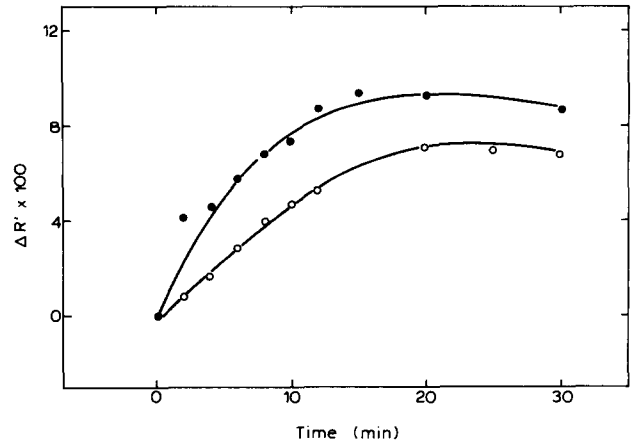

Fig. 7. Time-course of cell fusion measured by the resonance energy transfer method. Effect of pre-treating the cells with phospholipase C. Open circles, control; full circles, enzyme-treated cells.

pretreatment of the cell monolayer with SDS or Triton X-100 has on the fusion process. When the cell monolayer is pretreated with $5 \cdot 10^{-5}$ M SDS for 5 min before adding PEG 50%, the slope of the curve, m = 2.018 (Fig. 6A), is larger ($\approx$ 2.3-times) than in the control cells, not subjected to a detergent pre-treatment. The time required to reach the saturation value is not affected, being approx. 20 min for both cell monolayers. However, the fusion index is affected by the pre-treatment process, increasing by about 100% with respect to the control monolayer. The results obtained when the cells are pre-treated with Triton X-100 are shown on Fig. 6B. In previous experiments [1] this non-ionic detergent was observed not to be able to increase the fusion index obtained with PEG alone as seen under the light microscope. The results of these fluorescence experiments show that the pre-treatment of cells with non-toxic concentrations of Triton X-100 does not increase or actually decreases (Fig. 6B) the extent of cell fusion.

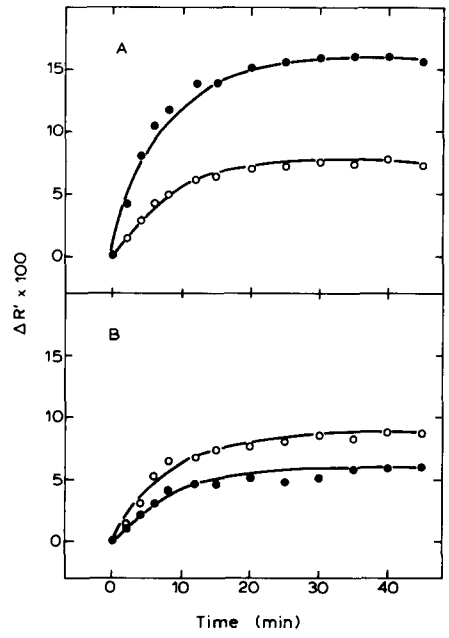

Fig. 6. Time-course of cell fusion measured by the resonance energy transfer method. Effect of pre-treating the cells with surfactants. Open circles: control; full circles: detergent-treated cells. (A) Sodium dodecylsulphate; (B) Triton X-100.

The effects observed for the pre-treatment with phospholipase C ($3 \cdot 10^{-3}$ U/ml) for 10 min are given in Fig. 7. The enzyme concentration was chosen after measurements of the cytotoxic effects of phospholipase C under our conditions [9]. The initial rate of lipid mixing is largely increased for the enzyme-treated monolayer (m = 2.060 vs. the m = 0.748 control value). The extent of fusion is also somewhat increased in the presence of the enzyme. When phospholipase C is previously incubated with *o*-phenanthrolin, a known enzyme inhibitor, the addition of phospholipase C does not modify PEG-induced fusion (data not shown).

4. Discussion

The above results show that the resonance energy transfer between the probes F18 and R18 is a suitable technique to characterize the kinetics and extent of PEG-induced cell fusion. Although the experimental data refer primarily to lipid mixing, the method can be calibrated with direct microscopic counting of multinucleate cells (Fig. 3). External calibration is also required because, in a scale of lipid mixing under our experimental conditions, a 0% point is easily determined, but a 100% cannot be fixed with certainty, mainly because of the presence of intracellular membrane systems.

Different procedures have been proposed previously for the study of cell fusion kinetics. However, those

involving mixing of aqueous contents (e.g., Refs. 10,11) are difficult to apply to living cells, while fluorescent phospholipid derivatives (e.g., Ref. 12) cannot be used in the presence of phospholipase C. Methods based on the dequenching of R18 have also been proposed [13] but this probe has a tendency to exchange spontaneously between bilayers and is better used, in combination with F18, in RET experiments. In view of these limitations, the procedure described by Keller et al. [5] appears more appropriate for our aims; it has been applied previously, e.g., to monitor fusion of microsomal vesicles [14]. Our results demonstrate that, at least under our conditions, spontaneous probe transfer between membranes does not significantly alter the experimental results (Fig. 4). In addition, the use of the corrected fusion parameter $\Delta R'$ avoids changes in fluorescence properties of R18 and F18 due to factors other than cell fusion, as shown by the good correlation with syncitia counting (Fig. 3).

Moreover, the RET method has shown its usefulness in confirming a variety of published and unpublished results concerning PEG-induced cell fusion. Fusion starts immediately after PEG is removed by dilution, if not beforehand, and reaches a plateau after 20–30 min. Phospholipid vesicles fuse readily without the dilution step [16], and the same may happen with cells [17]; however, we have followed in the present study the standard protocol for PEG-induced cell fusion, that includes PEG dilution [17]. Very similar data were obtained by Huang and Hui [12] for PEG-induced fusion of erythrocytes in monolayers. In addition, we have found that the initial rate of lipid mixing is higher the higher the molar mass of the polymer (Fig. 5). The results obtained with different concentrations of PEG 1500 may also be related to the dehydrating capacity of this polymer (Fig. 2). Studies carried out by Blow et al. [15] showed that the maximum percentage of cell fusion occurred when the concentration of free water in the polymer solution was zero. For PEG 1500 this happens at 50%, i.e., the concentration giving optimum fusion. Recent results on PEG–liposome interaction [16] also support the idea that the absence of free water is a pre-requisite for fusion to occur.

The results obtained when the cells are pre-treated with SDS confirm that this detergent enhances the initial rate of fusion (Fig. 6), which does not occur when the cell monolayer is pre-treated with Triton X-100, as described previously [3]. The influence of phospholipase C on PEG-induced cell fusion (Fig. 7) has not been mentioned previously, although the enzyme is known to promote liposome fusion [18]. Its effect on cells may be equally due to the ability of endogeneous diacylglycerol to perturb the bilayer through the formation of non-bilayer structures [19–21].

Acknowledgments

This work was supported in part by the Basque Government (grant No. 9002) and by a grant from DGICYT (PB91–0441). The authors thank M. Mencía and Dr. A. Prado for their help with preliminary experiments. M.A.P. was a post-doctoral fellow of the Basque Government.

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