EVIDENCE FOR REVERSIBLE MICROCLUSTERING OF LENTIL LECTIN MEMBRANE RECEPTORS ON HeLa CELLS

Alain B. SCHREIBER, Johan HOEBEKE+, Bernard VRAY† and A. Donny STROSBERG*

Laboratory of Biochemical Pathology, +Laboratory of Microbiology and Hygiene, VU Brussels, B-1640-St Genesius-Rode, †Laboratory of Microbiology and Immunology, Faculty of Medicine, UL Bruxelles, B-1000 Bruxelles, Belgium and **Molecular Immunology Group, Institut de Recherche en Biologie Moléculaire, CNRS, Université Paris 7, Tour 43-2, Place Jussieu-75221 Paris Cedex 05, France*

Received 17 January 1980

1. Introduction

The lateral mobility of membrane receptors and their clustering upon binding of ligands play an essential role in the process of transmembrane signaling in various biological systems [1–7].

Physicochemical techniques were developed for the quantitative study of membrane receptor lateral movements at the molecular level. Fluorescence photobleaching recovery [8,9] allows the calculation of diffusion coefficients and of the fraction of immobilized receptors. Fluorescence resonance energy transfer (RET) was applied for evaluating the proximity of lectin receptors on cell surface [10,11]. Both techniques focused on single cells and their results were correlated with the qualitative observations of fluorescence microscopy.

We reported the use of RET for a large number of cells as a statistical approach for the study of the redistribution of lentil lectin membrane receptors [12]. Using this approach, the kinetics of the redistribution of lentil lectin receptors on HeLa cells are presented here. At 20°C and 37°C, redistribution equilibria are rapidly reached and do not correspond to receptor patching observed by fluorescence microscopy. At lower temperatures, different equilibria are reached more slowly. The receptor–receptor interaction is initially reversible but becomes irreversible when redistribution is allowed to proceed over longer incubation periods.

2. Materials and methods

HeLa cells were grown in modified Eagle's medium supplemented with calf serum 5% in spinner bottles [13]. Cells were pre-washed 4 times with phosphate-buffered saline–bovine serum albumin 0.1% (PBS–BSA).

Fluorescein isothiocyanate (FITC) and rhodamin β-isothiocyanate (RITC) were purchased from British Drug Houses. Lentil lectin (LL), FITC–LL and RITC–LL were prepared as in [14]. The dye/protein ratio was calculated as 1.32 for FITC–LL and as 1.37 for RITC–LL from absorbance measurements. Stock solutions of LL, FITC–LL and RITC–LL were divided in aliquots, thawed once and centrifuged before use. Binding assays and inhibition studies were performed by quantitative fluorometry as in [14].

For fluorescence resonance energy transfer (RET) studies, 5×10^5 cells were added to a series of Eppendorf tubes in which a mixture of LL or RITC–LL and FITC–LL 9:1 was made in 0.5 ml final vol. PBSA–BSA; the total lectin conc. was 10^{-6} M. After incubation for various times and at different temperatures (0, 10, 20 and 37°C) with constant shaking, cells were fixed with 1% paraformaldehyde in PBS, centrifuged for 15 s in an Eppendorf table centrifuge, washed once with PBS–BSA and resuspended in 1 ml PBS before spectroscopic measurements.

In some experiments, cells were pretreated with 10 mM methylamine for 40 min at 37°C or prefixed with 1% paraformaldehyde before LL binding. The reversibility of the energy transfer process was studied in the following way: cells were incubated with the lectin mixtures for various times at 37°C as above, spun down, resuspended in 0.5 ml cold PBS–BSA and further incubated at 0°C, before fixation.

The turbidity of the cell suspensions was measured at 480 nm in a Zeiss DM4 spectrophotometer. Fluorescence spectra were recorded on a Jobin-Yvon JY3D spectrofluorometer. The excitation wavelength was 480 nm and scanning was performed from 500–600 nm. Slits were 10 nm for lamp and photomultiplier and 4 nm for excitation and emission. Fluorescence microscopic observations were performed on a Zeiss Universal model microscope.

3. Results

Fluorescence values were corrected for light scattering due to the presence of cells (5–25% of the signal) and for the variation in no. cells/sample. As both light scattering and turbidity varied in a linear relationship with the number of cells in the used conditions (5×10^5 cells, $A_{480} = 0.20 \pm 0.02$) the corrected fluorescence value, F_{corr} in arbitrary units, was calculated as:

$$F_{corr} = \left[\frac{F_{obs}}{A_{obs}} - \frac{F_o}{A_o}\right]$$

were F_{obs} and A_{obs} are, respectively, the fluorescence and absorbance measured for a given sample and F_o and A_o the fluorescence and the absorbance measured for a blank (cells incubated without lectin).

The binding of LL to HeLa cells was essayed by quantitative fluorometry [14]. The specific binding was saturable and revealed 8×10^7 sites/cell with K_a 10^7 M^{-1}, with no significant change for different temperatures. Equilibrium was reached after ~3 min incubation and non-specific binding was <10% of the specific binding. Lectin binding was calculated to be essentially monovalent [14]. Unconjugated and conjugated lectin preparations had the same binding characteristics, as measured by inhibition of FITC–LL binding.

RET could be followed by quenching of the donor (FITC–LL) fluorescence and sensitization of the receptor (RITC–LL) fluorescence. As the former gave a higher and thus more reliable signal, we chose to express the value of RET as % quenching of the donor fluorescence at 520 nm (Q) with:

$$Q = \frac{F_{corr}\,(\text{RITC–LL})}{F_{corr}\,(\text{LL})} \times 100$$

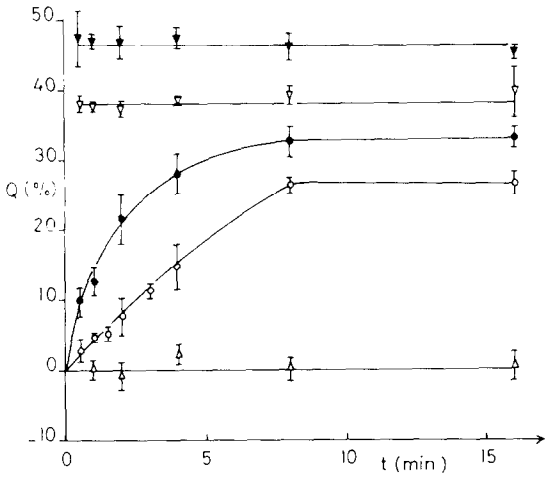

Fig.1. Evolution of the donor fluorescence quenching Q (see text) in function of the incubation time at different temperatures. (○) 0°C; (●) 10°C; (▽) 20°C; (▼) 37°C; (△) prefixed cells at 37°C.

where F_{corr} (RITC–LL) is the corrected fluorescence value of the sample with the mixture of RITC–LL and FITC–LL and F_{corr} (LL) the value for the sample with LL and FITC–LL in the same incubation conditions.

In fig.1, the evolution of Q is plotted in function of the incubation time at different temperatures. At 20°C and 37°C plateaus of 38.3 ± 1.1% and 46.5 ± 1.9%, respectively, were reached even at 30 s incubation. At 0°C and 10°C, lower plateaus of 26.9 ± 1.2% and 33.0 ± 1.4% were reached more slowly, after ~8 min incubation. No significant RET was observed for prefixed cells although the amount of bound lectin was the same as for non-fixed cells.

At the total LL concentration used (10^{-6} M), 91% of the binding sites are occupied, but even at 3×10^{-8} M LL when 17% of the sites are occupied, the values for Q remained identical; this observation rules out the possibility that the lower Q values at 0°C and 10°C for short incubation periods are due to a lower receptor occupancy.

Fluorescence microscopy revealed patching of LL membrane receptors at 20°C and 37°C for incubations of ⩾8 min. With shorter incubation periods, at lower temperatures, or with prefixed cells, a diffuse staining of the cell membranes was observed. Patching was totally inhibited when cells were pretreated with 10 mM methylamine but the binding of LL and the

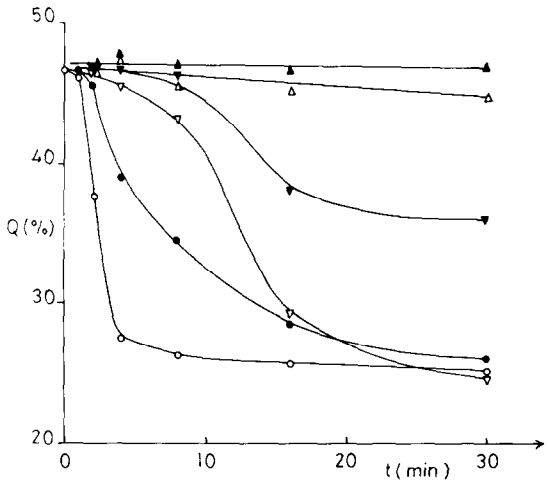

Fig.2. Time evolution of the donor fluorescence quenching Q (see text) at 0°C after preincubation at 37°C for different time periods ((○) 0.5 min; (●) 1 min; (▽) 2 min; (▼) 4 min; (△) 8 min; (▲) 16 min preincubation). Results are the mean of at least 3 independent expt.

value of Q remained unchanged in comparison with untreated cells.

The reversibility of the RET process was assayed by LL binding to cells at 37°C for various incubation times and further incubation at 0°C. Results are shown in fig.2. After ⩾8 min incubation at 37°C, the value of Q did not change significantly. For shorter incubation periods, a progressive decrease to the equilibrium value of Q at 0°C was observed; the rate of decrease was inversely correlated with incubation time at 37°C.

4. Discussion

Visible aggregation of cell membrane receptors in patches and caps upon binding of specific ligands has been correlated with the triggering of biological activity [3,4]. There is, however, an increasing body of evidence [5–7,15], that microclustering, the initial aggregation of a few receptor molecules, is of major importance in transmembrane signaling. This putative microclustering of receptors was postulated to be a fast and reversible process [16].

RET provides an ideal biophysical tool for probing the early events of membrane receptor redistribution. Changes in RET correspond to variations of the relative distances separating donor and acceptor molecules and it has to be emphasized that these are 0–100 Å. RET has thus been widely used as 'spectroscopic ruler' for intra- and intermolecular distances (reviewed [17]) and was recently applied to measure the proximity of con A receptors on single cells [10,11]. Here, the dynamics of cell surface redistribution of LL receptors was approached by the study of the temporal variation of RET between FITC–LL and RITC–LL bound on a large number of HeLa cells.

No significant RET occured when cells were prefixed, confirming the absence of receptor mobility in this conditions. This observation also indicates that the average separation between LL receptors on HeLa cells is larger than the distance allowing RET.

At 20°C and 37°C plateaus of RET are reached rapidly (<30 s incubation). At lower temperatures, different equilibria were reached more slowly. The redistribution evidenced by RET is clearly not what was observed by fluorescence microscopy as visible patching only appeared at 20°C and 37°C, after ⩾8 min incubation. This point was further confirmed by the study of methylamine-treated cells. Methylamine was shown to inhibit clustering of epidermal growth factor [15] and enkephalin membrane receptors [18] and also inhibited the patching of LL receptors on HeLa cells; RET values remained however identical to those of untreated cells.

The redistribution equilibrium at 37°C is initially reversible. The rate of 'dissociation' was inversely related to the time lapse during which receptors were allowed to redistribute at 37°C. It is interesting to note that irreversibility is reached concomitantly with the formation of visible patches.

We propose that our results indicate the existence of reversible microclustering of LL membrane receptors on HeLa cells. The kinetics of this process are fast and temperature-dependent. The microclustering does not necessitate bivalent linking of the receptors as LL binding was calculated to be essentially monovalent. The different RET equilibria obtained suggest a difference in size and/or in no. receptors of the microclusters as a function of temperature. At 37°C the redistribution process becomes irreversible as microclusters further aggregate resulting in visible patches.

Acknowledgements

We thank Mrs M. De Pelsmaeker for help with cell

culture and Dr A. Boeyé for constant support. This work was supported by grants from the FGWO, the Solvay-Tournay Foundation for Medical Research, the Paul Cams Foundation, the INSERM and the DGRST.

References

[1] Singer, S. J. and Nicolson, G. L. (1972) Science 175, 720–731.
[2] Frye, L. D. and Edidin, M. (1970) J. Cell. Sci. 7, 319–335.
[3] Edelman, G. M. (1976) Science 192, 218–226.
[4] Schreiber, G. F. and Unanue, E. R. (1976) Adv. Immunol. 24, 38–166.
[5] Segal, D., Taurog, J. D. and Metzger, H. (1977) Proc. Natl. Acad. Sci. USA 74, 2993–2997.
[6] Kahn, C. R., Baird, K. L., Jarrett, D. B. and Flier, J. S. (1978) Proc. Natl. Acad. Sci. USA 75, 4209–4213.
[7] Shechter, Y., Hernaez, L., Schlessinger, J. and Cuatrecasas, P. (1979) Nature 278, 835–838.
[8] Schlessinger, J., Koppel, D. E., Axelrod, D., Jacobson, K., Webb, W. W. and Elson, E. M. (1976) Proc. Natl. Acad. Sci. USA 73, 2409–2413.
[9] Edidin, M., Zagyansky, Y. and Lardner, T. J. (1976) Science 191, 466–468.
[10] Fernandez, S. M. and Berlin, R. D. (1976) Nature 264, 411–415.
[11] Chan, S. S., Arndt-Jovin, D. J. and Jovin, T. M. (1979) J. Histochem. Cytochem. 27, 56–64.
[12] Hoebeke, J., Vray, B., Foriers, A. and Strosberg, A. D. (1980) in: Protides of the Biological Fluids (Peeters, H. ed) vol. 27, Pergamon, London, in press.
[13] Van den Berghe, D. and Boeyé, A. (1973) Arch. Ges. Virusfors. 41, 216–228.
[14] Hoebeke, J., Foriers, A., Schreiber, A. B. and Strosberg, A. D. (1978) Biochemistry 17, 5000–5005.
[15] Maxfield, F. R., Davies, P. J. A., Klemperd, Willingham, M. C. and Pastan, I. (1979) Proc. Natl. Acad. Sci. USA 76, 5731–5735.
[16] Schlessinger, J. (1979) in: Physical Chemical Aspects of Cell Surface Events in Cellular Regulation (Delisi, C. and Blumenthal, R. eds) 89–111, Elsevier/North-Holland, Amsterdam, New York.
[17] Stryer, L. (1978) Ann. Rev. Biochem. 47, 819–846.
[18] Hazum, E., Chang, K.-J. and Cuatrecasas, P. (1979) Nature 282, 626–628.