FEBS Letters 586 (2012) 416–421

journal homepage: www.FEBSLetters.org

Single-particle tracking of immunoglobulin E receptors (FcεRI) in micron-sized clusters and receptor patches

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ARTICLE INFO

Article history:
Received 22 December 2011
Revised 4 January 2012
Accepted 9 January 2012
Available online 18 January 2012

Edited by Sandro Sonnino

Keywords:
FcεRI
Mast cell
Single-particle tracking
Supported lipid bilayer
TIRF microscopy
Monte Carlo calculation

ABSTRACT

When mast cells contact a monovalent antigen-bearing fluid lipid bilayer, IgE-loaded FcεRI receptors aggregate at contact points and trigger degranulation and the release of immune activators. We used two-color total internal reflection fluorescence microscopy and single-particle tracking to show that most fluorescently labeled receptor complexes diffuse freely within these micron-size clusters, with a diffusion coefficient comparable to free receptors in resting cells. At later times, when the small clusters coalesce to form larger patches, receptors diffuse even more rapidly. In all cases, Monte Carlo diffusion simulations ensured that the tracking results were free of bias, and distinguished biological from statistical variation. These results show the diversity in receptor mobility in mast cells, demonstrating at least three distinct states of receptor diffusivity.

1. Introduction

Mast cells are immune cells found in tissues throughout the body, including the skin and mucosal surfaces. When activated, they protect the body from parasitic infections, but are also responsible for allergic responses. In mast cells and basophils, a crucial player in this process is the high affinity immunoglobulin E (IgE) receptor (FcεRI). In allergic responses, multivalent ligand, e.g. a pollen grain, binds to IgE-loaded receptors (IgE–FcεRI) causing receptor aggregation also known as receptor cross-linking. Aggregation of these transmembrane receptors leads to receptor phosphorylation [1,2] and the subsequent initiation of signaling cascades that result in the release of inflammatory mediators such as histamine and serotonin [2].

To study immune signaling by mast cells, the rat basophilic leukemia 2H3 (RBL) cell line is typically used as a model [3–5]. In previous studies, it was observed that RBL cells loaded with fluorescent IgE form receptor aggregates when allowed to settle under gravity [6,7], or when pipette-pressed [8], onto fluid bilayers containing monovalent ligands. These receptor aggregates are not cross-linked and hence are different in character from clusters formed by multivalent ligands. However, RBL cell signaling still occurs on these fluid lipid membrane substrates [6,7]. Our recent work [8] showed that receptor clusters on ligand-presenting fluid bilayers originate from cell surface protrusions that form the initial contact points with the substrate. Receptor accumulation at these contact points was shown to be kinetically consistent with diffusion limited trapping; moreover, the cell membrane was far from the substrate *except* at receptor clusters, as shown by a dye exclusion study. After initial IgE–FcεRI cluster formation, small clusters diffuse slowly and coalesce to form a large central patch, termed the mast cell synapse, in which IgE–FcεRI were qualitatively observed to be laterally mobile [7]. The ability of monovalent ligands presented on fluid membranes to stimulate RBL cells speaks to a longstanding debate on the relationship between IgE–FcεRI mobility and signaling. Recently, it has been demonstrated that small antigen-induced IgE–FcεRI clusters can induce signaling while retaining mobility [9]. The principal aim of this paper is to quantify the mobility of IgE–FcεRI within initial cell–substrate contact points (receptor clusters), and in the larger patches, in order to address the role of IgE–FcεRI mobility in RBL cell activation and more fully characterize the diffusional behavior of this receptor.

Because the receptor clusters are typically smaller than a micron, methods such as photobleaching recovery or far-field fluorescence correlation spectroscopy are ill-suited for measuring receptor diffusion. Instead, we have turned to single-particle tracking, using the fluorescent dye Atto647, which yielded receptor

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0014-5793/$36.00
doi:10.1016/j.febslet.2012.01.013

trajectories with ca. 50 nm localization precision. To ensure that tracked receptors were in clusters, the majority of the receptor complexes were labeled with Alexa488; both dyes were imaged using a two-color total internal reflection fluorescence (TIRF) microscope. Analysis of single-particle trajectories showed that receptors maintain their diffusivity even when confined within receptor clusters, and increase their diffusivity (above that of monomeric unliganded IgE–FcεRI) in central patches. Together with the observation that weak signaling occurs when FcεRI on mast cells is presented with mobile, bilayer-incorporated ligand [7], this study shows clearly that signaling occurs under conditions where a majority of receptors (~70%) remain mobile.

2. Materials and methods

2.1. Two color labeling of RBL Cells

RBL-2H3 cells were maintained in Minimal Essential Medium (MEM) (Invitrogen) with 10% Fetal Calf Serum. At the day of the experiment, MEM with Fetal Calf Serum was exchanged with MEM supplemented with 10% Fetal Bovine Serum, 1% Penicillin–Streptomycin, and 1% L-glutamine, which will be referred to as media in the remainder of this manuscript. Anti-DNP IgE was purified as previously described [10,11]. Fluorescent anti-DNP IgE conjugates were created using Alexa488 (Invitrogen) and Atto647 (ATTO-TEC GmbH). Prior to microscopy, cells were fluorescent IgE primed by first incubating with 35 or 50 pM Atto647-IgE anti-DNP in media for 10 min at 37 °C and then washed 5 times with 2 ml media obtaining a final aliquot of 2 ml. Next 5 μl of Alexa488-IgE anti-DNP at a concentration of 0.7 μg/ml was added and incubated for 10 min at 37 °C. The primed cells with both fluorescent markers were then washed 4 times with 2 ml media and divided into 0.5 ml aliquots (~50 000 cells per aliquot) stored in 1 ml tubes at 37 °C in a humidified chamber with 5% CO_2 until later use.

2.2. Supported lipid bilayers

Prior to use, microscope glass cover slips were cleaned of organic residues with a mixture of sulfuric acid and hydrogen peroxide (“piranha” solution). Supported lipid bilayers [7] were made by spontaneous liposome fusion [12]. Lipids (Avanti) were dissolved in chloroform, dried under N_2, and then placed under vacuum for 1 h. The lipid film was then suspended in PBS + 2 mM Mg^{2+} to 1.3 mM and sonicated for 5 min using a probe sonicator. Laterally mobile bilayers were formed from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 12 mol% *N*-dinitrophenyl-aminocaproyl phosphatidylethanolamine (DNP-Cap PE) on piranha-cleaned cover glass for 15 min on a slide warmer at 37 °C. Each bilayer coated coverslip was kept immersed during transfer to the imaging chamber. Prior to adding cells to the bilayer, the chamber was flushed with 500 μl of media. Lipid mobility was checked using single-particle tracking as described elsewhere [7].

2.3. Fluorescence microscopy

Objective-based total internal reflection fluorescent microscopy was performed on an Olympus IX 71 (Olympus America Inc.) inverted microscope with a 150 × 1.45 NA oil objective using a 472 nm laser (CrystaLaser) to excite Alexa488 and a 635 nm laser (Coherent Inc.) to excite Atto647 with an evanescent wave. Two-color fluorescent images were collected at a frame rate of 20 frames/s using an electron multiplying CCD camera (Andor iXon + 897; Andor Technologies Inc.) and spectrally separated by an image splitter (Quad-View™, Optical Insights, LLC). The camera was cooled to −70 °C with a detector gain of 200. Sample temperatures were maintained at ~37 °C with an objective heater (Bioptechs Inc.) and images were collected with in-house software implemented in MATLAB (The MathWorks Inc.). Images were processed using MATLAB in conjunction with DIPImage [13], an image processing library. Two-color fluorescent images were collected in two channels. The red channel recorded the lower concentration of Atto647-IgE in the single-particle regime. Images in the green channel (Alexa488) recorded the fluorescent label of higher concentration to outline the spatial extent of receptor clusters and central patches. To overlay these two channels a dilute sample of 0.1 μm diameter fluorescent microspheres (yellow/green FluoSpheres, Molecular Probes Inc.) emitting spectral components detectable in both channels was imaged. The images of these microspheres were used to align the two channels.

2.4. Single-particle tracking

Single-particle trajectories of fluorescent receptor clusters and IgE–FcεRI receptor complexes were obtained by using a single-particle tracking algorithm implemented in MATLAB as previously described in Ref. [14]. IgE–FcεRI were tracked only if they were located within a receptor cluster or a central patch as determined from the two-color image overlay. The particles were tracked in a 50 ms time interval for at least 65 time steps. The average track length was ~100 time steps. The mean-squared displacement (MSD) was calculated from all n available displacements of a given duration $n\Delta t$ in the track record [15–17]. To characterize the motion, the MSD plot was computed up to Δt<1/4 of the total number of acquired time frames [16,18]. The MSD graph for IgE–FcεRI inside receptor clusters and in the central patch showed a downward curvature and asymptotically approached a finite value, which is a signature for confined diffusion. As the exact shape of the confinement (if it is not too eccentric) has a negligible effect on the form of the MSD [19], we fit to a circular confinement zone. The exact solution [20] contains an infinite sum of exponentials, but the second term is two orders of magnitude smaller than the first (and each subsequent term at least another order of magnitude smaller), so that a good approximation is obtained from the first exponential only:

$$\mathrm{MSD}(\Delta t) = 4\sigma^2 + R^2[1 - 0.99\exp(-3.393D\Delta t/R^2)].$$

Fitting parameter D is the diffusion coefficient and R is the confinement zone radius. σ is the sum of the static and dynamic localization (measurement) uncertainty [17,21], determined by fitting a straight line through time lags $2\Delta t$, $3\Delta t$, and $4\Delta t$. The offset determined by this method avoids using the part of the MSD plot between times 0 and $2\Delta t$ which is known to be complicated and times longer than $4\Delta t$ after which the confinement effects were apparent [22]. The average localization uncertainty for IgE–FcεRI diffusing inside clusters and central patches was $\sigma = 47 \pm 18$ and 30 ± 38 nm, respectively, where the error represents one standard deviation.

Cluster diffusion was also estimated from MSD plots of the cluster center, as determined from a 2D Gaussian fit to intensity. The MSD graph of receptor cluster trajectories was linear and fit to $\mathrm{MSD}(\Delta t) = 4\sigma^2 + 4D\Delta t$ to estimate cluster diffusivity. The average localization uncertainty for clusters was $\sigma = 32 \pm 13$ nm. In MSD plots, all points were equally weighted, which has been shown to give unbiased parameters if all available displacements are used [16,17].

2.5. Monte Carlo calculations

To determine statistical uncertainties (and possible biases) in fitting for diffusivity, model diffusion tracks were constructed