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PHOTOINDUCED CROSSLINKING OF MEMBRANE PROTEINS BY FLUORESCEIN ISOTHIOCYANATE

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SUMMARY

SDS polyacrylamide gel electrophoresis protein profiles of cell membranes labelled with fluorescein isothiocyanate (FITC) and bleached with 488 nm laser light show a broadening and loss of protein bands and the appearance of high molecular weight material indicating crosslinking of membrane proteins. The crosslinking probably proceeds through an FITC free radical intermediate and singlet oxygen produced upon exposure to light. This crosslinking may seriously affect many fluorescent staining experiments, particularly the membrane protein diffusion coefficients determined by fluorescence photobleaching recovery.

INTRODUCTION

The movement and location of proteins in biological membranes are commonly monitored by attaching fluorescent molecules to membrane proteins and observing the movement and location of fluorescence. Recently a modification of this approach, termed fluorescence photobleaching recovery (FPR), has been used by several investigators to measure the diffusion rate of proteins in the plasma membrane of whole cells (1-6). Two general methods are used for attaching the fluorescent molecule. One is to label the cell directly with an isothiocyanate derivative, such as fluorescein isothiocyanate (FITC) (1-3). FITC covalently attaches to amine and sulfhydryl groups and can label proteins and possibly phosphatidylethanolamine. The other is to indirectly label specific receptors through the use of FITC or tetramethyl rhodamine isothiocyanate conjugated ligands, such as concanavalin A (Con A) or antibodies to membrane proteins (3-6). The binding of the labelled Con A or antibody is assumed to be tight enough that the movement of fluorescence is controlled by the diffusion of the receptor complex. A specially designed microscope is

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used to focus an intense beam of light onto the plasma membrane. The light bleaches the fluorescent dye, yielding a small circular region of decreased fluorescence. This creates a fluorescence gradient and if the membrane proteins can diffuse, there will be an increase in fluorescence with time in the bleached area. The fluorescence intensity is monitored and from its rate of increase the diffusion coefficient of the labelled proteins can be calculated (7).

The experiments described above assume that the labelling with FITC and the intense illumination required for bleaching do not affect the membrane structure in such a way as to alter the rate of protein diffusion. Very little has been done to prove that these are valid assumptions, especially since it is known that fluorescein can sensitize the photo-oxidation of many molecules (8). Peters et.al. (1) compared the SDS polyacrylamide gel electrophoresis (SDS-PAGE) protein profiles of unlabelled and FITC labelled erythrocyte ghosts. They found the gel patterns to be similar, but the effects of bleaching were not investigated. We report the effects of FITC labelling and photobleaching on the protein banding pattern of human erythrocyte ghosts and baby hamster kidney (BHK) cell plasma membranes.

MATERIALS AND METHODS

Erythrocyte ghosts were isolated from fresh human blood by the method of Steck et.al. (9). Plasma membranes were isolated from BHK cells by the technique of Brunette and Till (10). Both were labelled with FITC in a manner similar to that used by Edidin et.al. to label mouse fibroblasts (3). One volume of a membrane suspension (2 mg/ml protein) in phosphate buffered saline (PBS), pH = 7.4, was mixed with one volume of FITC (0.2 mg/ml) in PBS, pH = 9.5, giving a final FITC concentration of 0.1 mg/ml. The membranes were incubated for 15 min. at room temperature in the dark, washed twice at 4°C in PBS, pH = 7.4, and suspended in PBS at a concentration of 1 mg/ml. The FITC labelled membranes were bleached with an argon ion laser (Spectra Physics) at 488 nm and a power level of 1.5 watts. The beam was expanded to about 1.0 cm diameter and the membranes suspended in a cuvette of 0.8 cm internal diameter. Exposures of 15 sec. and 165 sec. were used producing 4% and 50% bleaching of the fluorescein. Fluorescence intensity after light exposure was measured with a Turner model 430 spectrofluorometer using an excitation wavelength of 488 nm and an emission wavelength of 516 nm. The membrane proteins were separated by SDS-PAGE using the method of Fairbanks et.al. (11). The membranes were solubilized by heating to 90°C for one hour in a solution of 1% SDS, 10% sucrose, 10mM Tris-HCl (pH = 7.5), 1mM EDTA, 40mM dithiothreitol and 10μg/ml pyronin Y (tracking dye). To insure that one hour was sufficient for solubilization, samples were occasionally held at 90°C for an additional six hours and sonicated in a bath sonicator for five minutes. This additional treatment had no effect upon the protein profiles.

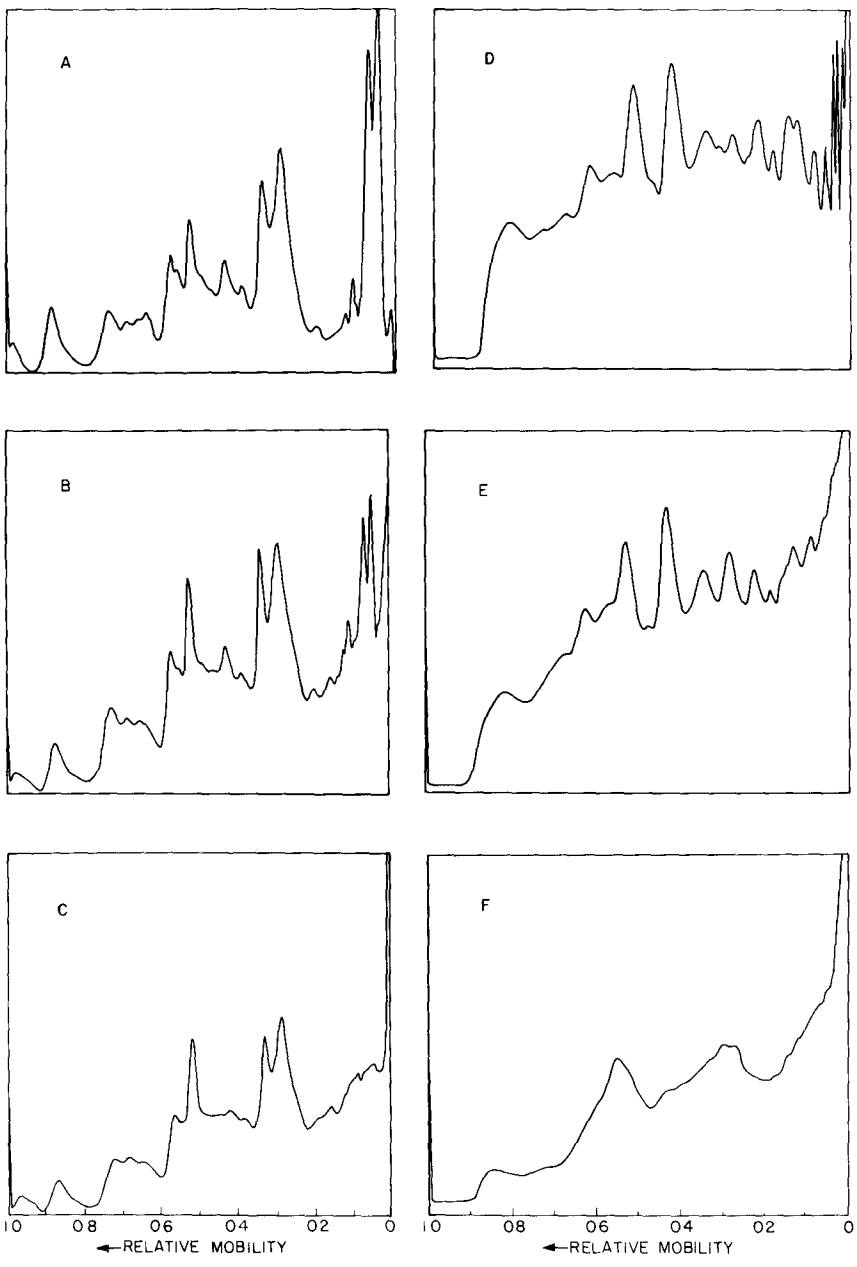

Fig. 1. SDS-PAGE protein profiles of human erythrocyte ghosts (A-C) and BHK plasma membranes (D-F). (A) Unlabelled control. (B) FITC labelled and irradiated at 488 nm for 15 sec. (4% bleaching). (C) FITC labelled and irradiated for 165 sec. (50% bleaching). (D) Unlabelled control. (E) FITC labelled and irradiated for 15 sec. (F) FITC labelled and irradiated for 165 sec.

RESULTS AND DISCUSSION

Densitometer tracings of the gels of irradiated and unirradiated membranes are shown in Fig. 1. For both erythrocyte ghosts and BHK plasma membranes, labelling with FITC had very little effect upon the gel patterns. Also irradiation of the unlabelled membranes did not change their gel patterns. Only irradiation of FITC labelled membranes had an effect. Exposure to light produced a buildup of high molecular weight material and the bands tended to broaden and run together. These effects are quite noticeable at 4% bleaching and by 50% bleaching most of the bands are missing for the erythrocyte ghosts and only two very broad bands remain for the BHK plasma membranes. These alterations in the gel patterns are indicative of some type of aggregation of the membrane proteins due to photobleaching. We strongly suspect that this is due to covalent crosslinking, since prolonged exposure to $90^{o}C$ and sonication did not dissolve the aggregates. There is evidence that bilirubin, another photosensitizer, can crosslink RBC ghost proteins (12).

An exposure to light producing roughly 50% bleaching is commonly used during FPR experiments. The intensity of the laser beam used in this work was approximately 1.9 watts/cm^2 and a 165 sec. exposure yields a total exposure of 315 joules/cm^2. For one photobleaching recovery experiment for which the necessary information is available (5), during bleaching the focused beam had a diameter of 2μm, an intensity of 1 mw and an exposure time of 3 sec. was used. This gives an intensity of 3.2×10^4 watts/cm^2 and a total exposure of 9.5×10^4 joules/cm^2. Thus our exposures are on the order of 300 times smaller but yield a comparable amount of bleaching.

An FITC free radical and oxygen are implicated in the crosslinking. Saturation of the FITC-BHK plasma membrane mixture with oxygen before photobleaching enhanced the crosslinking, while removal of oxygen with nitrogen reduced the amount of crosslinking. Crosslinking was also inhibited by sodium azide, a quencher of singlet oxygen. Fig. 2 gives the relative inhibition of crosslinking by sodium azide. BHK membranes were used and the height of the

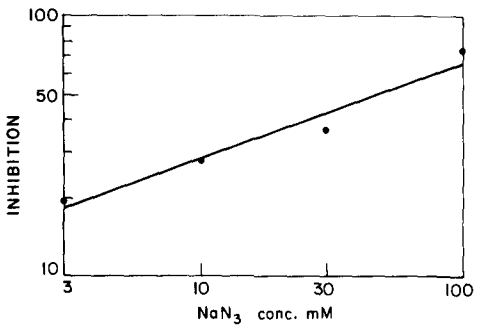

Fig. 2. Inhibition of crosslinking by sodium azide.

large peak with a mobility of 0.45 was used as a relative measure of crosslinking The parameter plotted is

$$\frac{h(x) - h(o)}{h(\text{control}) - h(o)} \times 100\%$$

where h(x) is the peak height in the presence of x concentration of sodium azide, h(o) is the height with no sodium azide present and h(control) is the height of the unbleached control. A value of 100 indicates no crosslinking and a value of 0 indicates complete disappearance of the peak (such as in Fig. 1 F).

Irradiation of FITC produces a free radical, the electron paramagnetic resonance (EPR) spectrum of which is shown in Fig. 3. The spectrum was obtained by irradiating a 20 mM solution of FITC in ethanol at room temperature with a 200 watt Hg lamp, filtered to remove the IR and UV radiation. The EPR spectrum is centered very near a g value of 2.003 and the fine structure is indicative of an organic free radical. The radical signal is quenched by oxygen.

Fluorescein is thought to sensitize the oxidation of many molecules through the production of a reactive oxygen species (8). The crosslinking appears to proceed by a similar mechanism since it can be inhibited by sodium azide. The FITC free radical may induce the formation of free singlet oxygen which may produce the crosslinking or an FITC moloxide (FITC-OO$^{\cdot}$) may be the reactive species. Alternatively the FITC free radical may react directly with

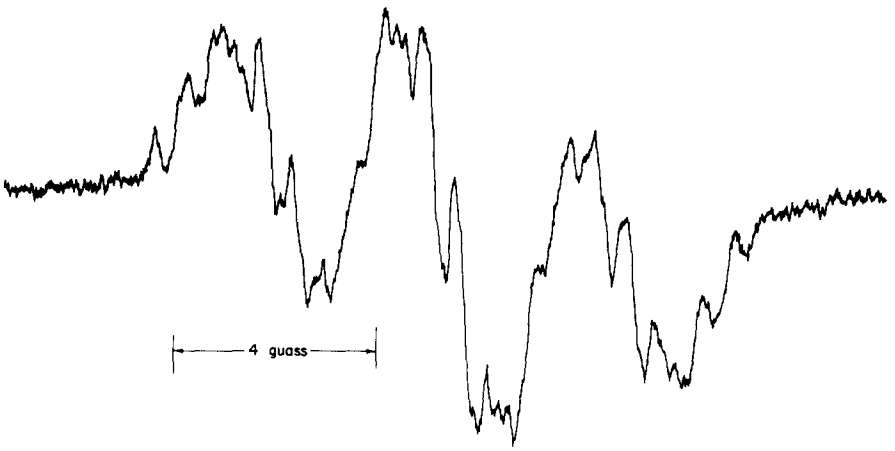

Fig. 3. The EPR spectrum of FITC (20 mM) in ethanol during irradiation with a 200 watt Hg lamp filtered to remove the IR and UV radiation. An Varian model E-12 EPR spectrometer was used. The following parameters were used: microwave frequency 9.35 GHz, modulation frequency 100 KHz, power 1.5 mw, modulation amplitude 0.2 guass, time constant 10 seconds and sweep time 2 hours.

proteins in the membrane. The sodium azide inhibition argues against the latter process, but it may still contribute to the crosslinking (12).

Several points must be considered before these results can be directly applied to FPR experiments. 1) Some photoinduced effects are dependent upon light intensity (8). The light intensity used in this study is on the order of 15,000 times less, due to beam diameter, than FPR bleaching intensities. This may affect the amount of crosslinking but an equal amount of crosslinking was observed using a 200 w high pressure Hg lamp (which has a much lower intensity at 488 nm than the laser used) as long as an equal amount of bleaching occurred. Thus crosslinking appears to be dependent upon the amount of bleaching, not light intensity or time. 2) A certain amount of crosslinking may be tolerated without significantly affecting FPR determined diffusion coefficients since they are proportional to $(MW)^{1/3}$ and thus change slowly with size. However, crosslinking to immobile membrane elements will stop diffusion, regardless of aggregate size. 3) Sodium azide at 10 mM has been reported to have no effect on diffusion rates (3,6). From Fig. 3 10 mM sodium azide reduced the cross-

linking by less than 30%. If it can be assumed that this corresponds to a 30% reduction in aggregate size, then there would be only a 10% change in diffusion coefficient because of the $(MW)^{1/3}$ dependence. The error of the diffusion measurements is generally much greater than 10% and thus one would not expect to see an effect with 10 mM sodium azide (6). A much greater inhibition of crosslinking, requiring a concentration of sodium azide of 100 mM or more, might be necessary to observe an effect. 4) Most recent FPR experiments have used indirect labelling, which results in a smaller amount of fluorescein being bound to surface of the cell. Thus, indirect labelling should reduce the level of crosslinking.

These results have been discussed with regard to FPR becasue of the large amount of bleaching that occurs. However, bleaching often occurs during microscopic observation of fluorescently stained cells and, hence, crosslinking may also occur under these conditions.

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