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INTRACELLULAR DYE HETEROGENEITY DETERMINED BY FLUORESCENCE LIFETIMES

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The cellular localization of a fluorescent probe molecule depends on both the chemical structure of the dye and the cellular environment. To study the number and types of environments in an epithelial cell line, we have measured in Madin-Darby canine kidney (MDCK) cells the fluorescence lifetimes of three structurally distinct fluorescent dyes – rhodamine-B, 3,3′-dihexadecylindocarbocyanine-(C_3) (diI), and Collarein – incorporated into these cells. The latter is a rhodamine-cardiolipin conjugate that we designed and synthesized for the property of exclusive localization in the plasma membrane. The former two dyes required at least two exponential components to fit their fluorescence decay curves, while the decay of Collarein was characterized by a single exponential. These data are consistent with fluorescence microscopic observations, in which diI and rhodamine-B exhibit heterogeneous spatial distributions, while Collarein appears to be located on the cell surface.

Introduction

The use of molecular probes for studying cellular phenomena requires knowledge of probe locations [1]. For fluorescent molecules, locations are usually determined by inspection under a fluorescence microscope, steady-state fluorescence spectroscopy, and/or subcellular fractionation [2,3]. The first method of detection, within its resolution limit, presents the spatial distribution, but environmental information of a very limited nature, the second is often of insufficient environmental sensitivity to distinguish between cellular compartments, and the third requires destructive manipulations. Measurement of fluorescence lifetimes, a type of kinetic spectroscopy, is a noninvasive method for determining fluorescence compartmentalizations and may provide a complementary or additional class of information in cases where any or all of the currently used methods are deficient.

Fluorescence is the phenomenon of radiative decay from an excited molecular singlet state to the ground state. The detailed nature of the molecule's environment influences its rate of decay [4]. For example, increased proximity to or concentration of a molecular species which can accept energy from the excited state of a probe molecule results in a decrease of the latter's fluorescence lifetime; this is observed in photosynthetic organisms [5]. Additionally, characteristics of the surrounding medium such as polarity, dielectric constant, pH, metal ion concentration, viscosity or hydrophilicity may also affect excited-state lifetimes [6].

We have used this principle of variation in lifetimes with environment to measure quantitatively the fraction of fluorescence in different cellular compartments in parallel with visualization under the fluorescence microscope. We observed that after brief periods of incubation with different dyes, cells of the Madin-Darby canine kidney (MDCK) line exhibited particular patterns when examined under the fluorescence microscope. Based on the known sensitivities of fluorescent

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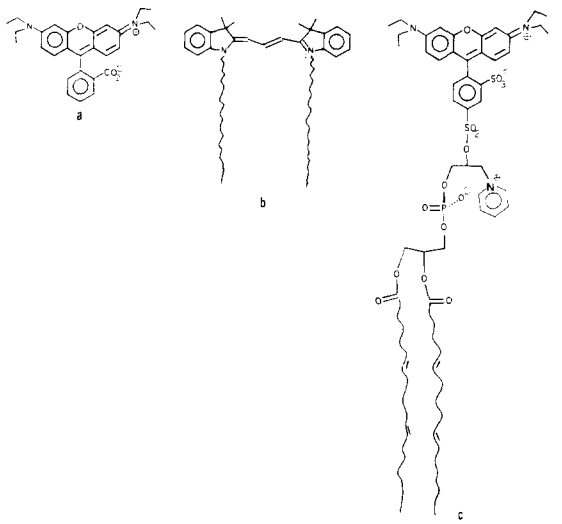

Fig. 1. Structures of three fluorescent dyes: (a) rhodamine-B; (b) the carbocyanine dil with two C_{16} chains and a three-carbon bridge; (c) Collarein.

molecules to their environments, we predicted that partitioning of such molecules among physically and/or chemically distinct cellular compartments would give rise to a distribution of fluorescence lifetimes.

We now report that subsequent to incubation with rhodamine-B (Fig. 1a) or 3,3′-dihexadecylindocarbocyanine-(C_3) (dil) (Fig. 1b), MDCK cells exhibit spatial heterogeneity under the fluorescence microscope and multiple component lifetimes. In contrast, Collarein (Fig. 1c), a rhodamine-cardiolipin conjugate that we designed and synthesized for the property of localization in the plasma membrane, appears to be homogeneously and exclusively distributed over the cell surface; this dye exhibits a single-component lifetime. In addition, we report the long-term time-dependent behavior of these fluorescence distributions.

Thus, effective monitoring of intracellular fluorophore compartmentalization and its time evolution in viable cells is possible by use of the presently reported technique.

Materials and Methods

Cells. Monolayer cultures of Madin-Darby canine kidney (MDCK) cells, obtained from the Laboratory of Cell Biology, Lawrence Berkeley Laboratory, University of California, Berkeley, CA, U.S.A., were grown to confluence on glass coverslips (approx. 10×22 mm) in Modified Eagle's Medium (GIBCO, Grand Island, N.Y., U.S.A.) containing 10% fetal calf serum (GIBCO) and 20 mM Hepes buffer (Sigma) at pH 7.4, 310 mosM and 37°C. After two washes with serum-free medium, cells were exposed to serum-free media containing fluorescent dyes for periods of time specific for each dye and dye concentration. After six washes with phosphate-buffered saline, coverslips were either examined and photographed under a Zeiss fluorescence microscope or transferred to cuvettes, which were then placed in the fluorescence lifetime apparatus.

For radioactive tracer studies, cultures were exposed to 0.05% trypsin (Difco, Detroit, MI, U.S.A.) for 15 or 25 min after isotope exposure and subsequent washings. (The number of adherent cells remained constant pre- and posttrypsin-treatment due to the fact that MDCK cells contain tight junctions and the trypsin solution was free of any chelating agent.) Following six more washes, cells were exposed to 2.0% sodium dodecyl sulfate for 2 min, removed with a rubber policeman, and counted for ^{14}C incorporation.

Dyes. Rhodamine-B (Sigma Chemical Co., St. Louis, MO. U.S.A. or laser grade from Eastman Kodak Chemicals, Rochester, NY, U.S.A.) was diluted to a final concentration of 8 μg/ml. DiI, synthesized by the method of Sims et al. [7], was either diluted via ethanol (final concentration 0.4%) or complexed to lipid-free albumin (Sigma and/or ^{14}C-labeled from Miles Laboratories, Elkhart, IN, U.S.A.) at a ratio of two dye molecules to one protein [8]; the concentration of dil in both incubation media was 2 μg/ml.

Collarein synthesis Lissamine rhodamine-B sulfonyl chloride (Molecular Probes, Junction City, OR, U.S.A.) and cardiolipin (PL Biochemicals, Inc., Milwaukee, WI, U.S.A. or Sigma) in a 2 : 1 ratio were stirred in dry pyridine for 24 h at room temperature. After removal of the solvent, the product was spotted on silica-gel plates, which were developed in chloroform/methanol/water (65 : 25 : 4). The single spot with an R_F of 0.70 was scraped and showed spectral properties consistent with the structure drawn in Fig. 1c.

Lifetime measurements. Fluorescence lifetimes were measured by a single-photon timing system using a previously described apparatus [9,10] which contains a Spectra Physics synchronously pumped mode-locked dye laser (SP 171 argon ion laser, SP 362 mode locker, and modified SP 375 dye laser) operating with rhodamine 6G with a 12 ns pulse separation and a pulse full-width at half-maximum of approx. 8 ps. The limit of resolution of the instrument is approx. 25 ps. Excitation of samples was at 580 nm and emission was detected at 650 nm with a cooled RCA C31034A photomultiplier. Cells were counted long enough to acquire a set number of photons in the peak channel of a 1024-channel Northern NS636 multichannel analyzer. The response function (curves labeled E in Figs. 3, 5, 6 and 7) of the apparatus was determined by measuring the scattering of the pulsed laser light at the excitation wavelength (580 nm) from an unlabeled cell sample or a colloidal suspension of Ludox particles. Background scattering by the Ludox particles at the emission wavelength rarely exceeded the dark count rate. The scattering function was deconvolved from the raw data leading to the true decays. The latter were then fit with an assumed decay law approximated by a sum of exponentials.

$$I(t) = \Sigma \alpha_i \exp(-t/\tau_i)$$

where α_i is the fraction, normalized to 1, of molecules with lifetime τ_i. Minimizations of the deviations from this sum were guided by determining the least number of terms for convergence [11]. Deviations from attempted fits are indicated by th · pattern and magnitude of ΔI.

Results

Incubation of MDCK cells with rhodamine-B. Cells, incubated with rhodamine-B for 3 min at room temperature, indicated the presence of more than one fluorescent component both under the fluorescence microscope (Fig. 2a) and by measurement of the fluorescence decay in the fluorescence lifetime apparatus (Fig. 3). Under the microscope, fluorescence appeared to be distributed mainly in two locations: the apical plasma membrane and vesicular accumulations exclusive of the nuclear

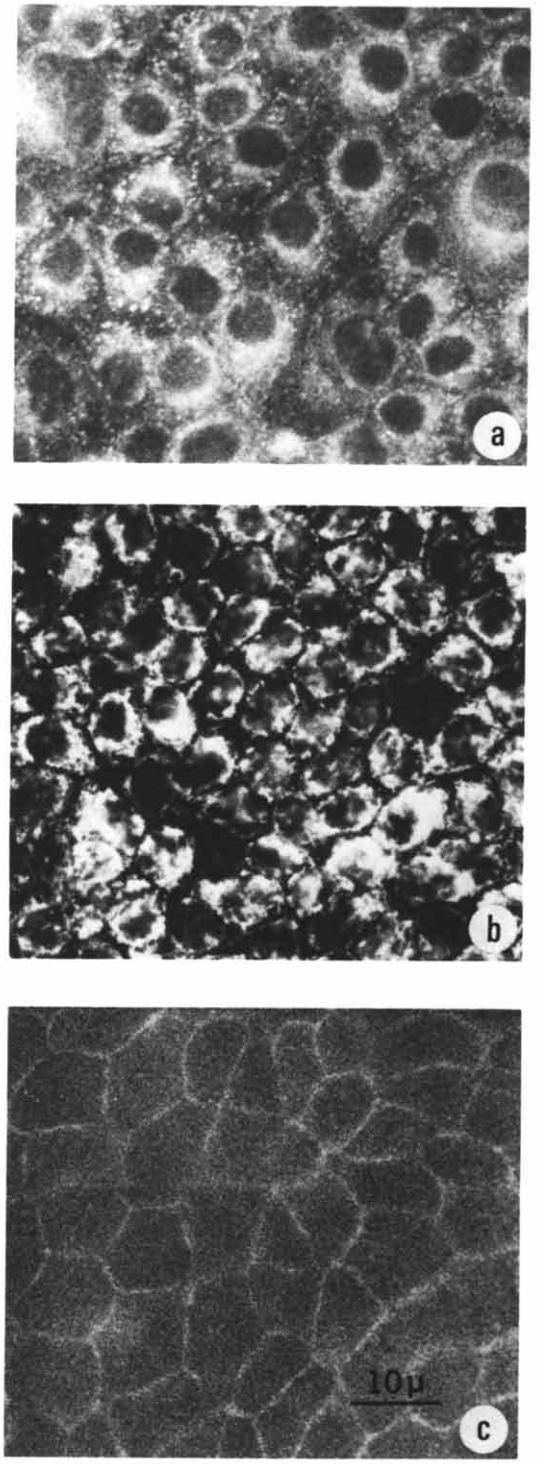

Fig. 2. Fluorescence micrographs of MDCK cells stained with (a) rhodamine-B, 60 min after incubation; (b) diI; and (c) Collarein.

region. A single exponential component fit to the measured fluorescence decay curve gave large deviations (Fig. 3a). (ΔI, units of 1000). A two-exponential component fit (Fig. 3b) resulted in a significant improvement as indicated by the pattern and decrease in ΔI units from 1000 to 400. Inclusion of a third component did not statistically improve the fit. Thus, the molecule has at least two environments which lead to two resolvable lifetimes.

A time-dependent study showed (Fig. 4) that the normalized fraction of the long-lifetime component decreased linearly over a 120 min timecourse, while the short lifetime component showed the inverse behavior. (The sum of amplitudes of the two components, α_1 and α_2, are normalized to unity at each time point.) Additionally, the total fluorescence yield decreased for the first 60 min, after which it leveled off. The latter may be due to a catabolic mechanism which approaches saturation at 60 min.

MDCK cells plus dil. As dil is water insoluble, it requires a carrier. We tried two: ethanol and lipid-free albumin. Time of incubation for both was 5 min at room temperature. Delivery via either carrier resulted in a similar fluorescence distribution under the fluorescence microscope (Fig. 2b).

A single exponential decay component fit for data obtained from cells incubated with dil delivered via ethanol showed large deviations (Fig. 5a). We employed the exponential series mode to fit these data since the computer was unable to

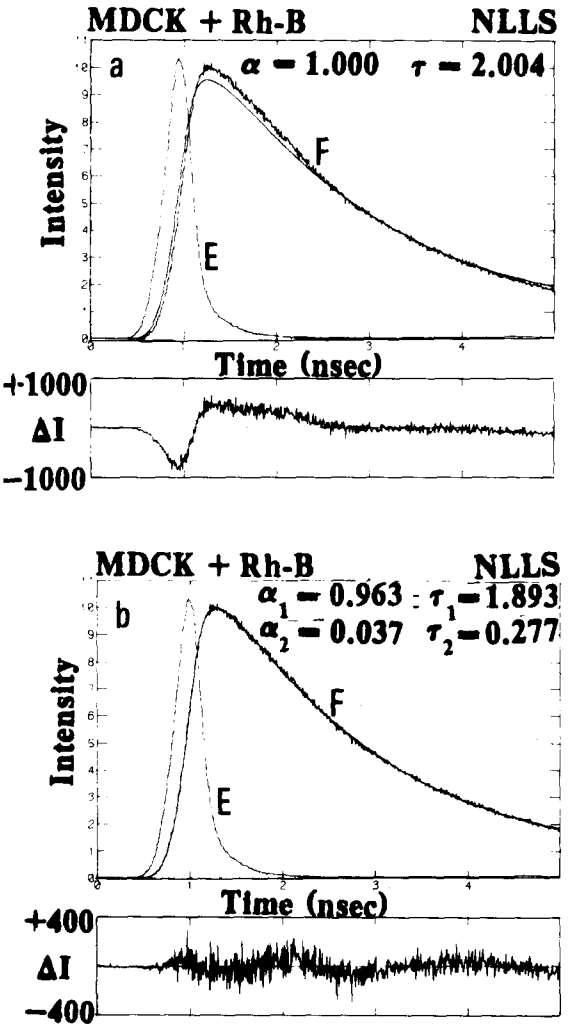

Fig. 3. Fluorescence decay curve of MDCK cells subsequent to incubation with rhodamine-B. Curves labeled E are the excitation profiles induced by the laser pulse and the response of the system. Curves labeled F are the experimental fluorescence decay (noisy) and the calculated fit (smooth) essentially superimposed. Deviations plots are below. Fits for: (a) single exponential; and (b) sum of two exponentials. The ordinates in all cases are in photon counts.

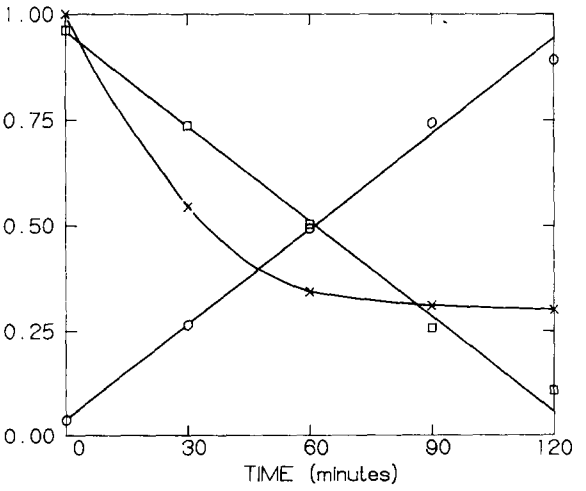

Fig. 4. The normalized amplitudes of long-lifetime (α_1) (□——□), short-lifetime (α_2) (○——○) and total (normalized to $t = 0$ value) fluorescence yield (FFY) (×——×) as functions of time after incubation with rhodamine-B. (Amplitudes are normalized such that $\Sigma\alpha_i = 1$ at each time point.) (The long-lifetime component at time $t = 0$ is always present in greater abundance than the short; however, exact slopes vary depending on cell culture passage number.)

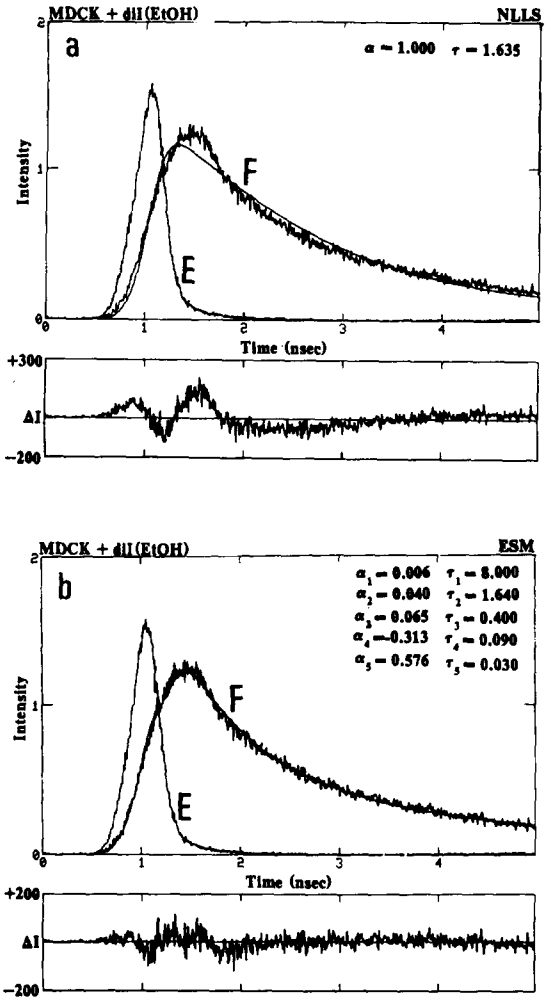

Fig. 5. Fluorescence decay curve for MDCK cells plus dil introduced via ethanol. (a) single exponential and (b) sum of five exponentials.

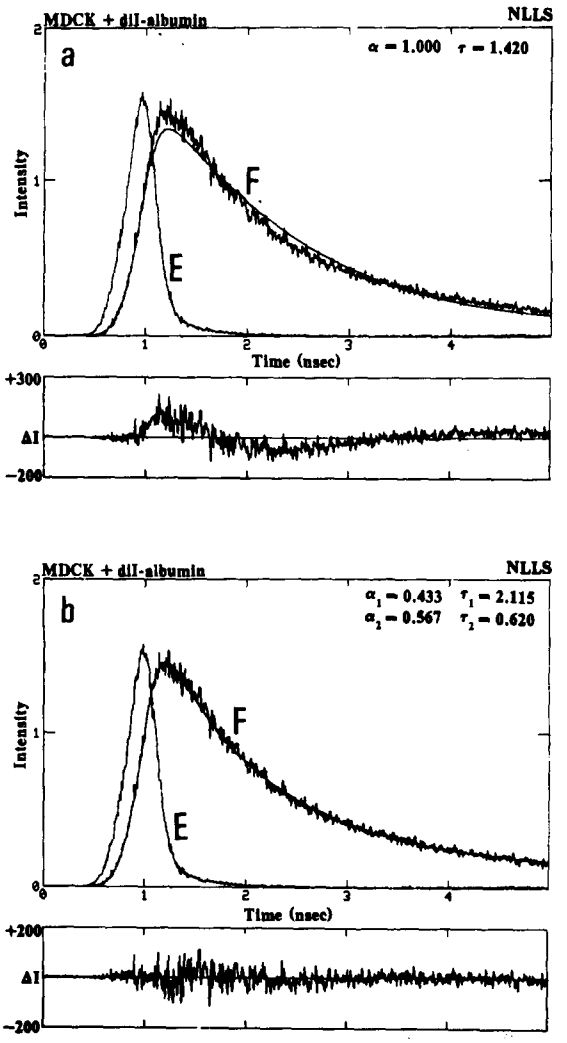

Fig. 6. Fluorescence decay curve for MDCK cells plus dil introduced via albumin complexation. (a) single exponential and (b) sum of two exponentials.

attain a reasonable fit in the normal nonlinear least-squares mode due to its limit of resolving four-exponential components. Fig. 5b shows the most reasonable fit attained – five components.

A single component showed large deviations for data from dil delivered via albumin (Fig. 6a). However, the computer was able to minimize effectively deviations with two components (Fig. 6b).

For dil delivered via either system the number and relative fractions of molecules with each lifetime remained fairly constant for 120 min, with spectra taken every 30 min. (The lifetimes of dil in acellular phosphate-buffered saline, solubilized via either carrier, was predominantly 45 ps.) However, total fluorescence yields were dramatically different between the two systems. For ethanol delivery the fluorescence yield remained constant, increasing just slightly over the 120 min time-course. In contrast, dil delivered via albumin, showed a 3-fold increase in fluorescence yield, leveling off between 75 and 120 min.

The dil-albumin experiment was performed four times. Qualitatively, data were the same, i.e., two

lifetime components and the fluorescence yield increasing, finally leveling off; quantitatively, lifetimes varied within limits: for the long-lifetime component, 1.18 to 2.50 ns and for the short-lifetime component 0.36 to 0.79 ns, with fluorescence yield leveling off at 75 to 120 min.

To probe the fluorescence yield increase further, dil was delivered via ^{14}C-spiked albumin. Following a 5 min incubation with this radioactive complex, cells were exposed to 0.05% trypsin for 15 or 25 min. After six washes with phosphate-buffered saline, 50–60% of the radioactivity remained with the cells and fluorescence data were fit by two exponentials. However, the fraction of molecules with the long lifetime was significantly decreased (less than 10% as opposed to 20–46% without trypsinization) and the lifetime of this component was significantly increased. (In some cases, decay was not completed by the next laser pulse.)

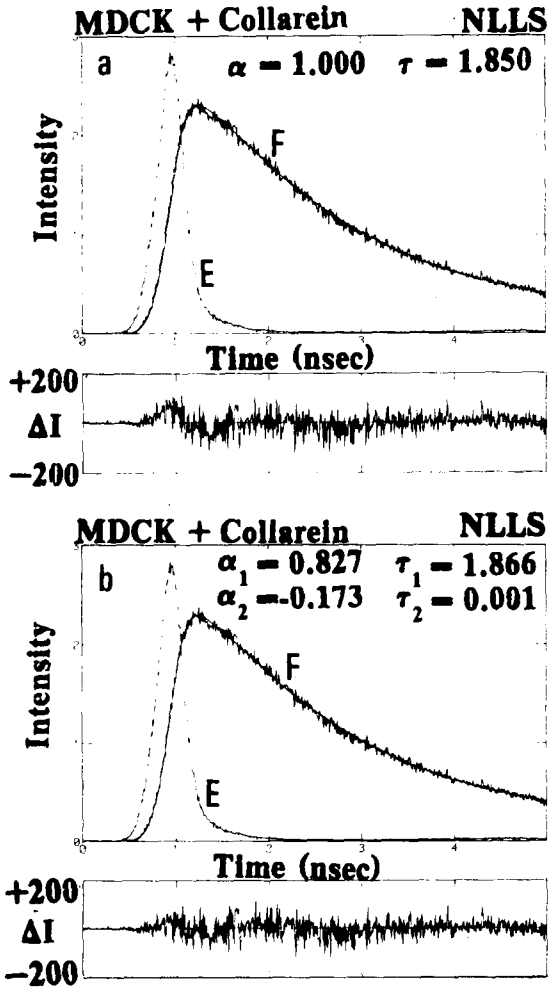

Fig. 7. Fluorescence decay curve for MDCK cells after exposure to Collarein. (a) single exponential and (b) two exponentials. (The lifetime of Collarein in phosphate-buffered saline is approx. 1.5 ns.)

Additionally, when medium containing the dil-albumin complex was pipetted onto the cellular monolayer and removed immediately, a small but finite amount of radioactivity, that was trypsin insensitive, remained associated with the cells. Examination under the fluorescence microscope indicated that all fluorescence appeared in vesicular structures, exclusive of the nuclear region.

MDCK cells plus Collarein. Exposure of cells to Collarein resulted in a homogeneous distribution of fluorescence over the cell surface, both the apical and basolateral aspects (Fig. 2c). The fluorescence decay curve of Collarein is fit with a single exponential (Fig. 7a). When a two-component fit was attempted, a second component with a lifetime (1 ps) below the limit of resolution of the instrument and a negative amplitude appeared (Fig. 7b). Single exponentials showed deviations due to just statistical noise to data acquired at 30-min intervals over a 4.5 h period. The total fluorescence continuously decreased over time.

Discussion

Fluorescence lifetime is a function of environment. Thus, the number of spectroscopically distinguishable environments a fluorophore occupies can be reflected in the multiplicity of its lifetime components. We have used the functional dependence of fluorescence lifetimes on environment to study the kinetics and multicomponent behavior of three fluorescent molecules.

When cells that have been incubated with rhodamine-B for three minutes are examined under a fluorescence microscope, the fluorescence appears to be on the apical surface and in intracellular vesicles. (The initial entry of dye into the cells is probably by diffusion as evidenced by the rapid time-course.) The fluorescence lifetime decay curve is best fit by two exponentials, with time constants of about 1.9 and 0.3 ns (Fig. 3b). As time progresses, the plasma membrane compo-

nent is endocytized (probably by an adsorptive mechanism) [12], and the concentration of fluorescence in the vesicular compartment increases (Fig. 2a); the decay curves are fit in temporal sequence by an increasing fraction of short lifetime component (Fig. 4). It is reasonable to ascribe the decrease in lifetime when going from the cellular surface to the intracellular vesicles to the increasing proximity of dye molecules to each other, making quenching more facile. This mechanism is suggested by the decrease in lifetime observed when these measurements are performed on a series of acellular, graded (from 8 to 800 μg/ml) solutions of rhodamine-B in phosphate-buffered saline. (Packard, B., unpublished observations: a five-solvent study in which the concentration of rhodamine-B was varied over this range and was studied by several spectroscopic methods supports this interpretation.) The decrease in overall fluorescence is probably due principally to cellular catabolism as the fluorescence yield is also diminished in dye containing cells that have been kept in the dark.

When diI was delivered by either ethanol dilution or as part of an albumin complex, the spatial distribution of fluorescence was not significantly different (Fig. 2b). However, ethanol may induce sufficient cellular alteration that the dye molecules take up a near continuum of environments and, hence, exhibit a corresponding distribution of lifetimes. Nonexponential behavior and a negative amplitude of one component (α_4) indicative of energy transfer are suggested in the data analysis. (Nonrandom behavior in the deviations plot over the time interval corresponding to the exciting pulse may be a reflection of a rise time and/or some Raman scattering.) This type of reasoning would help to account for the large number of components (five) required for a reasonable fit (Fig. 5). However, as the computer program is inefficient at resolving more than four exponential components, we question the significance of these data and fit.

In contrast, data from cells to which diI was delivered as part of an albumin complex were fit by a sum of two exponentials (Fig. 6b); curves were almost superimposable (slight shortening of the decay time) over a 120 min time-course. This two-component behavior in conjunction with the increasing fluorescence yield is consistent with there being a total of three cellular compartments in which the dye can reside. Initially, it can bind to both the plasma membrane and intracellular vesicles. With time the surface component may be endocytized into vesicles. (As with rhodamine-B, the fraction of vesicular dye at time $t = 0$ probably has entered the cell by a diffusive process with later internalization due to adsorptive endocytosis.) Fluorophores can then redistribute into intracellular membranes from which a small fraction may recycle back to the plasma membrane, perhaps as part of the normal cellular membrane recycling [12]. The environments within the two membrane compartments may be expected to be fairly similar and, thus, when populated at similar concentrations, it is reasonable to expect lifetimes to be fairly close. In nontrypsin-treated cells lifetimes within a 1.18–2.50 ns range are observed. The increase in the long-lifetime component observed in cells treated with trypsin, an enzyme thought to act only on surface proteins, can be explained by a decrease in fluorophore concentration in the plasma membrane compartment, resulting in a decrease in fluorophore-fluorophore proximity and, thus, less efficient quenching. As with rhodamine-B, the increased mutual proximity of dye molecules in the vesicles would be expected to result in decreased lifetimes. If the fluorescence yields in the various compartments differ and fluorophores are traversing from one to another, then only when steady-state levels are approached would the fluorescence yield be expected to level off [13].

In contrast to rhodamine-B and diI, Collarein, a fluorescent dye that we designed and synthesized for the property of exclusive localization in the plasma membrane, exhibits homogeneous fluorescence over the cell surface under the fluorescence microscope (Fig. 2c); decay curves up to 4.5 h after removal of the dye containing medium were fit by single exponentials with deviations due only to statistical noise (Fig. 7). These data are consistent with this synthetic, fluorescent phospholipid having a single environment when in a fluorescent state. (The appearance of the 1 ps lifetime is physically meaningless in an instrument with a limit of resolution of approx. 25 ps.) The observed decrease in fluorescence yield is probably due to catabolism or conversion to an otherwise nonfluorescent state.

We are planning experiments to differentiate among the mechanisms of lifetime variation, which are operative in the several cellular compartments.

We are applying lifetime measurements to molecules known to transmit biologic information [14]. There are many indications in the literature that transmittance is strongly affected by the environment of the transmitting medium [15–18]; we are interested in defining the degree of environmental heterogeneity necessary for resolution of lifetimes.

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