Molecular Perspective of Antigen-mediated Mast Cell Signaling

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Antigen-mediated cross-linking of the high affinity receptor for IgE (FcεRI), in the plasma membrane of mast cells, is the first step in the allergic immune response. This event triggers the phosphorylation of specific tyrosines in the cytoplasmic segments of the β and γ subunits of FcεRI by the Src tyrosine kinase Lyn, which is anchored to the inner leaflet of the plasma membrane. Lyn-induced phosphorylation of FcεRI occurs in a cholesterol-dependent manner, leading to the hypothesis that cholesterol-rich domains, or “lipid rafts,” may act as functional platforms for IgE receptor signaling. Testing this hypothesis under physiological conditions remains challenging because of the notion that these functional domains are likely transient and much smaller than the diffraction limit of optical microscopy. Here we use ultrafast fluorescence dynamics to investigate the correlation between nanostructural changes in the plasma membrane (labeled with 1,1'-diodoctadecyl-3,3,3',3'-tetramethylindocarbocyanine (diI-C18)) and IgE-FcεRI cross-linking in adherent RBL mast cells stimulated with multivalent antigen. Time-dependent two-photon fluorescence lifetime imaging microscopy of diI-C18 shows changes in lifetime that agree with the kinetics of stimulated tyrosine phosphorylation of FcεRI, the first identifiable biochemical step of the allergic response, under the same conditions. In addition, two-photon fluorescence lifetime imaging microscopy of Alexa Fluor 488-labeled IgE indicates that Förster resonance energy transfer occurs with diI-C18 in the plasma membrane. Our live cell studies provide direct evidence for the association of IgE-FcεRI with specialized cholesterol-rich domains within ~4-nm proximity and with an energy transfer efficiency of 0.22 ± 0.01 at maximal association during IgE receptor signaling.

Cholesterol-rich lipid domains in the plasma membrane, also known as “lipid rafts,” are believed to be engaged in various cellular functions, such as IgE receptor (FcεRI) signaling in mast cells and basophils (1–5). Antigen-mediated cross-linking of these cell surface receptors leads to their translocation into these domains prior to phosphorylation by the Src family kinase, Lyn, in a process that ultimately initiates the exocytotic release of histamine in the allergic response (1, 6). Because of the inherent complexity of cellular membranes, there has been major interest in understanding the thermodynamics and kinetics regulating the formation of lipid domains in model systems, such as giant unilamellar vesicles (7–9) and supported bilayers and monolayers (10–12), as well as in cells or blebs derived from plasma membranes (13–23). Forming model membranes with physiological proportions of cholesterol and various lipids, however, has produced conflicting evidence of whether segregated lipid domains actually form at 37 °C (24). In addition to imaging such lipid microdomains with optical microscopy (7–12, 21), translational diffusion (ms to s) has been the main observable in various studies using single particle tracking (17, 18), fluorescence recovery after photobleaching (10, 20, 21), and fluorescence correlation spectroscopy (7, 16, 20).

Many studies of cholesterol-rich lipid domains associated with IgE receptor signaling have mainly focused on RBL mast cells in suspension that are substantially (i.e. nonphysiologically) perturbed by cross-linking with a secondary antibody at 4 °C (6, 20, 21, 25, 26). In such studies, ordered lipid microdomains within the plasma membrane (labeled with a fluorescent lipid analog such as 1,1’-diodoctadecyl-3,3,3’,3’-tetramethylindocarbocyanine (diI-C18)3 (25)) have been found to co-redistribute with patches of cross-linked receptor-bound IgE (labeled, for example, with Alexa Fluor 488 (A488) (25)). Previously, we reexamined existing spatial relationships between lipid microdomains and extensively cross-linked IgE-FcεRI patches at the single cell level by probing the spatio-temporal dynamics (ps to ns) of the molecules that comprise these domains (25). Using ultrafast excited state dynamics and rotational diffusion (25), we observed that the lipid order (i.e. packing) or nanostructure of the plasma membrane is enhanced within cross-linking-induced lipid microdomains, or patches, as evidenced by increases in both fluorescence lifetime and...
rotational diffusion of diI-C₁₈. Similar trends in the same dynamic properties were also observed for cross-linked, co-localized A488-IgE-FcεRI. These results supported the hypothesis that lipid molecules are recruited into more ordered domains that serve as platforms for IgE-FcεRI signaling. Furthermore, the gross perturbations inherent under these non-physiological cross-linking conditions provided a simple platform on which to test and optimize our fluorescence dynamics imaging assay (25, 27) for probing molecule-molecule interactions and structural changes relevant to cell signaling.

Nonetheless, under physiological conditions, cholesterol-rich lipid domains in live cells have been hypothesized to vary in composition, dimension, and life span, making them a challenge to directly interrogate with relevant spatial and temporal resolution (28, 29). The spatial scale of diffusion-limited rotational imaging and the time scale of translational diffusion-based spectroscopic techniques (μs to s) present an obstacle toward understanding the molecular bases regulating domain formation and biological function. Fluorescence recovery after photobleaching and fluorescence correlation spectroscopy have been used to monitor the translational dynamics of carboxylic probes in adherent RBL cells as a function of cholesterol depletion (16) and in suspended cells subjected to nonphysiological IgE-FcεRI cross-linking (20, 21). Furthermore, electron-spin resonance spectroscopy (23) and Förster resonance energy transfer (FRET) (22) have been used to investigate the phase or order of suspended cell membrane lipids and their lateral distributions, but not within the context of IgE-FcεRI cross-linking and signaling.

Here, in individual mast cells under more physiological conditions, we test the hypothesis that IgE-FcεRI signaling occurs in specialized, cholesterol-rich regions of the plasma membrane, using the same fluorescence dynamics assay (25, 27). Our rationale is that membrane-associated molecular events on the ps to ns timescales translate to interrogating structural changes on the spatial order of 100 nm (at 100-ps resolution) (30), which is below the diffusion limit of traditional optical microscopy. By interrogating the fast molecular dynamics and order of the plasma membrane as mast cells are stimulated with multivalent antigen at room temperature (23°C), where domains may be transient and smaller than the diffusion limit, we could relate these changes directly to the stimulated tyrosine phosphorylation of FcεRI, the earliest biochemical step in the pathway (under identical conditions). To the best of our knowledge, the work described here represents the first time that these ultrafast dynamics methods have been used to monitor membrane-protein relationships during physiological cell signaling.

EXPERIMENTAL PROCEDURES

Cell Preparation—RBL-2H3 mast cells were maintained and harvested as described previously (6, 31). Adherent cells (1.5 × 10⁵ cells/ml) were plated onto glass-bottomed 35-mm Petri dishes (MatTek) and sensitized overnight with 0.75 μg of mouse anti-2,4-dinitrophenyl IgE (α-DNP IgE, provided by Dr. David Holowka, Cornell University) per 1 ml of cell suspension. IgE was used either unlabeled or conjugated to Alexa Fluor 488 (A488-IgE) via a commercially available protein labeling kit (Invitrogen) (dye/IgE = 4). The day of the experiment, the cells were washed three times with bovine serum albumin (BSA)-containing buffered saline solution (BSA/BSS: 20 mM HEPES, pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 1 mg/ml BSA). Cells were then labeled for 3 min at 37°C with 1 mg/ml diI-C₁₈ (Invitrogen) stock solution prepared in dimethyl sulfoxide (Me₂SO) that had been diluted 100-fold in BSS, and then washed three times with BSA/BSS. RBL cells were stimulated on the microscope at room temperature (23°C) with 1 μg/ml multivalent DNP-BSA antigen. Cells were either imaged as only diI-C₁₈-labeled, only A488-IgE-labeled, or dually labeled with both probes.

For solution experiments, A488-IgE was diluted in phosphate-buffered saline (PBS: 10 mM Na₂HPO₄, pH 7.4, 150 mM NaCl) and diI-C₁₈ in Me₂SO. Absorption spectra of 10 μM Alexa Fluor 488 in water, 0.052 μM A488-IgE in PBS, and 10 μM diI-C₁₈ in Me₂SO were measured with a diode array spectrophotometer (Hewlett-Packard 8453A) equipped with Chemstation software (Agilent). A fluorimeter (HORIBA Jobin Yvon Fluorolog FL3-21) was used to record the fluorescence emission spectra of 0.1 μM Alexa Fluor 488 in water, 0.01 μM A488-IgE in PBS, and 2.5 μM diI-C₁₈ in Me₂SO.

In Vivo Tyrosine Phosphorylation Assays—Adherent, IgE-sensitized RBL cells were plated overnight in 48-well culture plates. The cells were washed three times with BSA/BSS and stimulated at 23°C for the indicated times with 1 μg/ml DNP-BSA, lysed by addition of SDS sample buffer (10% glycerol, 0.05 M Tris, pH 6.8, 1% SDS, 0.1% bromphenol blue), boiled for 5 min, and centrifuged for 5 min at 13,000 × g. Equal numbers of cell equivalents of lysate (typically 3.5 × 10⁵ cell eq) were electroblotted on 12% nonreduced SDS-polyacrylamide gels, transferred to BioTrace polyvinylidene difluoride membrane (PALLGelman Laboratory), and blotted with horseradish peroxidase-conjugated anti-phosphotyrosine (4G10-HRP) (Millipore). Enhanced chemiluminescence (Pierce Biotechnology) was used to detect phosphorylated proteins and quantified from scanned blots using Un-Scan-It (Silk Scientific) and Igor-Pro (WaveMetrics) (6, 26).

Confocal Fluorescence Microscopy—The experimental setup and data analysis have been described in more detail elsewhere (25, 27). Briefly, a confocal microscope consisting of a fiber-coupled laser system, a scanner (Olympus FluoviewView300), differential interference contrast optics, an inverted microscope (Olympus IX81), and an Olympus 60 ×, 1.2 NA water-immersion objective was used for three-channel imaging. A488-IgE was imaged using 488 nm argon ion laser excitation and a 525/30 bandpass filter, whereas diI-C₁₈ was imaged with 543 nm HeNe laser excitation and a 605/70 bandpass filter. Differential interference contrast imaging was used to monitor cell viability before and after all experiments to ensure that no photodamage had occurred. Unlabeled RBL cells showed negligible autofluorescence (data not shown).

Fluorescence Lifetime Imaging—A femtosecond laser system (~120 fs, 76 MHz) provided 960 nm excitation, which was conditioned and steered toward the sample, through the confocal scanner and Olympus 60 ×, 1.2 NA water-immersion objective for 2P-FLIM. The epi-fluorescence was isolated from the excitation laser (~0.5–5 milliwatt or ~10–100 pJ/pulse at the
sample) using a dichroic mirror and filters (690SP for all samples, plus a 600LP to detect only diI-C18 or plus a 525/50 bandpass to detect only A488-IgE; Chroma) prior to detection using microchannel plates (Hamamatsu R3809U-50). For 2P-FLIM, measured at the magic angle (54.7°), and polarization imaging, measured at polarizations parallel and perpendicular relative to the excitation laser, the epi-fluorescence polarization was analyzed using a Glan-Thompson polarizer before each of two detector channels. The signal was amplified, and fluorescence lifetime images were constructed with a time-correlated single-photon counting module (Becker & Hickl SPC830) (27). 2P-FLIM images were recorded using 256 × 256 pixels with 65 time bins per pixel (i.e. 259 ps/bin) and a total acquisition time of 120 s, which is slower than membrane ruffling of antigen-stimulated cells. However, these studies remain relevant because of the three-dimensional resolution inherent in 2P microscopy, the polarized excitation selectivity, and the distinct differences in time scales of ruffling (s) versus excited state dynamics (ps to ns). Experiments were performed over several days on different cell preparations. Complementary 1P (480 nm, 4.2 MHz) time-resolved fluorescence measurements were carried out to enhance the time resolution and signal-to-noise ratio as described previously (25, 27). In these studies, the laser beam was strategically positioned at areas of interest on the cell membrane. In some cases, single cell averaging measurements using 2P-FLIM (pseudo-single point measurements) were also carried out to enhance the signal-to-noise ratio and temporal resolution using low repetition rate laser pulses (960 nm, 4.2 MHz) scanned over the entire cell.

Fluorescence Polarization Anisotropy Imaging—Steady-state and time-resolved fluorescence anisotropy measurements were performed using 2P and 1P excitation, respectively. For steady-state 2P anisotropy imaging, the parallel (0°) and perpendicular (90°) fluorescence polarizations were recorded simultaneously in a two-channel, laser-scanning mode. For time-resolved fluorescence anisotropy, 1P laser pulses were positioned on regions of interest on the cells, or a single cell was averaged via scanning 2P-FLIM, and the epi-fluorescence polarizations were resolved and detected simultaneously as described earlier. Both polarization images and time-resolved fluorescence polarization decays were constructed using the SPC830 module.

Theory and Data Analysis—The fluorescence lifetime of a fluorophore provides information about its molecular structure and the surrounding local environment (32, 33). Generally, the magic angle fluorescence decay of a fluorophore, located at pixel (x,y), can be described as a sum of exponentials with time constants (τi) and amplitudes (αi) as shown in Equation 1 (27),

$$l_{na,t}(x,y) = \sum_{j=1}^{3} \alpha_j(x,y)exp(-t/\tau_j(x,y)) \quad \text{(Eq. 1)}$$

The magic angle fluorescence decays were analyzed by a non-linear, least squares fitting routine with deconvolution of the system response function (FWHM ~ 60 ps) in the SPCImage software package (Becker & Hickl GmbH). The average fluorescence lifetime was calculated as $\langle \tau \rangle = \sum_i \alpha_i \tau_i / \sum_i \alpha_i$, and the residual and reduced $\chi^2$ were used to assess the goodness of fit, with $\chi^2 = 1.0 - 1.3$ considered satisfactory. The error of the average lifetime value for a given 2P-FLIM image was determined by fitting the total image histogram to a Gaussian with IgorPro to obtain the width or standard deviation. Unpaired, two-tailed Student’s t tests were performed using Excel (Microsoft) to determine whether variations in fluorescence decay parameters as a function of antigen stimulation were statistically significant ($p \leq 0.05$).

Following photoselective excitation of a fluorophore at pixel coordinates (x,y) in a cell, the time-resolved fluorescence polarization anisotropy, r(t;x,y), can be described as indicated in Equation 2 (27),

$$r(t;x,y) = \frac{(l_i(t;x,y) - l_\perp) - G \times (l_i(t;x,y) - l_\perp)}{(l_i(t;x,y) - l_\perp) + 2G \times (l_i(t;x,y) - l_\perp)}$$

$$\quad = \sum_{j=1}^{3} \beta_j(x,y) exp(-t/\phi_j(x,y)) \quad \text{(Eq. 2)}$$

where the parallel ($l_i$) and perpendicular ($l_\perp$) fluorescence emission intensities per pixel were corrected for background signals ($l_\parallel$ and $l_\perp$, respectively). The G-factor (0.74) was calculated using the tail matching approach (32, 33) to account for the polarization dependence of the detection efficiency (33). The rotational correlation time ($\Phi_j$) depends on the hydrodynamic volume of the fluorophore and the surrounding viscosity (33). The pre-exponential factor ($\beta_j$), whose sum is equal to the initial anisotropy, r0, can be used to assess the population fractions of fluorophores under different environmental restrictions (33). Depolarization because of the high NA objective was determined previously to be negligible when using an under-filled objective, as is the case for 1P measurements here (25). The degree of orientational constraint of a fluorophore is described by the ratio, r0/rp, which describes its range of angular rotation (34, 35). Using MATLAB (The Mathworks), we developed an image processing algorithm to calculate steady-state initial anisotropy images ($r_0(t;x,y)$) (25, 27) from parallel and perpendicularly polarized 2P-fluorescence images. The error in the average initial anisotropy value for a given image was determined by the method described for 2P-FLIM images above. For single point, time-resolved fluorescence polarization anisotropy, nonlinear least squares fitting (Equation 2) of these decays was carried out using either OriginPro 7 (OriginLab) or IgorPro without deconvolution of the system response function. The goodness of the fit was evaluated using $\chi^2$ calculated by Origin-Pro 7 or IgorPro, with $\chi^2 = 0 - 0.01$ considered satisfactory.

To probe lateral heterogeneity in the plasma membrane associated with IgE-FcεRII stimulation, we also carried out time-resolved FRET (24, 36–38). Based on the spectral overlap between the membrane (diI-C18) and IgE (A488), we examined the potential of these fluorophores to be a donor-acceptor pair for FRET. The efficiency of energy transfer, $E_T$, is defined as $E_T = 1 - (\tau_M/\tau_D)$, where $\tau_M$ is the lifetime of the donor (A488-IgE in the presence of acceptor (diI-C18) and $\tau_D$ is the donor lifetime in the absence of acceptor, both of which are obtained from time-resolved fluorescence decays (33)). Using $E_T$ in combination with the spectral overlap, the donor-acceptor separa-
Multiscale Dynamics of IgE Receptor Activation

FIGURE 1. Confocal microscopy demonstrates antigen (Ag)-mediated changes in morphology (A) and distribution of the lipid analog, dil-C_{18} (B), and A488-IgE-FceRI (C) over 30 min of stimulation with antigen at room temperature. No obvious co-localization of dil-C_{18} with cross-linked A488-IgE is observed. The cross hairs (and arrows) indicate the position of the focused laser beam for single point time-resolved fluorescence decay measurements (decays shown in Fig. 3, with corresponding data in Table 1). Bar, 20 μm; DIC, differential interference contrast.

RESULTS

Antigen-mediated Changes in Cell Morphology and IgE Distribution Are Demonstrated by Confocal Microscopy—We monitored changes in cell morphology, IgE-FceRI clustering, and plasma membrane structure as a function of antigen stimulation using three-channel confocal microscopy. RBL cells labeled with the lipid analog, dil-C_{18}, and sensitized with A488-IgE were imaged immediately prior to the addition of soluble multivalent antigen to establish an initial base line (“0 min”) and then subsequently imaged after the cells have been stimulated for 5, 10, 20, and 30 min. Fig. 1 shows a representative time series, in which A depicts cell morphology, whereas B and C show the corresponding dil-C_{18} and A488-IgE-FceRI fluorescence images, respectively. (Note that the fluorescence background observed only in Fig. 1B is because of dil-C_{18} nonspecifically adhering to the glass coverslip, despite extensive cell washing or varying labeling conditions.) Activated cells spread and ruffle in an actin-dependent manner as observed previously (26, 40).

Phosphorylation of IgE-FceRI has been hypothesized to result from cross-linking-induced coalescence of nanoscopic, cholesterol-rich membrane domains (41) that are smaller than the diffraction limit of optical microscopy (λ/2), thereby explaining the absence of clear dil-C_{18}-labeled membrane domains (Fig. 1B) that co-localize with A488-IgE-FceRI (Fig. 1C). This physiological observation contrasts with non-physiological studies on RBL cells at 4°C (6, 20, 21, 25, 26), plasma membrane blebs below 20°C (42), and model systems such as giant unilamellar vesicles (7–9, 16), in which micron-sized domains were clearly observed. Although antigen-mediated changes in cell morphology and antigen-induced receptor cross-linking and their associated dynamics can easily be visualized with fluorescence microscopy (Fig. 1 and supplemental Movie 1), the putative sub-diffraction changes of membrane nanostructure are not readily apparent in conventional imaging. To overcome this limitation, we performed excited state dynamics experiments and 2P-FRET on dil-C_{18} and/or A488-IgE-labeled mast cells, which report on the immediate local environment of the fluorophore (≤10 nm) (33).

Antigen-mediated IgE-FceRI Cross-linking Alters the Membrane Nanostructure as Reported by Ultrafast Excited State Dynamics—We investigated changes in 2P-fluorescence lifetime and time-resolved FRET that occur as IgE receptor signaling proceeds. It is worth noting that stimulated plasma membrane ruffling by the actin cytoskeleton is much slower (s to min time scale) than the excited state lifetime (ps to ns), allowing us to effectively separate these two processes. Even though the acquisition times for 2P-FLIM images (~120 s) are on the same time scale as membrane ruffling, the inherent three-dimensional resolution of 2P excitation and the photoselectivity associated with polarized excitation validates the potential of this approach for probing molecular events of the plasma membrane and associated proteins during the cellular response to antigen stimulation (i.e. ruffling).

2P-FLIM images of dil-C_{18} in RBL cells, which were dually labeled with dil-C_{18} and A488-IgE, were fit to a biexponential and are shown before and after antigen stimulation in Fig. 2A. Corresponding A488-IgE confocal images were sequentially recorded before each dil-C_{18} FLIM image to observe how changes in IgE-FceRI distribution correlate with changes in membrane nanostructure, as reported by the fluorescence life-
Nanostructural changes in the plasma membrane occur upon antigen stimulation. A, 2P-FLIM imaging of diI-C18 shows antigen-stimulated changes in membrane nanostructure and 4A88-IgE-FcεRI distribution (binning = 3). When compared with diI-C18 in the membrane, the background features in the diI-C18 FLIM images have a distinctively shorter lifetime (~0.3 ns), which can be fit to a single exponential (25), confirming that these features are unincorporated free diI-C18 aggregates on the coverslip surface. B, total FLIM image histograms, with 0 min (blue), 5 min (green), 10 min (yellow), 20 min (red), and 30 min (purple) time points, are shown to represent the overall changes in average lifetime distributions as a function of antigen stimulation. The most noteworthy shift in average lifetime is between 0 and 5 min. Bar, 10 μm.

Time-resolved fluorescence decays indicate variations in membrane and protein environment as a result of antigen-mediated stimulation. A, pseudo-single point time-resolved fluorescence decays of diI-C18, in which the entire cell is scanned and the signal is binned into one “pixel,” are shown as a function of antigen stimulation time (0 min (blue), 5 min (green), 10 min (yellow), 20 min (red), and 30 min (purple)) and serve as a comparison to the single point measurements. Note that the specific spatial information is lost because of the binning, although we accounted for the overall changes that occur upon antigen stimulation. Table 1 shows data for several of these pseudo-single point time series, revealing a transient increase in the average fluorescence lifetime of diI-C18 within 5 min of stimulation. B, single point time-resolved fluorescence decays of diI-C18 are displayed as a function of stimulation time (0 min (blue), 5 min (green), 10 min (yellow), 20 min (red), and 30 min (purple)). The location of the focused laser beam on the dually labeled cell is indicated by the arrow and cross hairs shown in Fig. 1. The average fluorescence lifetime transiently increases after 5 min. The statistics for several of these time series are shown in Table 1. C, average lifetime (τp) from single point measurements (Table 1) are plotted as a function of stimulation time.

As signaling continues, (τp) returns to its original value for the experimental duration (Fig. 3C). Furthermore, the data shown in Table 1 for single point lifetime measurements of diI-C18-labeled cells at 0 min (n = 5) agree with additional data from cells that were not subsequently stimulated (n = 25; data not shown).
Stimulated Tyrosine Phosphorylation of FceRI Supports Fluorescence Dynamics Observations—The functional relevance of the transient increase in the lifetime of dil-C18 was examined by following the time course of tyrosine phosphorylation of cells stimulated with the same conditions as the micro-spectroscopy experiments (1 μg/ml antigen, 23°C). The first identifiable biochemical step that occurs upon cross-linking of IgE-FceRI by multivalent antigen is the phosphorylation of the β and γ subunits of FceRI by the tyrosine kinase Lyn (6). Fig. 4A shows a representative immunoblot (n = 4) of time-dependent cell stimulation, and Fig. 4B shows the corresponding densitometric trace for the phosphorylation of FceRI β. Under these conditions, phosphorylation is maximal at 5 min and remains sustained for the experimental duration. The phosphorylation kinetics agree with the kinetics of the changes in dil-C18 fluorescence lifetime (Fig. 3C) and further strengthen the relevance of the lifetime results.

Time-resolved FRET between A488-IgE- and dil-C18-labeled Membrane Reveals Lateral, Nanoscale Heterogeneity Associated with Antigen Stimulation—To examine the potential interaction between ordered membrane domains (dil-C18 and IgE receptor (A488-IgE)) during antigen-induced stimulation, we carried out time-resolved FRET measurements on dually labeled RBL cells. This approach enabled us to probe lateral heterogeneities at the nanoscale (i.e. 0.1–10 nm), well below the diffraction-limited resolution (~λ/2) inherent in optical microscopy. Steady-state spectroscopy of A488-IgE and dil-C18 (supplemental Fig. 1) indicated the potential of these fluorescent labels as a donor and acceptor, respectively, in a FRET pair, and based on the spectral overlap of the donor emission and acceptor absorption, the estimated Förster distance (R0) is 3.33 ± 0.04 nm.

We measured the fluorescence lifetime of A488-IgE in RBL cells as a function of antigen stimulation, in the presence and absence of dil-C18 (acceptor). Using pseudo-single point measurements, we observed a reduction in the excited state fluorescence lifetime of A488-IgE in the presence of dil-C18, as compared with A488-IgE in the absence of dil-C18. (When detecting A488-IgE lifetime in the presence of dil-C18, we ensured that the cross-talk between the donor and acceptor emission was negligible by using the appropriate emission filters prior to detection.) Representative fluorescence decays with two exponential components are shown in Fig. 5A, and the lifetimes (τ) and amplitudes (α) of all decay components are summarized in Table 1. In unstimulated cells labeled solely with A488-IgE, (τp) is 2.94 ± 0.05 ns (n = 5) as compared with 2.41 ± 0.07 ns (p = 2.73 × 10−7) in the presence of dil-C18 (n = 6). Using higher time resolution (4-nsec time scale), the reduction in A488-IgE lifetime in the presence of acceptor is further emphasized (data not shown). Based on the measured reduction of the donor fluorescence lifetime in resting cells (i.e. prior to stimulation), in the presence of acceptor, the estimated EFRET, and R0 were determined to be 0.18 ± 0.01 and 4.3 ± 0.1 nm and 0.07 ± 0.02 ns−1, respectively (Table 2).

Following antigen stimulation, the reduction of donor (A488-IgE) (τp) is significant in the presence of dil-C18, going from 2.7 ± 0.1 to 2.1 ± 0.1 ns (p = 8.88 × 10−4) at 5 min of stimulation, and from 2.5 ± 0.2 to 2.15 ± 0.01 ns (p = 5.93 × 10−5) at 10 min. By 20 min, the average lifetime of A488-IgE in the presence of dil-C18 is no longer statistically significantly

### Table 1

Summary of fluorescence lifetime parameters obtained from pseudo-single point and conventional single point, time-resolved fluorescence decays of dil-C18 and A488-IgE in adherent mast cells

| Condition | pseudo-single point | conventional single point |
|-----------|----------------------|---------------------------|
| Time (min) | τp (ns) | α | τp (ns) | α | τp (ns) | α |
| 0 | 0.05 (1) | 0.7 (1) | 0.39 (2) | 0.2 (1) | 1.7 (2) | 0.1 (1) | 0.26 (2) | 4 |
| 5 | 0.06 (1) | 0.6 (1) | 0.39 (4) | 0.3 (1) | 1.66 (2) | 0.1 (1) | 0.32 (4) | 4 |
| 10 | 0.048 (7) | 0.7 (1) | 0.42 (7) | 0.2 (1) | 1.85 (8) | 0.1 (1) | 0.28 (6) | 4 |
| 20 | 0.061 (1) | 0.6 (1) | 0.45 (3) | 0.3 (1) | 2.0 (2) | 0.1 (1) | 0.36 (9) | 4 |
| 30 | 0.062 (1) | 0.6 (1) | 0.41 (6) | 0.3 (1) | 1.9 (4) | 0.1 (1) | 0.38 (8) | 4 |

### Summary

- **Summary**: This study examines the kinetics and heterogeneities at the nanoscale during antigen-induced stimulation of IgE receptors. The phosphorylation of FceRI β and γ subunits is maximal at 5 min and remains sustained for the experimental duration. Time-resolved FRET measurements show a reduction in the fluorescence lifetime of A488-IgE in the presence of dil-C18, indicating a decrease in the Förster distance and confirming the lateral, nanoscale heterogeneity.

- **Key Findings**:
  - The fluorescence lifetime of A488-IgE decreases significantly in the presence of dil-C18, with a dramatic drop from 2.94 ± 0.05 ns to 2.41 ± 0.07 ns at 5 min of stimulation.
  - This reduction is further emphasized at higher time resolution, suggesting a significant change in the donor lifetime in the presence of dil-C18.

- **Implications**: These findings highlight the importance of lateral heterogeneities in the function of IgE receptors and suggest potential mechanisms for antigen-induced signaling and receptor distribution within the cell membrane.
different from that with A488-IgE alone. Interestingly, \( \langle \tau_p \rangle \) decreases significantly between 0 and 5 min, both for cells labeled only with A488-IgE (2.94 ± 0.05 ns at 0 min and 2.7 ± 0.1 ns \( (p = 5.52 \times 10^{-3}) \) at 5 min) and for those dually labeled with A488-IgE and diI-C\(_{18}\) (2.41 ± 0.07 ns at 0 min and 2.1 ± 0.1 ns \( (p = 3.16 \times 10^{-3}) \) at 5 min). This trend of decreasing \( \langle \tau_p \rangle \) as a function of stimulation time is substantiated by our observations of A488-IgE labeled cells obtained from 2P-FLIM at lower signal-to-noise and temporal resolution. When the 2P-FLIM images of A488-IgE are fit with high binning to improve histogram signal-to-noise and better resolve lifetime components, the pixel image histograms reveal a decrease in \( \langle \tau_p \rangle \) by 5 min and at all subsequent time points relative to 0 min (supplemental Fig. 2). (At low binning, there were no resolvable, significant shifts in \( \langle \tau_p \rangle \) of A488-IgE with antigen stimulation; data not shown.) The major peak of \( \langle \tau_p \rangle \) at \( \sim 2.5 - 3.0 \) ns (supplemental Fig. 2) further agrees with the \( \langle \tau_p \rangle \) acquired via pseudo-single point measurements (Table 1). When we calculate \( E_T \) as a function of stimulation time (Fig. 5B), we find that the FRET efficiency increases from 0.18 ± 0.01 prior to stimulation to 0.22 ± 0.01 \( (p = 0.008) \) after 5 min of stimulation, followed by a gradual decrease with 0.14 ± 0.04 at 10 min, 0.11 ± 0.04 at 20 min, and 0.09 ± 0.01 at 30 min (see Table 2 and Fig. 5B). The kinetics of energy transfer efficiency agree with those of stimulated tyrosine phosphorylation and changes in the average lifetime of diI-C\(_{18}\).

These pseudo-single point measurements, with high temporal resolution as compared with 2P-FLIM, indicate that A488-IgE interacts with the membrane label, diI-C\(_{18}\) (acceptor), via energy transfer. Such interactions only occur when A488-IgE and diI-C\(_{18}\) are within 10 nm proximity, which is below the diffraction limit.

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**TABLE 2**

Summary of fluorescence resonance energy transfer parameters obtained from steady-state spectrophotometry of diI-C\(_{18}\) and A488-IgE and pseudo-single point time-resolved fluorescence decays of A488-IgE in adherent mast cells

| Time (min) | \( E_T \) | \( R/\text{nm} \) | \( k_{\text{FRET}}/\text{ns}^{-1} \) |
|------------|----------|-----------------|-----------------|
| 0 min      | 0.18 (1) | 4.3 (1)         | 0.07 (2)        |
| 5 min      | 0.22 (1) | 4.1 (1)         | 0.10 (2)        |
| 10 min     | 0.14 (4) | 4.5 (4)         | 0.06 (5)        |
| 20 min     | 0.11 (4) | 4.7 (5)         | 0.04 (5)        |
| 30 min     | 0.09 (1) | 4.9 (1)         | 0.03 (1)        |

\( a \) The donor (A488-IgE) fluorescence lifetime in the presence of acceptor (diI-C\(_{18}\)) \( (\tau_{\text{DA}}) \) and the donor fluorescence lifetime in the absence of acceptor \( (\tau_D) \), used in the calculation of energy transfer efficiency \( (E_T) \), were 2.41 ± 0.07 and 2.94 ± 0.05 ns, respectively (see Table 1). \( E_T \) values are statistically significant with \( p < 0.05 \). \( R_0 \) was calculated as 3.33 ± 0.04 nm and used to determine \( R \), as described under “Experimental Procedures.”

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**FIGURE 4.** The kinetics of antigen stimulation agree with nanostructural changes in the plasma membrane. RBL cells are stimulated with antigen under the same conditions as the micro-spectroscopy experiments (1 mg/ml DNP-BSA, 23 °C). A, representative anti-phosphotyrosine immunoblot shows stimulated in vivo tyrosine phosphorylation, and the band corresponding to the \( \beta \) subunit of Fc\( R \)\( \beta \) is indicated. B, level of tyrosine phosphorylation of Fc\( R \)\( \beta \) for the immunoblots shown in A is quantified using densitometry.

**FIGURE 5.** A488-IgE and diI-C\(_{18}\) interact transiently as RBL cells are stimulated with antigen, as measured by the excited state dynamics of A488-IgE-A, pseudo-single point fluorescence decays of the donor A488-IgE decays more quickly in the presence of the acceptor diI-C\(_{18}\) (2) as compared with the absence of acceptor (1). The representative decays were acquired prior to antigen stimulation; however, the fluorescence of A488-IgE decays faster in the presence of diI-C\(_{18}\) at all time points of stimulation (see Table 1). \( b \), efficiency of energy transfer \( (E_T) \) is plotted as a function of stimulation time to show that the probes experience maximal association 5 min after stimulation, corresponding to the largest increase in energy transfer efficiency (from 0.18 ± 0.01 before stimulation to 0.22 ± 0.01 at 5 min). The standard deviation of \( E_T \) is determined by the error in \( r_{\text{DA}} \) \( (n = 6; \) Table 1) and \( r_0 \) \( (n = 5; \) Table 1).
Multiscale Dynamics of IgE Receptor Activation

TABLE 3
Summary of rotational anisotropy parameters obtained from single point and pseudo-single point, time-resolved anisotropy decays of diI-C18 and A488-IgE in adherent mast cells

|                          | \( \phi_0 \)/ns | \( \beta_1 \) | \( r_{\infty} \) | \( r_0 \) | \( r_{\infty}/r_0 \) | \( n \) |
|--------------------------|----------------|-------------|--------------|----------|-----------------|------|
| diI-C18                  |                |             |              |          |                 |      |
| Single point             | 0.65 (9)       | 0.04 (2)    | 0.18 (6)     | 0.22 (5) | 0.8 (1)         | 8    |
| 0 min (pre-stimulation)  |                |             |              |          |                 |      |
| A488-IgE in absence of diI-C18 |            |             |              |          |                 |      |
| Pseudo-single point      | 0.6 (1)        | 0.17 (3)    | 0.11 (1)     | 0.38 (2) | 0.40 (7)        | 7    |
| 0 min (pre-stimulation)  |                |             |              |          |                 |      |
| 5 min                    | 0.6 (2)        | 0.14 (2)    | 0.12 (2)     | 0.26 (1) | 0.46 (9)        | 4    |
| 10 min                   | 0.6 (2)        | 0.17 (4)    | 0.11 (2)     | 0.28 (2) | 0.41 (1)        | 4    |
| 20 min                   | 0.7 (3)        | 0.17 (3)    | 0.11 (2)     | 0.28 (4) | 0.40 (4)        | 4    |
| 30 min                   | 0.5 (2)        | 0.16 (4)    | 0.14 (4)     | 0.30 (3) | 0.5 (1)         | 4    |
| A488-IgE in presence of diI-C18 |                |             |              |          |                 |      |
| Pseudo-single point      | 0.8 (2)*       | 0.18 (3)    | 0.13 (3)     | 0.31 (5) | 0.4 (1)         | 7    |
| 0 min (pre-stimulation)  |                |             |              |          |                 |      |
| 5 min                    | 0.61 (1)*      | 0.19 (1)*   | 0.11 (1)     | 0.30 (1)* | 0.36 (4)       | 3    |
| 10 min                   | 0.6 (1)        | 0.1 (1)     | 0.13 (1)     | 0.2 (1)  | 0.6 (2)         | 3    |
| 20 min                   | 0.50 (8)       | 0.15 (1)    | 0.15 (1)     | 0.30 (1) | 0.50 (1)*       | 3    |
| 30 min                   | 0.56 (8)       | 0.17 (4)    | 0.12 (3)     | 0.29 (6) | 0.43 (1)        | 3    |

* \( P < 0.05 \) was determined from unpaired, two-tailed Student’s \( t \) tests, meaning that the mean of the given fit parameter is statistically significantly different in the presence of diI-C18 as compared with the absence of diI-C18 at the same time point of stimulation.

** \( P < 0.05 \) was determined from unpaired, two-tailed Student’s \( t \) tests, indicating that the mean of the given fit parameter is statistically significantly different from the mean prior to antigen stimulation, i.e. at 0 min.

and may lend insight into the transient nature of cholesterol-rich nanodomains that may act as signaling platforms.

From the acceptor perspective of FRET, we would expect that the fluorescence of diI-C18 would increase at a rate of \( k_{FRET} \) and decay with a time constant corresponding to the excited state lifetime(s) of diI-C18 (33). To directly measure \( k_{FRET} \), we measured the fluorescence decay of diI-C18 at lower (16 ps/channel) and higher (5 ps/channel) temporal resolutions (data not shown). The acceptor fluorescence decay in resting RBL cells builds up instantaneously (data not shown). Similarly, little difference in the averaged \( r_0 \) was observed for A488-IgE over the entire cell, although individual A488-IgE-FceRI puncta that form as a result of ongoing cross-linking do have higher initial anisotropies than uncross-linked A488-IgE-FceRI (\( n = 9 \); data not shown). These results suggest that lipid and protein undergo negligible changes with respect to anisotropy as a function of stimulation and indicate the degree to which the membrane constrains rotational, or tumbling, motions of the probes, which could act as a fluorescence depolarization mechanism. However, pixel-to-pixel changes may be present during stimulation, and we are currently developing image processing algorithms to quantify further these possible variations.

Rotational diffusion of molecular dipoles directly probes fast conformational changes and possible restriction of the surrounding environment. Furthermore, these measurements also allow us to examine the validity of the randomization assumption in FRET calculations and, therefore, of using \( k^2 = 2/3 \). We performed 1P time-resolved fluorescence polarization anisotropy measurements on diI-C18 labeled cells (Table 3) to avoid sampling any free aggregated dye on the coverslip. With 1P excitation, the focal volume along the \( z \) axis is larger than the ruffling amplitude of antigen-stimulated cells. Thus, to avoid averaging over dipole moment ruffling and obscuring the interpretation of diI-C18 rotational dynamics within the plane of the plasma membrane, 1P time-resolved anisotropy measurements were performed without respect to stimulation. High signal-to-noise single point measurements reveal that diI-C18 decays as a single exponential (\( \phi_0 = 0.65 \pm 0.09 \) ns, \( \beta_1 = 0.04 \pm 0.02 \) and an additional residual anisotropy component (Table 3). The fast rotational correlation time reflects rapid depolarizations within our time scale of observation, suggesting temporal dipole-moment randomization of diI-C18 as a FRET acceptor. The degree of orientational constraint (\( r_{\infty}/r_0 = 0.8 \pm 0.1 \)) is high because diI-C18 is intercalated in the membrane.

The fast rotational correlation time of A488-IgE at all time points of stimulation (Table 3), in combination with that of diI-C18, validates the randomization assumption in our FRET calculations (i.e. \( k^2 = 2/3 \)). Prior to antigen stimulation, 2P pseudo-single point anisotropy decays of A488-IgE are somewhat slower in the presence of diI-C18 (supplemental Fig. 3). Upon stimulation, the rotational correlation time of A488-IgE in the presence of diI-C18 decreases by 5 min (as determined by
unpaired, two-tailed Student’s t tests), indicating enhanced association of the donor and acceptor (43) (supplemental Fig. 3). For A488-IgE on the membrane surface, the degree of orientational constraint is considerably less (e.g. 0.40 ± 0.07 prior to stimulation) than for diI-C18 in the membrane. Single point anisotropy decays of free fluorophores in solution (data not shown) generated fitting parameters that agree with Davey et al. (25), verifying the consistency in our experimental setup.

**DISCUSSION**

Antigen-stimulated phosphorylation of FceRI by Lyn has been shown to occur in a cholesterol-dependent manner during the initial biochemical step of the allergic response in RBL-2H3 mast cells (6), and these cholesterol-rich membrane domains have also been shown to protect Lyn from tyrosine phosphatases, thereby enhancing its activity (44). However, little direct information about the formation and chemical and physical nature of these domains within complex biomembranes is known, because of their transient nature with respect to space and time. In this work, we used a fluorescence dynamics assay to measure changes in membrane structure that occur as RBL mast cells are stimulated with multivalent antigen. Our experiments are performed at room temperature, which reduces the rate of signaling as compared with that occurring at 37 °C, and previous in vivo tyrosine phosphorylation assays performed on suspended RBL cells at 37 °C revealed maximal FceRI phosphorylation between 2 and 5 min after stimulation with the same concentration of DNP-BSA used in these studies (6, 26). Although ongoing cross-linking of IgE-FceRI continues throughout the experiment, its phosphorylation decreases with time as the receptors compete for limited amounts of Lyn (45), and as stimulated actin polymerization separates Lyn from IgE-FceRI as signaling proceeds (26, 46), resulting in down-regulation. We found that changes in the fluorescence lifetime of diI-C18 (Fig. 3) and the efficiency of energy transfer between IgE-FceRI and diI-C18 (Fig. 5) agree with the kinetics of the earliest biochemical step in the pathway, that is the stimulated tyrosine phosphorylation of FceRI (Fig. 4). This agreement leads us to suggest that these changes in the fluorescence dynamics are functionally relevant to antigen-mediated stimulation in mast cells.

The first indication of the association of IgE-FceRI with low density cholesterol-enriched membrane domains upon cross-linking was provided by sucrose gradient ultracentrifugation experiments of RBL cells lysed with Triton X-100 detergent (47). Furthermore, cholesterol depletion with methyl-β-cyclohexadextrin was shown to reduce the degree of stimulated tyrosine phosphorylation of FceRI as assessed by immunoblots (6). When suspended mast cells are cross-linked with secondary antibody at 4 °C, co-localization of a fluorescent lipid analog, which partitions preferentially into cholesterol-rich regions, with fluorescently labeled IgE-FceRI into micron-sized patches has been observed using conventional optical microscopy (21, 25). However, imaging cholesterol-rich domains in vivo, without substantial nonphysiological perturbation (i.e. low temperatures or extensive IgE-FceRI cross-linking (6, 21, 25, 26)), has remained a challenge, and therefore, the functional role of these domains in signaling is poorly understood and speculative at best. Such difficulty in characterization of these domains arises from their perceived small size and transient nature as well as the inherent diffraction-limited resolution of optical microscopy. Fortunately, time-resolved FRET can overcome the spatial limitation of conventional optical microscopy by probing nanoscale (0.1–10 nm) lateral heterogeneities of the membrane (i.e. associations of IgE-FceRI with cholesterol-rich domains) in real time. Recently, using steady-state FRET between carboxy-anine probes that preferentially partition into the liquid-ordered phase based on their alkyl chains, Sengupta et al. (22) demonstrated nanoscale lipid heterogeneity in the plasma membrane of RBL cells that was enhanced by nonphysiological cross-linking of IgE-FceRI.

To circumvent the temporal resolution of translational diffusion-based spectroscopic techniques (μs to s) (16–18, 20, 21), we used a fluorescence dynamics assay capable of probing molecular dynamics (fluorescence lifetime and rotational diffusion) on a ps to ns time scale to interrogate antigen-mediated changes in cell morphology (supplemental Movie 1). This fluorescence dynamics assay was previously tested on massively cross-linked, suspended mast cells, demonstrating increased fluorescence lifetime and rotational diffusion of diI-C18 and A488-IgE, and thus providing evidence for nanostructural changes in the plasma membrane within the nonphysiologically induced microdomains (25).

At room temperature, diI-C18-labeled membrane domains are transient, thermodynamically unstable, and smaller than the diffraction limit of differential interference contrast and confocal microscopy (Fig. 1). Despite the lack of visible domain formation during the course of antigen stimulation or co-localization between diI and the IgE receptor, 2P-FLIM revealed a distinct change in the membrane nanoenvironment of diI-C18 (Fig. 2), as observed by averaging over the entire FLIM images (binning = 3). The transiently increasing (τp) becomes more obvious when we interrogate lifetime with higher signal-to-noise and temporal resolution (Fig. 3 and Table 1), which follows the kinetics of stimulated tyrosine phosphorylation (Fig. 4). The effects of membrane structure on the isomerization and thus photophysics of diI-C18 likely results from increased local concentrations of saturated and mono-unsaturated phospholipids (48), or solvent protection by cross-linked membrane proteins and the underlying actin cytoskeleton (1, 49).

When the dipole-moment distribution of diI-C18 was monitored in cells as a function of antigen stimulation via steady-state 2P-fluorescence polarization anisotropy imaging, no gross changes in the averaged membrane order were observed over time, which is not surprising considering that there are no optically resolvable membrane domains. Changes in cellular morphology, which occur on the seconds time scale, may also obscure minor changes in the orientational order of the diI-C18 dipole moment during the ~120 s necessary for acquisition, and these may be reflected in the pixel-to-pixel variations we observed. At all time points of stimulation monitored, the average initial anisotropies obtained by 2P-fluorescence polarization anisotropy imaging were greater for adherent RBL cells (data not shown) than for cells in suspension (25), suggesting that lipid in adherent cell membranes may have an intrinsically higher packing order than those in the spherical, suspended cell membranes. Collectively, these diI-C18 results imply that there
are no functional domains at the steady-state, or resting, level under physiological conditions, but rather that such domains are small, dynamic assemblies, as proposed by Mayor and Rao (50). It is possible that the longer fluorescence and rotational dynamics of dil-C18 observed in stimulated adherent cells, as compared with cross-linked suspended cells (25), results from increased restriction because of confinement of dil-C18 into many nanoscopic domains with higher line tension (51) than the large microdomains of suspended cells.

The modest antigen-mediated changes in average A488-IgE fluorescence lifetime observed with 2P-FLIM can be attributed to the fact that the binding of DNP-BSA to IgE-FceRI follows a transient hapten exposure model (45, 52), in which the antigen and antibody interact at fast on/off rates. Populations of A488-IgE that are variously bound by DNP-BSA are suggested by the multiple Gaussian fits of pixel-lifetime histograms from total FLIM images (supplemental Fig. 2). At various time points of DNP-BSA stimulation, the average lifetimes of A488-IgE obtained with 2P-FLIM (supplemental Fig. 2) are longer than those we had observed in suspended cells that were extensively cross-linked with a secondary antibody (τρ = 0.9 ns and 1.35 ns (25)). The increase in environmental restriction for adherent cells may be explained by the steric hindrance of the DNP-BSA molecules transiently and electrostatically binding to IgE-FceRI, as compared with the more stable binding of secondary antibody to suspended cells. Increased restriction because of differences in cell treatment is supported by slightly higher average initial anisotropies obtained by 2P-fluorescence polarization anisotropy imaging of A488-IgE in antigen-stimulated adherent cells (data not shown), as compared with extensively cross-linked suspended cells (25). Notably, using either 2P-FLIM with high binning or single point lifetime methods, the average fluorescence lifetime (⟨τ⟩) of A488-IgE decreases within the first 5 min of antigen stimulation, in direct contrast with the transient increase of the lifetime of dil-C18, and it may be explained by the increased environmental disorder due to presenting multivalent antigen to IgE-FceRI.

Using spectrophotometric resonance energy transfer methods on RBL cell-derived membrane vesicles (i.e. ensemble averaging in a cuvette), Baird and Holowka (53) determined distances between various regions of IgE and the outer cell membrane surface ranging from ~4.5 to 10 nm. Their findings, in combination with the R0 obtained here (3.33 ± 0.04 nm), suggested that energy transfer would be feasible between A488-IgE and dil-C18. Here, the reduction of A488-IgE (donor) fluorescence lifetime in the presence of dil-C18 (acceptor) prior to stimulation, as reported by single point, single cell methods, can be attributed to FRET (Fig. 5A; Table 1) that occurs only when the donor and acceptor are within 10 nm and indicates that A488-IgE and dil-C18 are within 4.3 ± 0.1 nm (Table 2). Mathematical modeling studies of FRET data and electron microscopy images from cell membranes have implied that functional membrane domains are between ~5 and 20 nm in diameter (54).

Our results also show that A488-IgE and dil-C18 experience maximal association 5 min after stimulation begins, indicated by the increase in energy transfer efficiency (from 0.18 ± 0.01 prior to stimulation to 0.22 ± 0.01 at 5 min; Table 2) and also by the decrease in rotational correlation time of A488-IgE in the presence of dil-C18 between 0 and 5 min (supplemental Fig. 3; Table 3). The time of maximal FRET agrees with maximal tyrosine phosphorylation of FceRI by membrane domain-associated Lyn, suggesting that IgE-FceRI is recruited into ordered cholesterol-rich domains for high efficiency signaling. Even if such domains only coalesce transiently, as suggested by steady-state FRET studies by Kenworthy et al. (55), the ultrafast time resolution of our FRET studies provides an advantage for resolving sub-diffraction association of various molecules (e.g. IgE-FceRI) with cholesterol-rich domains (56). The FRET results, together with the dil-C18 lifetime results, support the hypothesis that cross-linked IgE-FceRI transiently translocates into regions of membrane with greater nanostructural order to facilitate signaling; however, our results do not rule out the possibility that receptor engagement may recruit functional lipid nanodomains (54).

The reduced FRET efficiency at 20 min post-stimulation (Table 1) indicates a reduction in lateral interactions between IgE-FceRI and dil-C18-labeled membranes and may be related to the hypothesis that actin microfilaments help to separate FceRI from ordered membrane domains (26, 46), resulting in the down-regulation of signaling. Antigen-induced IgE-FceRI cross-linking at 37 °C results in endocytosis of ~50% of the receptor complexes with a half-time of 5 min (57, 58), so it is unlikely that the reduced FRET observed here by 20 min is because of IgE-FceRI internalization, even with its much slower kinetics at room temperature. Moreover if IgE-FceRI is internalized, the reduction in FRET suggests that dil-containing domains are not co-internalized with the IgE receptor, and we are currently investigating this possibility.

In summary, we report a molecular perspective of the molecule-molecule interactions associated with antigen-stimulated IgE receptor signaling in RBL mast cells. Using confocal microscopy, we demonstrated antigen-mediated changes in cell morphology and IgE-FceRI cross-linking and reorganization that occur on the seconds time scale (Fig. 1; supplemental Movie 1). Fluorescence lifetime imaging of dil-C18 reveals nanostructural changes of the plasma membrane as a function of antigen stimulation time (Figs. 2 and 3; Table 1). These changes correlate with the kinetics of tyrosine phosphorylation of FceRI as assessed by immunoblotting (Fig. 4). These results suggest an IgE-FceRI stimulation-dependent change in the membrane nanoarchitecture at early times of the signaling process, which is further supported by the transient increase in FRET between A488-IgE-FceRI and dil-C18 within a 0.1–10-nm spatial resolution (Fig. 5; Table 2). The results presented here suggest that IgE-FceRI is recruited into transient, ordered membrane domains that serve as platforms for IgE-FceRI signaling. Furthermore, these studies offer an experimental approach that is applicable to other signaling pathways that depend upon localized membrane ordering.

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