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PHOTOINDUCED CELL KILLING AND CROSSLINKING OF FLUORESCEIN CONJUGATED CONCANAVALIN A TO CELL SURFACE PROTEINS

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SUMMARY

Bleaching of fluorescein conjugated concanavalin A (F-Con A) labelled plasma membranes with 488 nm laser light leads to some broadening and a loss of protein bands and the appearance of high molecular weight material as shown by sodium dodecylsulfate polyacrylamide gel electrophoresis, indicating membrane protein crosslinking. The F-Con A is found throughout the high molecular weight regions linked to other proteins in large aggregates. Irradiation of whole cells labelled with F-Con A leads to cell death. These effects are dependent upon total light exposure and F-Con A concentration.

INTRODUCTION

The use of fluorescently labelled ligands that can bind to receptors on the cell surface has become a widely used and powerful technique for the study of membrane receptor location and diffusion. A fluorescent molecule, usually fluorescein or rhodamine B, is covalently attached to the ligand which then binds to its cell surface receptor through weak bonds. The location of fluorescence then signifies the location of the receptor. A receptor-ligand diffusion coefficient can be obtained by bleaching a small area of the cell surface (2-3 μm diameter) with an intense laser beam, and monitoring the return of fluorescence to this area, presumably through receptor diffusion. This technique has been used extensively on a wide variety of labelled ligands and cell types (1,2). Visual observation of fluorescently labelled ligands allows one to directly observe their location on the cell surface and within the cell (3).

Fluorescence techniques have proved very useful because of their high sensitivity due to the relatively high energy of photons in the visible region. The interaction of these photons with a chromophore can produce

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reactive oxygen species, such as singlet oxygen, and alter covalent bonds inducing molecular rearrangements. The exposure of cells to low dye concentrations and light can be extremely toxic. Eosin Y (1-10 μM) is not taken up by yeast cells but leads to cell death in the presence of light, probably through damage to the cell surface (4). Human lung fibroblasts (WI-38) are killed by visible light interacting with endogenous flavins, which act as photosensitizers (5). Photosensitization by dyes has been used to study giant squid axons by photochemically modifying sodium channels (6). Protophorphyrin (7) and bilirubin (8) have been shown to induce membrane protein crosslinking in human erythrocytes during exposure to light. Fluorescein isothiocyanate (FITC) labelled human erythrocyte membranes and baby hamster kidney (BHK) cell plasma membranes shown extensive protein crosslinking after exposure to 488 nm laser light (9). Irradiation of F-ConA labelled erythrocyte membranes has recently been shown to crosslink membrane proteins (10). The damage produced by the above photosensitizers is to cellular membranes.

This study deals with the action of light upon fluorescein conjugated concanavalin A (F-Con A) labelled plasma membranes of cultured fibroblasts. Polyacrylamide gel electrophoresis banding patterns are used to monitor membrane protein crosslinking and F-Con A crosslinking to membrane proteins after light exposures producing considerable fluorescein photobleaching. Photo-induced damage was assessed by cell killing at lower light levels.

MATERIALS AND METHODS

Cultured V-79 Chinese hamster lung fibroblasts harvested during exponential growth were used. Plasma membranes were isolated by a modification of the technique of Brunette and Till (11). Zn^{2+} was omitted during isolation and the plasma membranes were still obtained in large sheets with comparable purity and enrichment to that obtained by Brunette and Till. The plasma membranes were suspended in phosphate buffered saline (NaCl 136 mM, KCl 3 mM, Na_2HPO_4 8 mM, KH_2PO_4 1.4 mM, $MgCl_2$ 0.5 mM, $CaCl_2$ 0.5 mM), pH = 7.4, at 2 mg/ml protein and the fluorescein conjugated concanavalin A (F-Con A) (Miles Laboratories, Elkhart, Indiana) added at 1 mg/ml. The treated membranes were washed twice and resuspended at 1 mg/ml in phosphate buffered saline and irradiated as previously described with an argon ion laser at 488 nm (9). A power level of 1.5 watts and a beam diameter of 1.5 cm were

used. Exposure times of 0, 95, and 190 seconds photobleached 0, 26, and 53% of the fluorescein. The percent bleaching was determined by measuring the fluorescence intensity before and after bleaching with a spectrofluorometer using an excitation wavelength of 488 nm and an emission wavelength of 516 nm. The percent bleaching is exponential with time and can be used as a measure of light intensity. The above irradiation procedure gives a half-life of bleaching of 180 seconds. The membranes were solubilized by heating at 90°C for one hour in a solution of 1% sodium dodecylsulfate, 10% sucrose, 10 mM Tris-HCl (pH = 7.5), 1 mM EDTA, 40 mM dithiothreitol and 10 µg/ml pyronin Y (Tracking dye) and the membrane proteins separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Fairbanks et. al. (12). The gels were either stained with coomassie blue or sliced, the protein extracted from each slice with NCS (Amersham, Oakville, Ontario) and the fluorescence intensity of the extracted protein measured as described above.

Cell survival was determined by the colony assay method of Puck and Marcus (13). After removal with trypsin, cells were maintained in medium in suspension for three hours to repair trypsin damage. They were washed, resuspended at 1.5×10^4 cells/ml in phosphate buffered saline. The F-Con A was then added and the cells irradiated with a 200 watt Hg lamp filtered to remove ir and uv radiation. Irradiation with the Hg lamp gave a half-life of bleaching of about 60 minutes. Aliquots were removed at five minute intervals and plated to determine survival. All survival values were corrected for the plating efficiency of cells treated identically but maintained in the dark for the required time.

RESULTS AND DISCUSSION

SDS-PAGE protein profiles indicate extensive crosslinking of membrane proteins labelled with fluorescein isothiocyanate and photobleached (9). Fig. 1 shows the protein profiles obtained after labelling V-79 plasma membranes with F-Con A and photobleaching at 488 nm. Fig. 1A is unirradiated F-Con A alone (i.e. no plasma membrane) stained with coomassie blue. The major peak with a relative mobility (R_f) of 0.64 is the F-Con A monomer. The other peaks are impurities with the two major components having molecular weights of about 16,000 and 19,500.

The protein profile of V-79 plasma membranes treated with F-Con A but not exposed to light is shown in Fig. 1B. Peaks which may largely consist of the F-Con A monomer and two major impurities can be seen with R_f values of 0.64, 0.78, and 0.85. The profile of the same membranes after a 95 second exposure to laser light of 488 nm producing 28% bleaching of the fluorescein is shown in Fig. 1C. There are definite changes in the banding pattern; specifically the disappearance of the high molecular weight bands and a

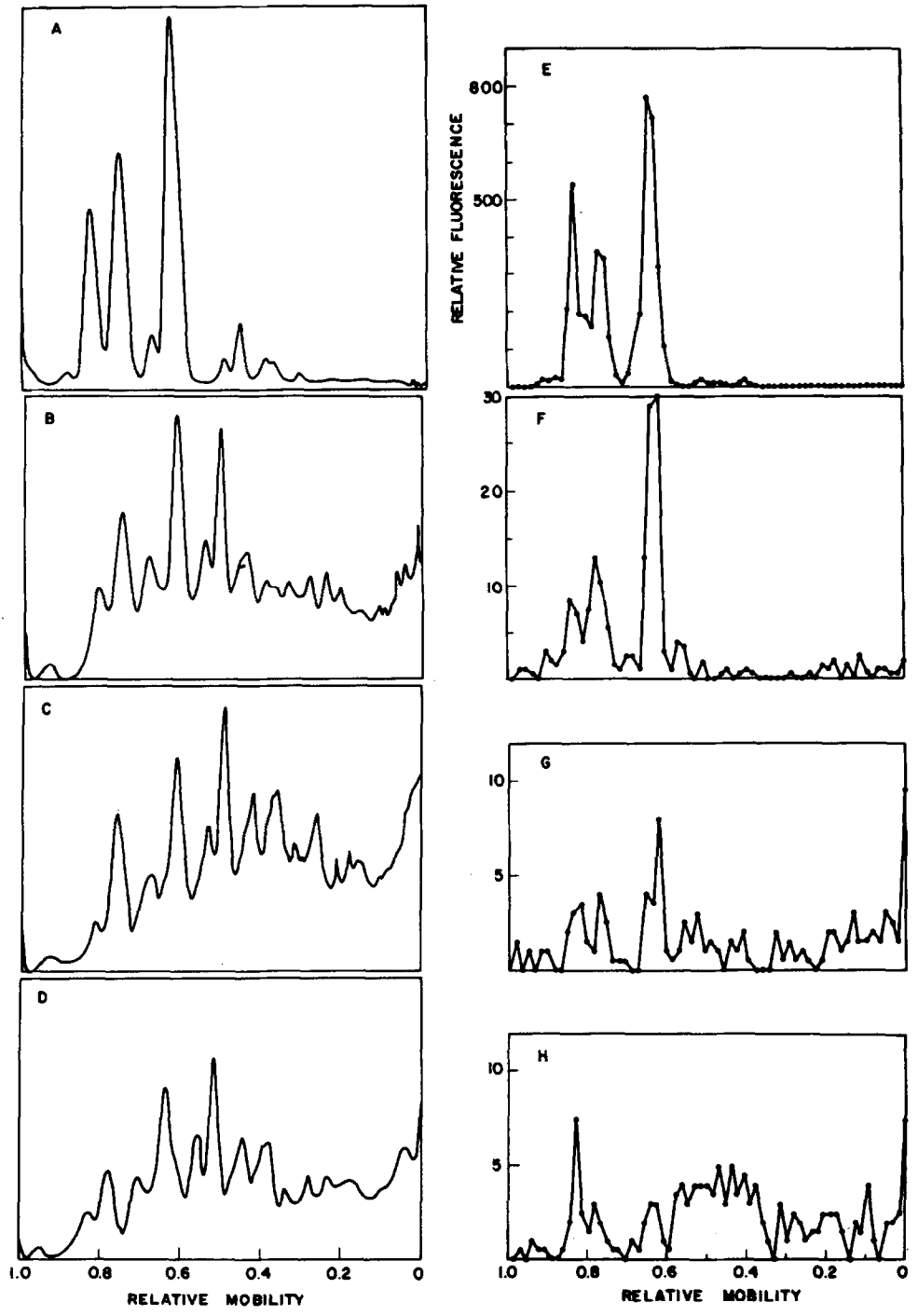

Fig. 1. SDS-PAGE profiles of F-Con A (A and E) and F-Con A labelled V-79 plasma membranes (B→D and F→H). Gels A→D are stained with coomassie blue and the absorbance is measured; gels E→H are sliced and the fluorescence of each slice measured. A and E, F-Con A; B and F, F-Con A labelled plasma membrane control; C and G, F-Con A labelled plasma membrane irradiated at 488 nm for 95 seconds; D and H, irradiated for 190 seconds.

Table I. The amount of fluorescently labelled material with R_f less than 0.58.

	Exposure, sec.	Total fluorescence, R_f less than 0.58,%
F-Con A	0	2.8
F-Con A labelled plasma membranes	0	15
F-Con A labelled plasma membranes	95	58
F-Con A labelled plasma membranes	190	75

slight build up of material at the top of the gel. This indicates some protein aggregation, probably covalently linked, which is not broken up by the SDS-heat treatment. There is also a decrease in the height of the peak with R_f = 0.64 corresponding to the F-Con A monomer. Thus the F-Con A may compose some of the crosslinked material. Fig. 1D is from a 190 second exposure producing 56% bleaching. The protein profile is similar to that in Fig. 1C with an additional decrease in the peak with R_f = 0.64. Similar crosslinking has been observed with erythrocyte membranes (10).

The gels shown in Fig. 1E-H are the same as the corresponding gels shown in Fig. 1A-D except that the fluorescence intensity along the gel is given rather than coomassie blue absorbance. The location of fluorescence should correspond to the location of the F-Con A and impurities. Fig. 1E is the fluorescence profile of F-Con A in the absence of membrane. Three major peaks are again seen as with the coomassie blue staining at the same mobilities. There is very little fluorescence (2.5%) with R_f less than 0.58. This corresponds to a molecular weight of about 34,000. Table I gives the integrated fluorescence intensity with R_f less than 0.58 from the gels shown in Fig. 1E-H.

Fig. 1F is a gel of plasma membranes labelled with F-Con A and not bleached. Most of the fluorescence is found in the F-Con A peak. The components with molecular weights of 16,000 and 19,500 are still present, but in a relatively smaller amount compared to the F-Con A. Thus these two major impurities appear able to bind to plasma membranes, but less effectively

than the F-Con A. About 15% of the fluorescence is found at an R_f less than 0.58 (Table I).

A light exposure of 95 seconds yields the gel shown in Fig. 1G. The height of the F-Con A peak is considerably reduced in intensity and 58% of the fluorescence is now found in the higher molecular weight region. The peak with greatest fluorescence is found at the origin and consists of material of very high molecular weight. A 190 second exposure produces a similar result (Fig. 1H) with a greater reduction in the F-Con A peak and more fluorescence (75%) in the higher molecular weight region.

The appearance of fluorescence after light exposure at higher molecular weight than the F-Con A indicates that the F-Con A is being cross-linked to other proteins. The crosslinking is most likely covalent since it is not broken by the SDS-heat treatment. The F-Con A appears to be cross-linked more effectively than the two lower molecular weight impurities. Crosslinking could be occuring between F-Con A molecules or between F-Con A and membrane proteins. A combination of the two processes would seem most probable, with a high degree of crosslinking between F-Con A and its membrane receptor.

The effect of a lower light intensity than that produced by a laser was investigated using cell death as an assay of damage. The 200 watt Hg lamp used produced a light intensity sufficient to bleach fluorescein with a half-life of about 60 minutes.

The percent survival of V-79 cells as a function of time of exposure to visible light after F-Con A treatment is shown in Fig. 2. There is some killing of the control cells not treated with F-Con A. This has been observed with WI-38 human fibroblasts and is probably due to photosensitization by intrinsic cellular chromophores that absorb in the visible region (5). The addition of F-Con A considerably increases the rate of cell killing. This is dose dependent with the cells treated with 100 µg/ml F-Con A killed faster than those treated with 10 µg/ml.

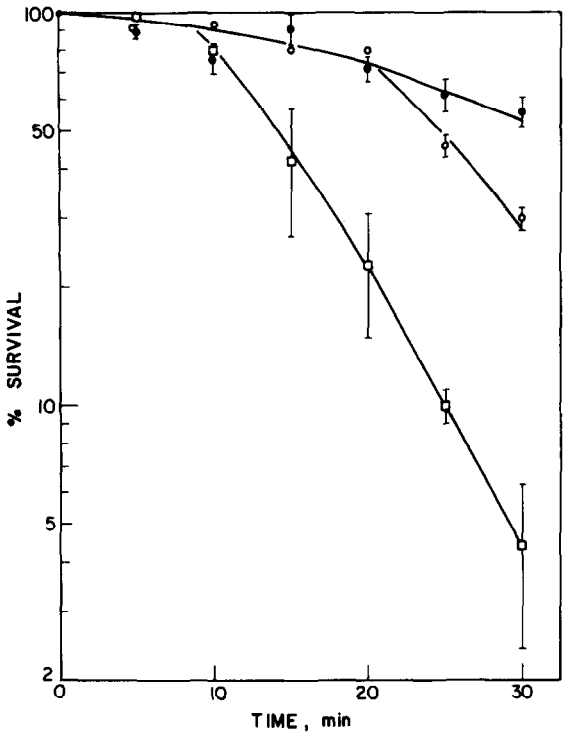

Fig. 2. Percent colony survival as a function of time of irradiation. •, Control Cells; o, Cells treated with 10 μg/ml F-Con A; □, Cells treated with 100 μg/ml F-Con A.

The survival curves are classical in shape with a linear and a shoulder region. The shoulder indicates that the cells can accumulate some damage before death ensues. For the 100 μg/ml F-Con A treated cells killing does not begin until they have been exposed for ten minutes (12% bleaching), but they are still being damaged during the first 10 minutes. This is a much lower light intensity than that used in photobleaching experiments and is comparable to that used for microscopic observation. The cells treated with 10 μg/ml F-Con A have a shoulder of about 20 minutes. A method other than cell killing would be required to detect this sub-lethal damage.

The F-Con A must attach to the cell for killing to occur. Exposure of cells treated with 100 μg/ml F-Con A and 4 mM D(+) mannose gives a survival curve similar to the control with no F-Con A present (not shown).

The irradiation of dye molecules can produce highly reactive compounds. One of these is singlet oxygen which oxidizes macromolecules (14)

and participates in the crosslinking of membrane proteins (7-10). These effects can lead to cell death (4,5) and should occur to some degree whenever fluorescently labelled ligands are used. The amount of cell killing is proportional to the concentration of fluorescent label and total light exposure. Thus damage can be limited by minimizing the amount of dye used and the light intensity. For visual observation, low light levels can be overcome with an image-intensified video camera (3). This may be particularly important for following ligand-receptor movement since covalently linked ligand receptor complexes may behave differently than ligands weakly bound to their receptors.

Because of the large amount of bleaching required for diffusion measurements some crosslinking should occur during photobleaching experiments, particularly of the fluorescently conjugated ligand to membrane proteins. Whether this will affect the diffusion coefficients obtained is not clear since some damage may be tolerated. Jacobson et. al. could detect no visual change in F-Con A labelled cells after bleaching a small spot on the surface (15). This and other observations led them to conclude that photo-induced damage is not a problem during photobleaching. But whenever fluorescent molecules are exposed to light there is the possiblity of photodamage which should be taken into account when planning and performing experiments.

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