Oxidized phospholipids in serum initiate severe pathophysiological responses during the process of atherogenesis. On the cellular level it is known that these lipids induce apoptosis; however, the uptake mechanism remains enigmatic. We investigated here the behavior of the fluorescent oxidized phospholipid 1-palmitoyl-2-glutaroyl-sn-glycero-3-phospho-N-Alexa647-ethanolamine (PGPE-Alexa647) in the plasma membrane of various cell lines. The probe was taken up by the cells unspecifically via caveolae or clathrin-coated pits. Interestingly, we found the uptake to be facilitated by the overexpression of the scavenger receptor class B type I. Ultra-sensitive microscopy allowed us to follow the uptake process at the single molecule level; we observed rapid diffusion of PGPE-Alexa647 in the plasma membrane, interrupted by transient halts with duration of ~0.9 s at endocytotic sites. Scavenger receptor class B type I overexpression yielded a pronounced increase in the single molecule mobility, and in consequence an increased frequency of immobilization. Alternatively, the plasma membrane fluidity could also be increased by treating cells with high levels of the unlabeled oxidized phospholipid 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine; also in this case, the immobilization frequency of PGPE-Alexa647 was concomitantly increased. The data demonstrate the relevance of plasma membrane properties for uptake of oxidized phospholipids, and indicate a novel indirect mechanism for the control of endocytosis.

Oxidation of low density lipoprotein (LDL) is known to be a key step in atherogenesis, leading to inflammation, proliferation, and apoptosis of cells of the arterial wall. These effects are largely exerted by oxidatively fragmented phospholipids, which are highly exchangeable between cells, tissues, and lipoproteins. In particular, 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC) has been identified in minimally modified LDL and has been reported to elicit a wide range of pathophysiological responses in vascular cells, e.g. the activation of apoptotic signaling pathways. Fast uptake of synthetic PGPC analogues to the perinuclear region of vascular smooth muscle cells has been reported and discussed in view of its ability to activate acid sphingomyelinase. The uptake mechanism and sites of action, however, still remain enigmatic.

PGPC carries an acyl fragment in the sn-2 position, which represents the oxidation product of an unsaturated acyl chain. This hydrophilic side chain is expected to be folded to the polar region of the membrane, yielding a structure best described by a lysolipid-like inverted cone. Addition of such nonbilayer lipids to model membranes is known to bend lipid monolayers, and it may well assist in the formation of highly curved structures also in cell membranes. In turn, such lipids are expected to become energetically trapped and thereby enriched in bent membrane regions. It can therefore be expected that local enrichment of PGPC may exert dramatic changes onto the structure of the plasma membrane, whereas low amounts of PGPC may be a proper tool to sense highly curved plasma membrane regions.

In this study, we investigated the uptake mechanism of oxidized phospholipids in living cells at the single molecule level. The movement of individual fluorescent phospholipids could be readily imaged using ultra-sensitive fluorescence microscopy, yielding trajectories characteristic for free Brownian motion, which were frequently interrupted by transient halts. We provide evidence that the observed halts correspond to the unspecific trapping of the molecules in endocytotic structures such as caveolae and clathrin-coated pits; the association and dissociation kinetics were determined and related to the mobility of the diffusing probe in the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Synthesis of Reagents**—The synthesis of oxidized phospholipids PGPC and Alexa647-labeled analogues (1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine and PGPE-Alexa647) was conducted as described in the Experimental Procedures section.

**Abbreviations**

- LDL: low density lipoprotein
- CHO: Chinese hamster ovary
- HIE: human embryonic
- HILO: highly inclined and laminated optical sheet
- oxPL: oxidized phospholipid
- PGPC: 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine
- PGPE-Alexa647: 1-palmitoyl-2-glutaroyl-sn-glycero-3-phospho-N-Alexa647-ethanolamine
- SR-BI: scavenger receptor class B type I
- YFP: yellow fluorescent protein
- DMEM: Dulbecco’s minimal essential medium
- FCS: fetal calf serum
- MSD: mean square displacement
teroyl-sn-glycero-3-phospho-N-Alexa647-ethanolamine) was performed as described in Ref. 7.

Cell Lines and Tissue Culture—Chinese hamster ovary (CHO) cells, lacking LDL receptor activity (IdlA7 cells) and IdlA7 cells expressing high levels of recombinant SR-BI (IdlA7-SRBI), were the kind gift of M. Krieger, Massachusetts Institute of Technology. Cells were maintained in medium A (1:1 mixture of Dulbecco’s minimal essential medium (DMEM) and Ham’s F-12 medium with 1% penicillin/streptomycin sulfate (PAA Laboratories, Linz, Austria), supplemented with 5% FCS Gold (PAA Laboratories) and 0.25 mg/ml G418 (PAA Laboratories)). Human embryonic kidney cells (HEK), kindly provided by K. Groschner, Karl-Franzens-University Graz, Austria, were maintained in DMEM (PAA Laboratories) supplemented with 10% FCS Gold, 1% penicillin/streptomycin. HEK cells stably transfected with Caveolin-YFP were maintained in DMEM supplemented with 10% FCS Gold, 1% antibiotic-antimycotic (Sigma), and 0.8% G418.

Fluorescence Labeling of Cells—Cells were incubated at 37 °C with PGPE-Alexa647 at concentrations ranging from 0.1 to 50 nM directly from buffer solution (PBS: 170 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) on the microscope stage. Experiments were performed immediately after incubation. For colocalization studies with transferrin, HEK cells were incubated with 4 nM PGPE-Alexa647 at 4 °C for 30 min, rinsed with PBS before the measurement, mounted on the microscope stage, incubated with 0.05 µg/ml transferrin-Alexa555, and then imaged in two colors. To measure colocalization with Caveolin-YFP, HEK-Caveolin-YFP cells were incubated with 4 nM PGPE-Alexa647 at 37 °C for 30 min.

Microscopy and Image Processing—Single molecule experiments were performed as described in detail elsewhere (13). In brief, samples were mounted on a modified epifluorescence microscope (Axiovert 200 TV, Zeiss, Oberkochen, Germany) placed inside a home-built temperature-controlled incubator (T = 37 °C). The 647 nm line of a krypton laser (Innova 300, Coherent, Santa Clara, CA) was used for excitation of Alexa647, the 514 nm line of an argon laser (2020 series, Spectra Physics, Mountain View, CA) for excitation of YFP or Alexa555. Samples were illuminated with excitation intensities up to 15 kilowatts/cm² for illumination times t_ill ranging from 1 to 5 ms. Precise timing of t_ill and the delay between two consecutive illuminations t_delay was achieved using acousto-optical modulators (Isomet 1205C). Emitted fluorescence was collected using a ×100 oil-immersion objective (Plan-Apochromat NA = 1.4 or α-Plan Fluor NA = 1.45 for HILO microscopy, Zeiss). Emitted signals were split into two-color channels using a custom-made dichroic wedge (Chroma) and imaged on a back-illuminated, liquid-nitrogen cooled CCD-camera (Micro Max 1300-PB, Roper Scientific, Trenton, NJ). Dichroic emission filters and the wedge coating were optimized for simultaneous Alexa555 and Alexa647 imaging.

Excitation via HILO was set up as described (14). Starting from TIR excitation, the inclination of the excitation beam was reduced slightly below the critical angle, so that penetration into the measurement chamber became observable. A field stop was used to confine the range of the excitation light sheet. We used the HILO approach in Fig. 3, Fig. 4, A and C, Fig. 5, and Fig. 6B.

To facilitate spectroscopic separation for the identification of two different dyes in colocalization experiments, we used a timing protocol as described previously (15). In brief, we toggled the illumination between 514 and 647 nm excitation with a delay of 1 ms, so that vesicles essentially did not move between the two excitation pulses. Images were further split in emission using the dichroic wedge described above. By this, we measure four images corresponding to each time point on a macroscopic time scale. A sequence of such image quadruples was recorded with a time delay of typically 1 s.

All images were analyzed and processed using MATLAB algorithms. Probability density functions were generated using the built-in MATLAB function ksdensity (kernel “epanechnikov”). For determination of colocalization, images were corrected for autofluorescence as described previously (15). To correct for image distortions in two-color microscopy, TetraSpeck™ fluorescent microspheres (100 nm diameter, Molecular Probes, Eugene, OR) were used as fiducials to calculate an image correction matrix (16); all two-color images were corrected accordingly.

For vesicle colocalization (Fig. 1), we identified endocytosed and mobile PGPE-Alexa647 positive structures. Only those vesicles were counted as being colocalized, where co-movement over multiple frames was observed in the green and red color channel.

Single Molecule Analysis—Images were analyzed using in-house algorithms implemented in MATLAB (MathWorks). Individual diffraction limited signals were selected and fitted with a Gaussian intensity profile, yielding the single molecule position ħ(t) with accuracy σ. Single molecule trajectories were reconstructed according to previous studies (13), and the mean square displacement shown in Equation 1,

\[ \text{MSD}(t_{lag}) = \langle (\hat{r}(t + t_{lag}) - \hat{r}(t))^2 \rangle \quad \text{(Eq. 1)} \]

was calculated as a function of the time lag t_{lag} = t_{ill} + t_{delay}. Lateral diffusion constants D were determined according to MSD = 4D_{lag} + 4\sigma_{xy}^2 (17), with \sigma_{xy} specifying the localization precision of ~40 nm.

Periods of transient immobilization were identified based on a threshold criterion for the observed step-length l. Two consecutive observations of l < \sqrt{4 \cdot 0.03 \, \mu m^2/s \cdot t_{lag}} were classified as immobile and the rest as mobile. With this criterion, erroneous classifications of less than 0.1% are expected.

Photobleaching limits the total length of single molecule trajectories, thereby introducing a bias toward shorter lifetimes of the individual modes of motion. To correct for this effect, we derived an analytical description of the respective probability distribution of the measured immobilization times. Let us assume that the duration of the two modes of motion, t_{off} for periods of immobilization and t_{on} for free diffusion, can be described by a Poisson process, i.e., the probability density functions are given by Equation 2,

\[ p_{off, on}(t_{off, on}) = \frac{1}{t_{off, on}} \exp\left(-\frac{t_{off, on}}{t_{off, on}}\right) \quad \text{(Eq. 2)} \]

with t_{off, on} the respective expectation values. Photobleaching is introduced by an exponential distribution (18) with a characteristic bleaching time t_{ble} according to Equation 3,
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\[ p_{\text{off}}(t_{\text{bl}}) = \frac{1}{\tau_{\text{bl}}} \exp\left( -\frac{t_{\text{bl}}}{\tau_{\text{bl}}} \right) \]  
(Eq. 3)

which limits the total length of individual trajectories irrespective of the mode of motion. We can therefore expect the observation of trajectories of the following types: (i) no transition, i.e., one mode of motion for the whole observation period; (ii) one transition, i.e., different modes of motion in the beginning and in the end; (iii) two transitions, i.e., the same mode of motion in the beginning and in the end, interrupted by the second mode. If more than two transitions were observed, only the first two transitions were taken into account. Note that only case iii allows for the measurement of a duration \( \tau_{\text{off}} \) or \( \tau_{\text{on}} \).

The probability distribution for measuring transient immobilization within the time window \((t_{\text{off}}, t_{\text{off}} + dt_{\text{off}})\) according to case iii is given by Equation 4,

\[ p_{\text{off}}(t_{\text{off}})dt_{\text{off}} = \frac{\tau_{\text{on}}}{\tau_{\text{off}} + \tau_{\text{on}}} p_{\text{off}}(t_{\text{off}}) \cdot p_{\text{bl}}(t_{\text{on}} > t_{\text{off}} + t_{\text{on}}|t_{\text{off}})dt_{\text{off}} \]  
(Eq. 4)

It is the product of the probability for an observation starting with free diffusion, \( \tau_{\text{on}}/(\tau_{\text{off}} + \tau_{\text{on}}) \), for the occurrence of \( \tau_{\text{off}} \), \( p_{\text{off}}(t_{\text{off}}) \), and the probability that the bleaching time is long enough to enable such an observation as shown in Equation 5,

\[ p_{\text{bl}}(t_{\text{bl}} > t_{\text{off}} + t_{\text{on}}|t_{\text{off}}) = \frac{\tau_{\text{bl}}}{\tau_{\text{on}} + \tau_{\text{bl}}} \exp\left( -\frac{t_{\text{off}}}{\tau_{\text{bl}}} \right) \]  
(Eq. 5)

In total, we find Equation 6,

\[ p_{\text{off}}dt_{\text{off}} = \frac{\tau_{\text{on}}}{\tau_{\text{off}} + \tau_{\text{on}}} \cdot \frac{\tau_{\text{bl}}}{\tau_{\text{on}} + \tau_{\text{bl}}} \cdot \frac{1}{\tau_{\text{off}}} \exp\left( -\frac{t_{\text{off}}}{\tau_{\text{app}}} \right) dt_{\text{off}} \]

(Eq. 6)

The measured values for \( t_{\text{off}} \) yield the apparent expectation value \( \tau_{\text{app}} \) as observable, which depends solely on the “true” lifetime, \( \tau_{\text{off}} \) and the photobleaching time \( \tau_{\text{bl}} \). To obtain an independent measure of \( \tau_{\text{on}} \), we compared the probabilities for the occurrence of the trajectory classes. The probability for observing two transitions, one for immobilization and the second for release, in a trajectory starting with free Brownian motion provides the second observable value, which is given by Equation 7,

\[ P_{\text{type iii}} = \int p(t_{\text{off}})dt_{\text{off}} \]

\[ = \frac{1}{\tau_{\text{off}} + \tau_{\text{on}} + \tau_{\text{bl}}} \]  
(Eq. 7)

Finally, the ratio \( \alpha \) between the length of the mobile segments (\( \tau_{\text{on}} \)) and immobile segments (\( \tau_{\text{off}} \)) is equal to the ratio of the number of trajectories starting with a mobile segment and trajectories starting with an immobile segment, yielding the third observable value shown in Equation 9,

\[ \alpha = \frac{\tau_{\text{on}}}{\tau_{\text{off}}} \]  
(Eq. 9)

From Equations 6, 7, and 9, the three unknowns \( \tau_{\text{off}} \), \( \tau_{\text{on}} \), and \( \tau_{\text{bl}} \) were calculated.

RESULTS

Endocytosis of Oxidized Phospholipids via Caveolae and Clathrin-coated Pits—To investigate the uptake of oxidized phospholipids (oxPL), we used a fluorescent analogue of PGPC, 1-palmitoyl-2-glutaroyl-N-glucero-3-phospho-N-Alexa647-ethanolamine (PGPE-Alexa647), which has been recently described to mimic closely the behavior of unlabeled PGPC (7). Cells were incubated directly on the microscope with the fluorescent compound at 37 °C. Immediately upon incubation, we found accumulation in sites along the plasma membrane, internalization, and active transport to perinuclear regions (Fig. 1 and supplemental Movie 1, Movie 2, and Movie 4). Similar observations were made in HEK cells and CHO cells. To identify the uptake pathway, we measured the colocalization with markers for the clathrin-coated pit and caveolar pathway. In HEK cells, 42% of all endocytosed PGPE-Alexa647 positive structures were found to colocalize with transferrin-Alexa555,
which labels the clathrin-coated pit pathway (19). Active cotransport along cytoskeletal elements was observed using video microscopy (supplemental Movie 1 and Movie 2). Uptake via the caveolar pathway was addressed in a stably transfected CaveolinYFP HEK cell line; 57% of PGPE-Alexa647 positive structures colocalized with CaveolinYFP. We conclude that PGPE-Alexa647 is endocytosed via at least two independent uptake pathways.

Scavenger Receptor SR-BI Modulates Endocytotic Uptake of PGPE-Alexa647—Because cells in blood vessels may be exposed to oPL via lipoproteins, we tested the influence of the well characterized lipoprotein SR-BI (20–22) on the uptake efficiency of PGPE-Alexa647. SR-BI is known to facilitate cholesterol and phospholipid uptake directly by its ability to bind lipoproteins and to transfer lipids but also indirectly by altering the ultra-structure of the plasma membrane (23, 24). Indeed, when we applied PGPE-Alexa647 to CHO cells overexpressing SR-BI, we found a 3.4-fold increase in the average brightness of endocytosed structures when compared with control CHO cells (Fig. 2); the intracellular trafficking and subcellular location, however, was not affected. Apparently, SR-BI expression leads to an enrichment of oPL at endocytic sites. Such enrichment may be due to an increase in plasma membrane

insertion of oPL, an increase in association to endocytic sites, or a decrease in efflux from these sites. To discriminate the individual contributions, we investigated the behavior of PGPE-Alexa647 in the plasma membrane at the single molecule level.

Movement of Single PGPE-Alexa647 Molecules in the Plasma Membrane of Living Cells—For detection of single PGPE-Alexa647 molecules, we reduced the loading concentration by a factor of 50. Typically, the surface density was smaller than 1 molecule per μm². Because PGPE-Alexa647 unspecifically adhered to the glass surface, we performed all experiments on the top membrane of the cells. To reduce background signal arising from the glass surface, from endocytosed PGPE-Alexa647 or from cellular autofluorescence, we illuminated the cells with a HILO; by this wide field illumination configuration, the excitation volume can be confined to a narrow sheet roughly perpendicular to the optical axis (14) (Fig. 3A). Time-resolved imaging enabled us to follow single PGPE-Alexa647 molecules moving in the plasma membrane of living cells. Fig. 3, B and C, show a typical example of a single molecule trajectory. After 450 ms of rapid Brownian motion (Fig. 3, B and C, green) the molecule was transiently immobilized for 1.25 ms and got released again (see supplemental Movie 3). The distinct transitions between two different modes of motion were used later for quantitative dwell time analysis.

It is tempting to speculate that locations of immobilization are equivalent to sites of endocytosis; in this view, an equilib-
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![Image](61x485 to 289x734)

**FIGURE 4. PGPE-Alexa647 immobilization and uptake.** A, white trajectory shows the immobilization of a single PGPE-Alexa647 molecule in a CaveolinYFP-positive structure inside the plasma membrane of HEK-CaveolinYFP cells. The delay between two images was 200 ms. B, PGPE-Alexa647 is endocytosed via a caveola, observed in a HEK-CaveolinYFP cell at a delay of 1 s between two images (see supplemental Movie 4). The dashed line indicates the plasma membrane and the blue line the trajectory of the endocytosed caveola. C, load fluctuations of an endocytotic site. Variations in the fluorescence signal of an immobilization site for PGPE-Alexa647 recorded within a time span of 3.3 s. The magnitude of the fluctuations corresponds to the signal of single PGPE-Alexa647 molecules, which enter or leave the site; signal loss can also be caused by photo-bleaching. The width of the gray bars represents the standard deviation of corresponding single molecule signals. Bars, 1 μm.

![Image](2262x552)

**FIGURE 5. Single molecule analysis.** A, time duration for periods of transient immobilization \( t_{\text{off}} \) is plotted as a cumulative density function (c.d.f.). The offset in \( t_{\text{off}} \) originates from the threshold criterion for mobility classification and is taken into account by fitting with the function \( \text{cdf} = 1 - \exp((-t_{\text{off}} + \Delta t_{\text{off}})/t_{\text{app}}) \), with \( \Delta t_{\text{off}} = 0.3 \) s. For control CHO cells (circles) and SR-BI overexpressing CHO cells (diamonds), we find time constants \( t_{\text{app}} = 0.52 \) s and \( t_{\text{app}} = 0.24 \) s, respectively. After correction for photobleaching, we can specify \( t_{\text{off}} = 0.90 \) s for control cells and \( t_{\text{off}} = 0.85 \) s for the SR-BI-overexpressing cells. B, mobility of PGPE-Alexa647 in CHO cells. Periods of free diffusion were subjected to mobility analysis. The MSD was plotted versus the time lag for single PGPE-Alexa647 molecules in control cells (circles) and SR-BI-overexpressing cells (diamonds). Data were fitted according to MSD = \( 4Dt_{\text{mon}} + 4\alpha r_{\text{m}}^2 \), with \( \alpha r_{\text{m}}^2 \) specifying the localization precision. The fit yielded \( D = 1.76 \pm 0.05 \) μm²/s for control cells (dashed line) and \( D = 2.45 \pm 0.11 \) μm²/s for the SR-BI-overexpressing cells (solid line). A localization precision of 40 nm was found. No change in mobility over 1 order of magnitude in length scale has been observed as indicated by the inset, which shows data recorded at 38-fold higher frame rate.

The kinetic on-rate of the molecules \( k_{\text{on}} = \tau_{\text{on}}^{-1} \) is fully described by \( \tau_{\text{off}} \) and the equilibrium dissociation constant defined as the ratio of mobile and immobile molecules at a given instant of time, \( \alpha = \tau_{\text{on}}/\tau_{\text{off}} \). For determination of \( \alpha \), only the first five images of a recording sequence were taken into account. This ensures that the data are not biased because of photobleaching. \( \alpha \) was calculated as the ratio of the number of trajectories starting with a mobile segment to trajectories...
starting with an immobile segment, yielding $\alpha = 2.2$ and $\tau_{on} = \alpha \tau_{off} = 1.98$ s.

The mobile segments of each trajectory were further used for mobility analysis. We plotted the mean square displacement as a function of the time lag $t_{lag}$ yielding a linear increase according to MSD = $4Dt_{lag} + 4\sigma_{xy}^2$, a lateral diffusion constant $D = 1.76 \pm 0.05 \mu m^2/s$ was obtained (Fig. 5B, circles).

**Effect of SR-BI Expression on Single Molecule Properties**—To identify the mechanism of SRBI-mediated uptake enhancement, we studied the influence of SR-BI overexpression on the movement of single PGPE-Alexa647 molecules. The probe inserted rapidly into the plasma membrane of CHO cells overexpressing SR-BI, with a slightly increased surface density compared with the control cells by a factor of ~1.4. Analysis of single molecule trajectories in SR-BI-overexpressing cells revealed a decrease in the immobilization time yielding $\tau_{off} = 0.85$ s (Fig. 5A, diamonds). This value is similar to the respective value determined for the control cells. However, we found a 1.6-fold decreased equilibrium dissociation constant $\alpha (\alpha = 1.37)$, concomitant with a decreased time period of free Brownian motion $\tau_{on} = 1.16$ s. See Table 1 for a list of all determined parameters obtained from single molecule trajectories.

For diffusion-limited reactions, the kinetic off rate scales linearly with the diffusion constant and the surface density of adhesive sites (25). The motion of free PGPE-Alexa647 is characterized by the diffusion constant $D = 2.45 \pm 0.11 \mu m^2/s$ (Fig. 5B, diamonds). The 1.4-fold higher mobility of the fluorescent probe molecule upon SR-BI overexpression therefore mainly accounts for the more frequent immobilization events.

**Alteration of Membrane Properties by Oxidized Phospholipids**—We next investigated whether large excess of oxidized phospholipids, comparable with physiological levels found in blood vessels, induces macroscopically observable alterations. CaveolinYFP expressing HEK cells were treated with 10 $\mu$m PGPC, the nonfluorescent analogue of PGPE-Alexa647. Such concentrations have been reported to induce apoptosis in smooth muscle cells (6, 8). Immediately upon addition, patches of caveolae started to dissolve, and the cells began to round up (Fig. 6A). After 8 min, basically all cells observed showed significant morphological changes. Control experiments with the same cell line treated with buffer only did not undergo similar changes or caveolar redistribution.

Single molecule trajectories of PGPE-Alexa647 were used to sense the effect of PGPC on plasma membrane structure. Compared with nontreated control cells, we found a 1.9-fold decrease in the immobilization time yielding $\tau_{off} = 0.47$ s (supplemental Fig. S1), and a similar (2.3-fold) decrease in the association time yielding $\tau_{on} = 1.34$ s. Concomitantly, we observed a dramatic increase in the mobility of PGPE-Alexa647 in the plasma membrane of HEK cells upon treatment with PGPC from $D = 1.31 \pm 0.16 \mu m^2/s$ (untreated control cells) to $D = 3.30 \pm 0.33 \mu m^2/s$ (Fig. 6B). Again the increase in the on-rate

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**TABLE 1**

| dissociation constant $\alpha$, dwell times $\tau_{off}$, diffusion constant $D$, and number of analyzed trajectories $n$ for single PGPE-Alexa647 molecules recorded in the indicated cell lines |
|---|
| CHO control | CHO-SRBI | HEK-CavYFP | HEK-CavYFP + PGPC |
| $\alpha$ (s) | 2.2 | 1.37 | 3.5 | 2.9 |
| $\tau_{on}$ (s) | 1.98 | 1.16 | 3.1 | 1.34 |
| $\tau_{off}$ (s) | 0.90 | 0.85 | 0.89 | 0.47 |
| $D (\mu m^2/s)$ | $1.76 \pm 0.05$ | $2.45 \pm 0.11$ | $1.31 \pm 0.16$ | $3.30 \pm 0.33$ |
| $n$ (MSD analysis) | 235 ($t_{lag} = 4$ ms) | 147 ($t_{lag} = 4$ ms) | 230 ($t_{lag} = 4$ ms) | 208 ($t_{lag} = 4$ ms) |
| $n$ (MSD analysis) | 480 ($t_{lag} = 150$ ms) | 667 ($t_{lag} = 150$ ms) | 209 ($t_{lag} = 50$ ms) | 234 ($t_{lag} = 50$ ms) |
| $n$ (duration analysis) | 387 | 664 | 324 | 338 |

Cav means caveolin.
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can be explained by the increased mobility of PGPE-Alexa647 upon treatment with PGPC.

**DISCUSSION**

In this study we characterized the uptake pathway of oxidized phospholipids at the single molecule level. To optimize signal brightness and contrast against cellular autofluorescence, we chose Alexa647 as a fluorophore, which was conjugated to the headgroup of PGPE (7). Recent studies on this structure revealed no differences in intracellular localization compared with other fluorescent PGPC analogues, including head- and tailgroup-labeled lipids, suggesting that the fluorescent label does not significantly alter the uptake mechanism of the compound (7). This behavior was expected, as the polar fluorescent moiety in the headgroup just exaggerates the inverted cone-shaped structure of PGPC itself.

PGPE-Alexa647 rapidly inserted into the plasma membrane, when added to living cells at concentrations below the critical micelle concentration. Recent studies on model membranes have shown that the rate-limiting step for this insertion process is determined by the probability of encountering a free area in the membrane liquid surface (26). In the plasma membrane of different cell types (CHO and HEK), single molecules were observed to move at remarkably high diffusion constants \( D \sim 1.3\text{--}2.4 \mu m^2/s \) when compared with data reported in the literature for nonoxidized phospholipids and GPI-anchored proteins (\( D \sim 0.4\text{--}0.6 \mu m^2/s \)) (13, 27, 28). Indications for a strong influence of a bulky headgroup on lipid mobility have also been reported for model membranes, where BODIPY-labeled phospholipids within gel phase regions were found to locally increase the fluidity, resulting in mobility indistinguishable from fluid phase regions (29). It is likely that the nonbilayer lipid PGPE-Alexa647 imposes disorder on its local environment, thereby shifting the molecule slightly out of the membrane normal. This shift may be further facilitated by the decreased exposure of hydrophobic area to the surrounding buffer solution compared with double-chain lipids. Such a lipid, anchored virtually only via a single hydrophobic fatty acid, requires less free area for lateral diffusion, resulting in a higher mobility (30, 31).

In addition to free diffusion, our measurements show transient immobilization of PGPE-Alexa647. We have directly identified caveolae as sites for immobilization. This appears plausible as the inverted cone-shaped structure of the probe molecule leads to an energetic benefit at positions of high positive membrane curvature, e.g. in the neck regions of endocytic sites. Indeed, we found here that endocytosis of PGPE-Alexa647 proceeds via multiple independent pathways, indicating transient anchorage also in clathrin-coated pits. Accumulation of the oxidized lipid in caveolae and clathrin-coated pits for endocytosis is mediated via its increased lifetime in such structures. We were able to measure this lifetime directly from single molecule trajectories, yielding values ranging from 0.6 to 0.9 s in various cell types. It is interesting to compare these values with the time a freely diffusing lipid would reside in a region of the same size, \( \tau_{\text{diff}} \). Assuming a diameter of 100 nm for caveolae or clathrin-coated pits (32, 33) and a diffusion constant of \( \sim 2 \mu m^2/s \), we can estimate \( \tau_{\text{diff}} \sim 4 \) ms. The ratio shown in Equation 10,

\[
\hat{\tau} = \frac{\tau_{\text{diff}}}{\tau_{\text{diff}}}
\]

(Eq 10)

specifies the confinement strength and can be regarded as an estimate of the difference in free energy between the two local environments. Our data reveal in Equation 11,

\[
\hat{\tau} = 120 - 225
\]

(Eq 11)

concomitant with Equation 12,

\[
\Delta G = RT \log \hat{\tau} \approx SRT
\]

(Eq 12)

We also addressed the role of SR-BI for oxidized phospholipid uptake. This receptor regulates high density lipoprotein- and LDL-derived cholesterol and phospholipid homeostasis, and thereby most likely plays a role in the initiation of pathophysiological cellular responses. SR-BI is a member of the CD36 family of cell surface receptors (34), which has been described to be involved in the recognition of modified lipoproteins (35, 36), and in the progression of atherosclerosis (37). The location of SR-BI in caveolae indicates a direct influence on the process of endocytosis (38–41). Indeed, we found an increased PGPE-Alexa647 load of endocytosed vesicles in SR-BI-overexpressing cell lines by a factor of 3.4. Interestingly, this increase can hardly be explained by a direct interaction of the oxidized phospholipid with the receptor, as the off-rate from endocytotic sites was not altered. In contrast we found an increase in the kinetic on-rate by a factor of 1.7, which can be ascribed to a higher mobility of the probe molecule. This finding agrees with recent reports showing the influence of SR-BI on the lipid composition of the plasma membrane (23, 24). Moreover, the apparent increased membrane fluidity correlates with a larger free area for the diffusing probe lipid, which also affects the insertion of the oxidized phospholipids from bulk solution into the plasma membrane (26). 1.4-Fold higher insertion efficiency upon SR-BI expression was indeed found here and contributes to the enhanced load. Taken together, SR-BI alterations of the plasma membrane fluidity account for an increased occupancy of endocytotic sites by a factor of 2.4, which represents the major contribution to the observed enhanced PGPE-Alexa647 load.

We next investigated the effect of oxidized phospholipid concentrations, which \textit{in vitro} were found to induce apoptosis in vascular smooth muscle cells (8). Based on the insertion efficiency of PGPE-Alexa647, we can estimate a total PGPC-fraction of the total plasma membrane lipid content of \( \sim 0.5\% \). Immediately upon addition (within the first 30 s) cells responded by dramatic morphological changes; they rounded up and yielded a more homogeneous distribution of CaveolinYFP. Similar cellular responses have been observed in recent studies upon application of lysophosphatidylcholine (42). The fast response indicates that this effect is primarily caused by the alteration of the membrane structure because of change in lipid composition. A change in membrane structure has been directly observed by measuring the mobility of single PGPE-Alexa647 in PGPC-treated cells. A strong increase in the diffusion constant reflects a PGPC-induced reduction in the micro-
viscosity, an effect that has also been observed in lysophosphatidylcholine-treated cells (43). Interestingly, the increase in mobility fully accounts for the observed increase in the association rate constant with endocytic sites, indicating that the surface density of sites remains unchanged. Moreover, we found a 1.9-fold reduction in the residence time of PGPE-Alexa647 in endocytic sites upon PGPC treatment. Two interpretations appear plausible. First, assuming similar enrichment of PGPC in endocytic sites as PGPE-Alexa647, this high local concentration of nonbilayer lipids may alter the structure of the site itself and thereby its potency to retain the probe molecule. Second, the presence of PGPC outside of endocytotic sites may induce local curvature (44), thereby reducing the energy penalty for the escape of PGPE-Alexa647.

We conclude that oxidized phospholipids are trapped and thereby enriched unspecifically in endocytic sites, putatively due to their lysolipid-like, inverted cone-shaped structure. We presented strong indications that uptake of oxidized phospholipids is not receptor-mediated. Even in the case of receptor-enhanced endocytosis, the receptor does not directly interact but indirectly increases the association rate of the probe lipid with sites of endocytosis.

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REFERENCES

1. Leitinger, N., Tyner, T. R., Oslund, L., Rizza, C., Subbanagounder, G., Lee, H., Shih, P. T., Mackman, N., Tigi, G., Territo, M. C., Berliner, J. A., and Vora, D. K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12010–12015
2. Subbanagounder, G., Watson, A. D., and Berliner, J. A. (2000) Free Radic. Biol. Med. 28, 1751–1761
3. Subbanagounder, G., Leitinger, N., Schwenke, D. C., Wong, J. W., Lee, H., Rizza, C., Watson, A. D., Faull, K. F., Fogelman, A. M., and Berliner, J. A. (2000) Arterioscler. Thromb. Vasc. Biol. 20, 2248–2254
4. Watson, A. D., Navab, M., Hama, S. Y., Sevanian, A., Prescott, S. M., Stafforini, D. M., McIntyre, T. M., Du, B. N., Fogelman, A. M., and Berliner, J. A. (1995) J. Clin. Investig. 95, 775–782
5. Deigner, H. P., and Hermetter, A. (2004) Experientia 60, 87–90
6. Loidl, A., Claus, R., Ingolic, E., Deigner, H. P., and Hermetter, A. (2004) Biochim. Biophys. Acta 1690, 150–158
7. Mounizti, A., Tenker, M., Flicker, K., Zengmaier, E., Saff, R., and Hermetter, A. (2007) J. Lipid Res. 48, 565–582
8. Loidl, A., Sevcik, E., Riesenhuber, G., Deigner, H. P., and Hermetter, A. (2003) J. Biol. Chem. 278, 32921–32928
9. Mcmahon, H. T., and Gallop, I. L. (2005) Nature 438, 590–596
10. Cheremomordik, L. (1996) Chem. Phys. Lipids 81, 203–213
11. Farsad, K., and De Camilli, P. (2003) Curr. Opin. Cell Biol. 15, 372–381
12. Owstronski, S. G., Van Bell, C. T., Winograd, N., and Ewing, A. G. (2004) Science 305, 71–73
13. Schütz, G. J., Kada, G., Pastushenko, V. P., and Schindler, H. (2000) EMBO J. 19, 892–901
14. Tokunaga, M., Imamoto, N., and Sakata-Sogawa, K. (2008) Nat. Meth. 5, 159–161
15. Moertelmaier, M. A., Kögel, E. J., Hesse, J., Sonneleitner, M., Huber, L. A., and Schütz, G. J. (2002) Single Mol. 3, 225–231
16. Koyama-Honda, I., Ritchie, K., Fujiwara, T., Iino, R., Murakoshi, H., Kasai, R. S., and Kusumi, A. (2005) Biophys. J. 88, 2126–2136
17. Schütz, G. J., Schindler, H., and Schmidt, T. (1997) Biophys. J. 73, 1073–1080
18. Fürderer-Kitzmüller, E., Hesse, J., Ebner, A., Gruber, H. J., and Schütz, G. J. (2005) Chem. Phys. Lett. 404, 13–18
19. Sonnichsen, B., De Renzis, S., Nielsen, E., Rietdorf, J., and Zerial, M. (2000) J. Cell Biol. 149, 901–914
20. Acton, S., Rigotti, A., Landuschel, K. T., Xu, S., Hobbs, H. H., and Krieger, M. (1996) Science 271, 518–520
21. Stangl, H., Hyatt, M., and Hobbs, H. H. (1999) J. Biol. Chem. 274, 32692–32698
22. Swarnakar, S., Temel, R. E., Connelly, M. A., Azhar, S., and Williams, D. L. (1999) J. Biol. Chem. 274, 29733–29739
23. Parathath, S., Connelly, M. A., Rieger, R. A., Klein, S. M., Abumrad, N. A., de la Llera-Moya, M., Iden, C. R., Rothblat, G. H., and Williams, D. L. (2004) J. Biol. Chem. 279, 41310–41318
24. Williams, D. L., Wong, J. S., and Hamilton, R. L. (2002) J. Lipid Res. 43, 544–549
25. Bell, G. I. (1978) Science 200, 618–627
26. Sampiao, J. L., Moreno, M. J., and Vaz, W. L. C. (2005) Biophys. J. 88, 4064–4071
27. Kenworthy, A. K., Nichols, B. J., Remmert, C. L., Hendrix, G. M., Kumar, M., Zimmerberg, J., and Lippincott-Schwartz, J. (2004) J. Cell Biol. 165, 735–746
28. Murase, K., Fujiwara, T., Umemura, Y., Suzuki, K., Iino, R., Yamashita, H., Saito, M., Murakoshi, H., Ritchie, K., and Kusumi, A. (2004) Biophys. J. 86, 4075–4093
29. Burns, A. R., Frankel, D. J., and Buranda, T. (2005) Biophys. J. 89, 1081–1093
30. Gall, T. H., Marttina, W., Theilen, U., and Sackmann, E. (1979) J. Membr. Biol. 48, 215–236
31. Liu, C., Paprica, A., and Petersen, N. (1997) Biophys. J. 73, 2580–2587
32. Anderson, R. G. (1998) Annu. Rev. Biochem. 67, 199–225
33. Grunfelder, C. G., Engstler, M., Weise, F., Schwarz, H., Stierhof, Y.-D., Morgan, G. W., Field, M. C., and Overath, P. (2003) Mol. Cell Biol. 14, 2029–2040
34. Krieger, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4077–4080
35. Acton, S. L., Scherer, P. E., Lodish, H. F., and Krieger, M. (1994) J. Biol. Chem. 269, 21003–21009
36. Calvo, D., Gomez-Coronado, D., Suarez, Y., Lasuncion, M. A., and Vega, M. A. (1998) J. Lipid Res. 39, 777–788
37. Hazen, S. L., and Chisolm, G. M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 12515–12517
38. Frank, P. G., Marcel, Y. L., Connelly, M. A., Lublin, D. M., Franklin, V., Williams, D. L., and Lisanti, M. P. (2002) Biochemistry 41, 11931–11940
39. Graf, G. A., Connelly, P. M., van der Westhuyzen, D. R., and Smart, E. J. (1999) J. Biol. Chem. 274, 12043–12048
40. Graf, G. A., Matveev, S. V., and Smart, E. J. (1999) Trends Cardiovasc. Med. 9, 221–225
41. Matveev, S., van der Westhuyzen, D. R., and Smart, E. J. (1999) J. Lipid Res. 40, 1647–1654
42. Mogue, K., Nakashima, S., Tsuchie, A., Tokumura, A., and Fukuzawa, K. (2003) Chem. Phys. Lipids 126, 29–38
43. Ghosh, P. K., Vasanji, A., Murugesan, G., Eppell, S. J., Graham, L. M., and Fox, P. L. (2002) 4, 894–900
44. Kooijman, E. E., Chupin, V., de Kruijff, B., and Burger, K. N. J. (2003) Traffic 4, 162–174