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Nanodomains in Early and Later Phases of FcεRI Signaling

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Abstract

Our long term efforts to elucidate receptor-mediated signaling in immune cells, particularly transmembrane signaling initiated by the receptor (FcεRI) for immunoglobulin E (IgE) in mast cells, led us unavoidably to contemplate the role of the heterogeneous plasma membrane. Our early investigations with fluorescence microscopy revealed co-redistribution of certain lipids and signaling components with antigen-crosslinked IgE-FcεRI and pointed to participation of ordered membrane domains in the signaling process. With a focus on this function, we have worked along with others to develop diverse and increasingly sophisticated tools to analyze the complexity of membrane structure that facilitates regulation and targeting of signaling events. This essay describes how initial membrane interactions of clustered IgE-FcεRI lead to downstream cellular responses and how biochemical information integrated with nanoscale resolution spectroscopy and imaging is providing mechanistic insights at the level of molecular complexes.

Keywords

IgE receptors (FcεRI); mast cells; tyrosine phosphorylation cascade; receptor immobilization and clustering; phosphoinositide-dependent signaling; Ca^{2+} mobilization; store-operated Ca^{2+} entry; exocytosis; electrostatic association; BAR domains; syntaxin

INTRODUCTION

IgE is a soluble antibody protein that binds with high affinity to its transmembrane receptor, FcεRI, on mast cells and thereby becomes the recognition component of this cell surface receptor. Crosslinking of IgE-FcεRI by multivalent ligands (antigen) in IgE binding sites initiates transmembrane signaling by causing receptor coupling with a Src family tyrosine kinase, Lyn, which is anchored to the inner leaflet of the plasma membrane by means of saturated fatty acid chains(Figure 1).

Phosphorylation of clustered FcεRI by Lyn kinase in ordered membrane domains leads to recruitment and activation of the tyrosine kinase Syk, which then phosphorylates multiple substrates, including the transmembrane adaptor protein, LAT, as well as phospholipase Cγ1 and γ2 (PLCγ; [15]). Activation of these lipases by tyrosine phosphorylation results in hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate 2,3-diacylglycerol, which is the membrane-associated activator of protein kinase C, and inositol 1,4,5-trisphosphate (IP_3), which is the soluble activator of Ca^{2+} release from the endoplasmic reticulum (ER). IP_3-mediated release of Ca^{2+} from ER stores triggers the process known as store-operated Ca^{2+} entry (SOCE), in which the ER Ca^{2+} sensor protein, STIM1, oligomerizes in response to Ca^{2+} depletion within the ER and couples to the Ca^{2+} release-

activated Ca^{2+} (CRAC) channel, Orai1, resulting in sustained Ca^{2+} influx that is necessary for functional responses such as exocytosis and cytokine biosynthesis and secretion [16, 17]. These signaling pathways lead to secretion of histamine and other cellular responses associated with allergic reactions.

A) Nanodomains formed by antigen clustering of IgE/ FcεRI complexes

Early in our studies of cell signaling, using RBL-2H3 mast cells as a model system, we found with fluorescence microscopy that restricting IgE-FcεRI crosslinking to trimers caused much larger scale clustering of these receptors on the cell surface – the cell was somehow amplifying the original small cluster [1]. Our thoughts that this involves the plasma membrane structure was supported by later fluorescence microscopy experiments showing that antigen crosslinking of IgE-FcεRI causes certain types of lipids to co-redistribute with the clustered receptors [2]. This led us to a much larger realization: We had to consider how the plasma membrane structure contributes to cellular signaling. This is the subject of our essay.

Although the membrane is primarily a lipid bilayer, this simple view belies the richness of its structural composition, including a large and diverse variety of lipids, proteins, and carbohydrates. Moreover, in most cells, this outer, plasma membrane is structurally connected to the internal parts of the cell, including various internal membrane compartments, by the cytoskeleton and by membrane trafficking events.

Despite much of the focus on proteins in cell signaling over the years, membrane lipids are receiving increasing attention. This interest is based in part on the impact of lipid phase behavior, which has been studied rigorously by physical scientists in model membrane systems. In a biological context, the attractive concept has emerged that phase properties add a new axis to the regulatory and targeting capabilities of membranes. In addition to providing a surface where upon reactants can encounter each other more readily than in three-dimensional media, membrane lipid phase-like properties offer lateral heterogeneity and the possibility of selective partitioning. In this manner, the membrane can provide compartments that preferentially include (or exclude) particular reactants according to their physico-chemical properties.

A number of research groups contributed to the emerging notion that lipid phase-like behavior in membranes has an important functional role. In simple terms of model membranes, certain compositions of lipids with high melting temperatures (e.g., sphingolipids or phospholipids with saturated fatty acid chains), lipids with low melting temperatures (e.g., phospholipids with unsaturated fatty acid chains), and cholesterol separate into two distinguishable phases, typically termed liquid-ordered (Lo, rich in high Tm lipids and cholesterol) and liquid-disordered (Ld, rich in low Tm lipids). A common term currently used to describe the Lo-like domains in biological membranes is “lipid rafts,” in which receptors connect effectively with other membrane and signaling components that co-partition. However, this term is highly problematic because it does not provide a clear definition in terms of composition, size, and dynamic changes that accompany function in a biological membrane. Tools used initially to characterize these Lo-type or “ordered” membrane domains include resistance to detergent solubility and dependence on cholesterol

[3, 4]. These tools continue to provide useful correlative information. However, over-interpretation of results with these simple approaches has led to considerable controversy in the literature about the role of phase-like properties in membrane function. The interplay of lipids, proteins, and other structural elements is central to membrane function, and the membrane biophysics community is responding by developing a range of more sophisticated means of analysis, as amply demonstrated by this volume of *Essays in Biochemistry*.

Our strategy toward understanding plasma membrane participation in cell signaling has been to focus on particular interactions and functions initiated by clustering of IgE-FcεRI. Toward this end, our group has worked with collaborators to develop new approaches using mass spectrometry, electron spin resonance, and nanofabrication [5-7]. In this essay we will describe briefly our recent advances using super-resolution (nanoscale) imaging. Although our previous work had taken advantage of sophisticated quantitative fluorescence microscopy techniques, these are conventionally limited by the diffraction limit of light (≥250nm). To gain a nanoscale view, we first turned to scanning electron microscopy with IgE and selected signaling components labeled with gold particles by means of secondary antibodies. After overcoming multiple challenges to determine the appropriate analysis [8], we demonstrated that that antigen-clustering of IgE-FcεRI on RBL mast cells causes co-redistribution of Lyn kinase and other signaling components on the inner plasma membrane [9]. Pair correlation analysis showed the average radius of these co-clusters to be 70-90 nm within a minute of adding antigen. Further experiments with this approach showed that both reduction of membrane cholesterol and inhibition of Lyn kinase activity reduced the co-clustering. These results provided the first nanoscale view of these clusters and thereby provided important support for our current model: Antigen clustering of IgE-FcεRI facilitates coupling with Lyn in ordered membrane domains, and Lyn association is further amplified by FcεRI phosphorylation which yields additional binding sites for this kinase.

EM has proven powerful in providing nanometer resolution in cell studies for many years. However, EM imaging has a number of limitations, including requirements for sample fixation and immuno-gold for specific labeling. Development within the past decade of super-resolution fluorescence microscopy has provided exciting new opportunities for versatile imaging at the level of molecular complexes. Although a number of these new techniques have now been developed, including Stimulated Emission-Depletion (STED) microscopy [10], currently the most widely used are the localization microscopies, which are most readily built into standard fluorescence microscopes. These include Photo Activation Localization Microscopy (PALM/fPALM; [11, 12]) and Stochastic Optical Reconstruction Microscopy (STORM/dSTORM: [13]), which typically also utilize total internal reflectance fluorescence (TIRF) optics (see also chapter by K.Gaus in this volume). The angle of the excitation beam in TIRF microscopy restricts excitation, and thereby emission, of fluorophore probes to the ventral cell membrane, thereby minimizing cytoplasmic noise. Localization microscopy techniques enable nanoscale imaging of densely labeled cells by sequential activation and pin-pointing of a small subset of the fluorophores with ~20 nm resolution, and this is followed by compiling accumulated snapshots to reconstruct the image. Although this process can be carried out on fixed cells as for EM

imaging, an exciting new capability is imaging live cells and tracking individual fluorophores with time.

These new methods greatly expanded our opportunity to monitor early stages of antigen-induced clustering of IgE-FcεRI corresponding to the earliest transmembrane signaling events. Our collaborative studies with Sarah Veatch at the University of Michigan used fluorescently labeled IgE and dSTORM to image IgE-FcεRI on the cell surface, before and after addition of antigen [14]. Analyses of multiple images over time yielded average values and quantified changes both in spatial distributions (with pair correlation functions), and diffusion coefficients (with mean square displacement plots) of individual fluorophores as they became clustered (Figure 2). These experiments showed that within 2 min of antigen addition, IgE-FcεRI diffusion coefficients decrease by an order of magnitude, and single-particle trajectories become confined. Within 5 min of antigen addition, IgE-FcεRI organize into clusters containing ~100 receptors with average radii of ~70 nm. The antigen-induced changes in physical properties of IgE-FcεRI, i.e., clustering and mobility, can also be correlated to cell signaling events that are stimulated in parallel, and this study considered stimulated Ca^{2+} mobilization (vide infra). The results revealed two stages in the physical changes occurring after antigen addition. The first stage, which precedes stimulated Ca^{2+} mobilization, is characterized by decreased mobility of receptors while clusters remain small. This stage may represent interactions of small clusters with other components in the membrane initiating downstream signaling. During the second stage, beginning at about 1.5 min, receptors become tightly packed and confined. Although this stage likely represents multiple, stabilized interactions related to ongoing signaling, we found that both receptor clustering and mobility can be reversed by displacement with monovalent ligands within 7 min after antigen addition. We also found that these changes can be modulated through enrichment or reduction in cellular cholesterol levels, pointing again to participation of ordered membrane domains.

B) Functional evidence for PIP_2 nanodomains relevant to Ca^{2+} signaling

PIP_2 was previously implicated in regulating a wide range of ion channels at the plasma membrane [18]. Both positive and negative regulation have been observed in different cases, and the structural bases for this regulation have also been observed to vary widely. In some cases, such as for the KCNQ voltage-gated K^+ channel, a short sequence with a high percentage of positively charged, basic residues has been implicated in this regulation, suggesting an electrostatic association between PIP_2 clusters, which have a high negative change density [63], and an unstructured segment of this protein [19]. In other cases, basic residues scattered throughout a wider sequence of a cytoplasmic segment have been identified as crucial for regulation by PIP_2; it is likely that these basic residues are proximal in the three-dimensional folded structure of this segment. Interestingly, for the large majority of ion channels with PIP_2 regulation thus far described, activation is the most common role for PIP_2 association [18].

A previous study in our laboratory provided initial evidence for functionally distinct pools of PIP_2 that influence Ca^{2+} signaling in response to IgE receptor activation: We found that phosphatidylinositol 4-phosphate 5-kinase Iγ (PIP5K Iγ) contributes to the synthesis of PIP_2

that is hydrolyzed by antigen-stimulated PLCγ to initiate SOCE. In contrast, another isoform, PIP5K Iβ, does not contribute to this pool, but confers negative regulation of SOCE in a mechanism that depends on its catalytic activity [20]. Subsequently, we found that mild cholesterol depletion by methyl β-cyclodextrin strongly inhibits SOCE, indicating a role for cholesterol-dependent, ordered membrane domains in this process [21]. We observed parallel inhibition of the stimulated association between oligomeric STIM1 and Orai1, as monitored by fluorescence resonance energy transfer (FRET) between fluorescent protein-tagged constructs [21, 22]. This stimulated FRET was also decreased by 10 μM wortmannin, which inhibits several different phosphoinositide kinases important in Ca^{2+} signaling [23, 24].

These results, taken together, suggest that cholesterol-dependent lipid order plays a role in the mechanism by which functionally distinct pools of PIP_2 contribute to Ca^{2+} signaling, as indicated by our previous study. To test this hypothesis, we compared the effects of over-expression of PIP5-kinases and plasma membrane-targeted inositol 5-phosphatases, which hydrolyze PIP_2 to PI4P, on the distributions of PIP_2 in sucrose gradient-fractionated cell membranes following lysis by a low concentration of Triton X-100 that preserves cholesterol-dependent, detergent-resistant membrane domains [4, 25]. Under these conditions, over-expression of PIP5K Iβ enhanced PIP_2 levels in both detergent-resistant membranes and in detergent-soluble membranes, whereas PIP5K Iγ enhanced PIP_2 levels only in detergent-soluble membranes [21]. Expression of inositol 5-phosphatase with an ordered membrane targeting sequence (L10: the first 10 amino acids of Lck, which are myristoylated and palmitylated) substantially decreased the pool of PIP_2 in detergent-resistant membrane domains, whereas inositol 5-phosphatase with a disordered membrane targeting sequence (S15: the first 15 amino acids of Src) caused a decrease in the disordered membrane pool of PIP_2, and no significant decrease in the ordered membrane PIP_2 pool. Importantly, over-expression of PIP5K Iβ caused an increase in stimulated STIM1/Orai1 association, whereas over-expression of PIP5K Iγ caused a decrease in stimulated STIM1/Orai1 association. Consistent with this, expression of inositol 5-phosphatase containing an ordered membrane targeting sequence significantly reduced the pool of PIP_2 in the detergent-resistant membranes and it inhibited stimulated STIM1/Orai1 association, whereas expression of inositol 5-phosphatase with a disordered membrane targeting sequence significantly reduced the pool of PIP_2 in the detergent-soluble membranes and enhanced stimulated STIM1/Orai1 association.

These structural effects were paralleled by functional effects on SOCE: Ordered membrane-targeted inositol 5-phosphatase inhibited SOCE stimulated by the SERCA pump ATPase inhibitor, thapsigargin, whereas disordered membrane-targeted inositol 5-phosphatase enhanced this Ca^{2+} response [21]. These results, taken together, can be accounted for by a model in which a higher ratio (>1) of PIP_2 in ordered membrane domains to PIP_2 in disordered membrane domains enhances stimulated SOCE, whereas a lower ratio (<1) inhibits stimulated SOCE. The structural basis for this PIP_2-dependent regulation of stimulated SOCE was revealed by mutational analysis: We found that the positive role of PIP_2 in ordered membrane domains depends on the C-terminal polybasic tail of STIM1, whereas the negative role of PIP_2 in disordered membrane domains depends on a sequence of basic amino acid residues in the N-terminal cytoplasmic segment of Orai1 [21].

As depicted in Figure 3, these results support a model in which two different pools of PIP_2, determined by their preference for ordered or disordered membrane domains, play important roles in the regulation of SOCE: In the absence of STIM1 activation by ER store depletion, Orai1 preferentially associates with disordered membrane domains, and the basic residues in its N-terminal segment associate with PIP_2 in this milleu to facilitate this localization. In this unstimulated state, STIM1 is distributed throughout the ER. Upon activation by depletion of Ca^{2+}, STIM1 is induced to oligomerize [26] and undergoes a conformational change in its cytoplasmic segment ([27]; changes not shown in figure) that together facilitate the binding of its C-terminal polybasic sequence to PIP_2 in the cytoplasmic leaflet of the plasma membrane. For reasons that are not yet clear, this association occurs preferentially with PIP_2 in ordered membrane domains, thereby positioning STIM1 oligomers to couple with plasma membrane-associated Orai1 and driving the redistribution of this CRAC channel protein from a disordered membrane environment to an ordered one. In this model, the ratio of PIP_2 in ordered membrane domains to PIP_2 in disordered membrane domains determines the probability by which STIM1-Orai1 coupling occurs, and changes in this ratio influence the Ca^{2+} influx response.

C) Structural evidence for PIP_2 nanodomains relevant to Ca^{2+} signaling and exocytosis

Similar to the results in Calloway et al. [21], studies by Pike and colleagues identified a pool of PIP_2 in detergent-resistant membrane domains from A431 cells and Neuro 2a cells that is differentially altered by receptor activation compared to that in disordered membranes [28, 29]. One question raised by these biochemical identifications of a pool of PIP_2 in detergent-resistant membranes is whether this phosphoinositide exhibits an acyl chain composition that is consistent with the capacity to pack in ordered domains in live cells. Qualitatively supporting this view, a mass spectrometry analysis of PIP_2 acyl chain composition in human fibroblast cells detected a PIP_2 species with an acyl chain composition of C36:0 (two chains) as a significant component [30]. Interestingly, a biochemical study in plant cells, both from cultured cells and from tobacco leaves, provided evidence that more than half of the PIP_2 in the cultured cells fractionates with detergent-resistant membranes, and the composition of PIP_2 in these cells was found to be dominated by fatty acid species with saturated or monounsaturated acyl chains [31]. Furthermore, immuno-gold EM analysis of plasma membrane-derived vesicles from these cells found evidence for a clustered distribution of PIP_2 with an average diameter of ~50 nm.

In neither of the functional studies on PIP_2 distributions in sucrose gradient-fractionated membranes from mammalian cells cited above was the distribution of PIP_2 in the plasma membrane of intact cells characterized. Perhaps the most reliable way to specifically label PIP_2 in live cells is by the use of the fluorescent protein-tagged PH domain from PLCδ1 [32, 33]. By confocal imaging, this protein typically labels the plasma membrane exclusively, and this labeling is usually uniformly distributed at the resolution of light microscopy, unless morphological features contribute to the appearance of nonuniformity [34, 35]. In a recent study, we gained distributional information while investigating the effectiveness of inhibitors of PI-kinases in interfering with signaling responses downstream of FcεRI activation in RBL cells. In particular, we observed that re-synthesis of PIP_2 at the plasma membrane following its hydrolysis (stimulated by high concentrations of cytoplasmic Ca^{2+})

occurred in discrete puncta at or near the plasma membrane, as monitored by appearance of PLCδ1 PH-EGF at these sites on the timescale of several minutes at 37°C [24]. These PIP_2 puncta were often as large as several microns in diameter, were stably localized over this time period.The puncta were co-labeled by fluorescent cholera toxin B, often used as a marker for detergent-resistant, ordered membrane domains, similar to previous observations [65]. At longer times, the distribution of PLCδ1 PH-EGF became more uniform at the plasma membrane in most cells. Interestingly, both PI-kinase inhibitors, phenyl arsine oxide and quercetin, inhibited the reappearance of PLCδ1 PH-GFP binding to these puncta, suggesting that new synthesis of PIP_2 occurs in these membrane domains [24]. It is not yet clear whether these puncta represent physiologically relevant domains of new PIP_2 synthesis, or whether they result from non-physiological elevation of cytoplasmic Ca^{2+} by the Ca^{2+} ionophore, A23187, which induces large-scale hydrolysis and re-synthesis of PIP_2 in these cells.

Taking advantage of the specificity of the PLCδ PH domain for PIP_2, Fujimoto and colleagues developed a freeze-fracture EM method to characterize the nanoscale distribution of PIP_2 at the inner leaflet of the plasma membrane in cultured fibroblasts and smooth muscle cells [36; see also chapter by T. Fujimoto in this volume]. In addition to observing concentration of PIP_2 both at coated pits and around the rims of caveolae, they also observed weak clustering of PIP_2 throughout flatter regions of the plasma membrane with an average diameter of ~72 nm. The density of PIP_2 in these flatter regions of human fibroblasts was rapidly reduced by stimulation of PLC by angiotensin II, followed by a slower recovery to the original PIP_2 density in these regions, presumably due to resynthesis. Interestingly, both treatments, cholesterol depletion by methyl β-cyclodextrin or inhibition of actin polymerization by latrunculin A, caused the distribution of PIP_2 in these flatter membrane areas to become more random, suggesting that both cholesterol-dependent membrane domains and the actin cytoskeleton participate in the nanoscale clusters of PIP_2 observed.

Several studies used fluorescence to examine the distribution of PIP_2 at the inner leaflet of plasma membrane sheets prepared by sonication of well-attached cells to remove the dorsal plasma membrane, the bulk of the cytoplasm, and intracellular organelles. Using this approach on PC12 cells, Aoyagi et al. [37] detected fluorescent puncta by confocal microscopy in cells expressing PLCδ1 PHEGFP or labeled with an anti-PIP_2 mAb. They further showed partial co-localization of these puncta with the plasma membrane SNARE protein, syntaxin-1, and with large dense core secretory vesicles, suggesting that these PIP_2 puncta play a role in stimulated exocytosis [37]. Interestingly, about half of the PIP_2 puncta showed some co-localization with clusters of the GPI-linked protein Thy-1, consistent with association of these with ordered membrane domains as described above. Van den Bogaart et al. [38] used a similar plasma membrane preparation to examine the distribution of PIP_2 with nanoscale resolution STED microscopy. They found the organization of PIP_2 labeled by either the PLCδ PH domain or by an anti-PIP_2 mAb to be in clusters with average diameters of 73 and 87 nm, respectively. Like Aoyagi et al. [37], van den Bogaart et al. [38] also observed clusters of syntaxin-1 similar to those of PIP_2, and co-expression of the PI-phosphatase synaptojanin-1 reduced the size of these clusters by 3.7-fold. Furthermore, PIP_2 in giant unilamellar lipid vesicles (GUVs) caused binding and micron-scale clustering of the soluble C-terminal fragment of syntaxin-1, which contains PIP_2-associating basic residues.

Cholesterol was also found to cause clustering of this syntaxin-1 peptide in GUVs, but it was not necessary for clustering caused by PIP_2. These results, obtained with high spatial resolution, provide evidence for the existence of plasma domains of PIP_2 with functional implications in neuronal exocytosis.

The structural basis for the PIP_2 nanodomains observed in this study remains an open question. Although van den Bogaart et al. [38] demonstrate that PIP2/syntaxin-1 electrostatic association is sufficient to induce micron-scale clusters in GUVs, their data in plasma membrane sheets, and that of Aoyagi et al. [37], indicate that syntaxin-1 localizes to only a fraction (5-10%) of the observed PIP_2 clusters. Super resolution imaging with dSTORM by Wang and Richards [39] provided evidence that clusters of phosphoinositide 3,4,5-trisphosphate (PIP_3) are distinct from clusters of PIP_2, with average diameters 103 nm for the latter. While it is possible that different phosphoinositide nanodomains arise from basic binding motifs on distinctive proteins, it is not yet clear to what extent such electrostatic associations account for the range of phosphoinositide nanodomains that exist and the basis for structural heterogeneity.

Recent evidence points to BAR domain proteins as a general class that bind phosphoinositides to restrict their lateral diffusion, both in model membranes and in plasma membranes of yeast cells [40]. The BAR domain is typically a dimeric α-helical protein motif that interacts with membranes through a curved interface. These proteins frequently oligomerize into helical scaffolds that can promote membrane deformation [41]. They often exhibit a high concentration of basic residues on one surface that can electrostatically bind to negatively charged phospholipid head groups, including phosphoinositides, which have the highest charge density among this phospholipid subset [42]. BAR domain proteins are often implicated in cellular processes that require high degrees of membrane curvature, but some can stabilize planar membrane sheets [43]. The potential roles of this protein class in stabilizing phosphoinositde nanodomains in cells remain to be determined.

Segregation of PIP_2 and PIP_3 in micron-scale domains has been characterized in the process of phagocytosis. In studies on cultured macrophages by Grinstein and colleagues, phagocytic cup formation during the initial stage of this process was found to be accompanied by concentration of PIP_2 at the cytoplasmic side of the forming phagosome relative to the region of the plasma membrane outside of the phagocytic cup [44]. Over the course of several minutes, the concentration of PIP_2 declined and was replaced by enrichment of PIP_3 in the same region [45]. Subsequent to pinching off and complete internalization of the phagosome, PIP_3 returned to levels similar to that in the nonphagocytosed plasma membrane. In this dramatic manifestation of plasma membrane lipid heterogeneity, McLaughlin and colleagues found that the PIP_2 localized to the phagosomal cup is laterally segregated from PIP_2 in the rest of the plasma membrane by a diffusion barrier: Photobleaching of a fluorescent PIP_2 analogue in this region did not lead to fluorescence recovery, even though recovery occurred efficiently in regions of the PM distal from the phagocytic cup, whereas fluorescence correlation spectroscopy measurements indicated that this analogue is mobile in both regions [46]. Although the structural basis for this segregation was not determined, the authors speculate that the diffusion barrier detected may be mediated by the septin family of proteins. Septins have

been previously shown to participate in membrane diffusion barriers in budding yeast at mother-daughter bud neck junctions [47], and also at the midbody during mammalian cell cytokinesis [48].

In a recent siRNA screen for proteins that contribute to the regulation of STIM1/Orai1 coupling, Sharma et al. [49] identified several different septin family members as playing important roles in this process. They showed that septins 2,4, and 5 all contribute to efficient coupling of STIM1 and Orai1 under conditions of ER store depletion. Furthermore, these septins appeared to organize PIP_2 in domains around STIM1/Orai1 puncta [49]. Septins contain a conserved polybasic sequence that appears to be involved in this organization, possibly similar to the association of PIP_2 with syntaxin-1 via its polybasic cytoplasmic sequence as described above. Indeed, septins have been implicated in the regulation of syntaxin-dependent fusion events [50]. The details of the structural organization of septins with PIP_2 remain unclear, but, by analogy to the function of septins in budding yeast, these proteins may oligomerize to form filamentous barriers that retain concentrations of PIP_2 in nano- or micro-domains to facilitate associations of this phosphoinositide with STIM1 and Orai1 as detected in the biochemical and FRET experiments described above. Future studies that incorporate super resolution imaging methods should help to illuminate structural interactions among PIP_2 and septins within the heterogeneous plasma membrane.

D) Conclusions and Future Directions

Studies summarized in this review serve to illustrate how plasma membrane participation in cellular process, strongly implicated in biochemical experiments, can now be examined at increasing spatial resolution approaching the level of molecular complexes. As described in Section A, super resolution fluorescence imaging now provides not only “snapshots” of receptor redistribution during stimulated signaling at the nanometer scale in fixed cells, but also, in live cells, the dynamics of individual receptor redistributions during this process with robust statistical analysis. An important consequence is the capacity to relate directly time-dependent changes in receptor diffusion with changes in receptor interactions in response to an external stimulus. Our dSTORM measurements of IgE receptor clustering in response to antigen have revealed that loss of lateral diffusion occurs prior to large-scale receptor clustering, and comparison of the temporal relationships of these processes to the onset of Ca^{2+} signaling indicates that the latter occurs only after the stage of small receptor clusters that are likely interacting with other membrane associated components [14].

Key interactions involved in cell signaling initiated by clustered IgE-FcεRI remain to be investigated in detail, and two-color super resolution imaging offers exciting prospects [51]. For example, we expect it will be possible to monitor the dynamics of FcεRI association with Lyn tyrosine kinase and evaluate the role played by ordered membrane domains in facilitating this interaction. It also may be possible to observe dynamic association of Syk tyrosine kinase with phosphorylated FcεRI. Previous confocal imaging studies have failed to detect this interaction [52], even though its occurrence has been established by genetic and biochemical methods [53]. Well defined bivalent ligands, such as monoclonal anti-IgE [54] or paucivalent ligands based on DNA spacers [55, 56] can be used to limit clustering of IgE-FcεRI on the cell surface, and this should allow clearer interpretation of resulting signaling

events, for example, the relevance of receptor mobility and cluster properties. This information will be valuable in ongoing efforts to model early signaling events initiated by IgE-FcεRI and other multi-chain immune recognition receptors [57].

Downstream of IgE-FcεRI activation, Ca^{2+} mobilization is central to most consequent cellular responses. The initial steps in this process, the activation of PLC to produce IP_3, are common to many receptors, including both those of the multi-chain immune recognition receptors, as well as many different G protein-coupled receptors. Although these latter receptors utilize PLCβ rather than PLCγ to hydrolyze PIP_2, subsequent events are quite similar, and both receptor classes activate STIM1-Orai1 coupling as a major pathway to SOCE [23, 58]. As summarized in Section B, there is now strong evidence that PIP_2 is involved in the functional coupling of STIM1-Orai1, in addition to its role as the substrate for PLC in the activation of ER store depletion leading to SOCE. Several studies now implicate PIP_2 in regulating STIM1-Orai1 coupling, and the ratio of PIP_2 in ordered membrane domains to PIP_2 in disordered membrane domains appears to be a key feature (Figure 3). With the advent of super resolution methods to visualize nanoscale distributions of PIP_2 at the plasma membrane, as described in Section C, it should now be possible to investigate whether nanoscale clusters of PIP_2 in ordered *vs* disordered membrane domains can be distinguished. The use of rapidly recruitable, rapamycin-dependent association of FKP-inositol 5-phosphatase [19] with FRB binding domains attached to order- *vs* disorder-preferring protein motifs [59] should permit rapid modulation of PIP_2 pools in each of these domains, providing additional insight to spatial distribution. A prediction of our model (Figure 3) is that Orai1 clusters with PIP_2 in disordered membrane domains in the absence of stimulation, and with PIP_2 in ordered membrane domains following activation of STIM1-Orai1 coupling. Nanoscale imaging using these recruitment strategies should allow this hypothesis to be tested, and this approach should also allow examination of septin participation in PIP_2 distributions [49].

The predicted role of PIP_2 nanodomains in exocytosis discussed in Section C was recently evaluated by Honigmann et al. [60], who provided evidence that the Ca^{2+} binding C2A/2B fragment of secretory vesicle-associated synaptotagmin-1 binds to PIP_2 in syntaxin-1/PIP_2 clusters prior to Ca^{2+} elevation. This could facilitate plasma membrane/secretory vesicle docking and enhance the Ca^{2+}- and SNARE-dependent membrane fusion to mediate vesicle exocytosis. Regulation of secretory granule exocytosis in mast cells by the polybasic effector domain of the MARCKS protein has been previously demonstrated [61], and electrostatic binding of this peptide to PIP_2 at the plasma membrane has been implicated in this regulation. This 25-residue effector domain sequence contains three threonine residues that, upon phosphorylation by protein kinase C, have been shown to result in dissociation from PIP_2-containing membranes [62, 63]. Using super resolution imaging methods, it should be possible to test whether this MARCKS effector domain peptide exhibits localized binding to PIP_2/syntaxin clusters, and whether dissociation occurs under conditions of secretory vesicle exocytosis. With the recent advances in super resolution imaging highlighted in this review, the stage is now set for a plethora of new insights into cell membrane biology questions, including the many that involve phosphoinositides in cell signaling.

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Author Biographies

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My research interests and current work include long-term efforts to understand molecular mechanisms by which crosslinking of IgE receptors on mast cells triggers complex cellular signaling processes that lead to important functional responses in immune host defense. Central to mast cell and other cell signaling responses is the mobilization of intracellular calcium ions, and a component of my current work focuses on understanding this spatio-temporally complex process and its function roles in exocytosis, cytokine production, and host-pathogen interactions.

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I became fascinated by cell membrane function in college when the Singer-Nicholson model [66] came along to capture the interest of a diverse range of chemists, physicists and biologists. Since then, taking the route of biophysical chemistry, I have had the good fortune of linking up with Dave Holowka to investigate membrane participation in the functioning of IgE receptors on mast cells. Over the years, our joint research group at Cornell, together with our valuable collaborators, have steadily worked to develop higher resolution tools to elucidate both early and downstream signaling events initiated by activated IgE receptors, and how these are regulated and targeted by membrane interactions.

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Summary

- TIRF and super-resolution imaging reveals that crosslinking of IgE/FcεRI complexes by multivalent antigen results in the time-dependent formation of nanoscale clusters that rapidly lose laterally mobility over several minutes and more slowly form larger clusters that continue to activate Ca^{2+} mobilization leading to granule exocytosis.

- Super-resolution imaging reveal nanoscale clusters of phosphoinosities, primarily PIP_2, at the plasma membrane that appear to participate in exocytosis and other downstream signaling processes.

- Although electrostatic interactions between negatively charged phosphoinositides and proteins with spatial concentrations of positively charged, basic amino acids undoubtedly contribute to phosphoinositide clusters, the structural bases for these PIP_2 nanodomains are incompletely understood.

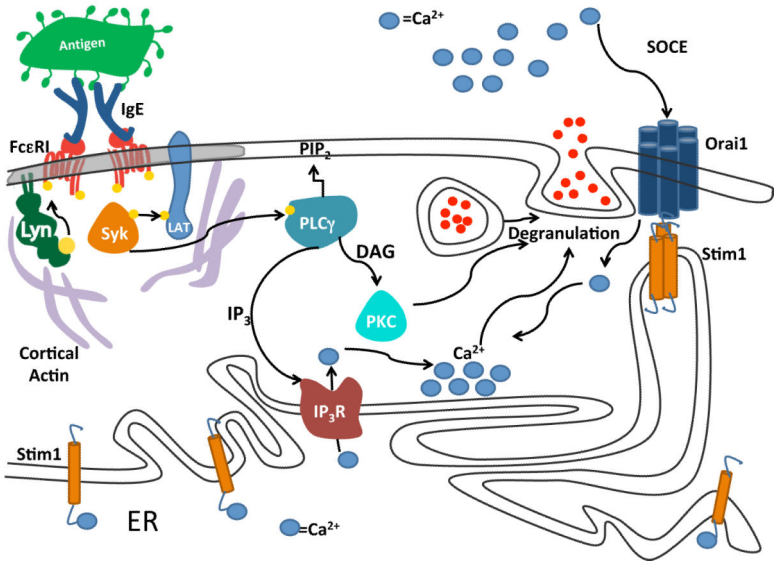

Figure 1.

Schematic of intracellular signaling after antigen-mediated crosslinking of IgE-FcεRI on the surface of mast cells. High affinity binding of antigen-specific IgE to FcεRI sensitizes these receptors to antigen-stimulated signaling that is initiated by a tyrosine phosphorylation cascade which utilizes Lyn kinase phosphorylation of FcεRI, recruitment and activation of Syk kinase, and consequent phosphorylation of adaptor proteins such as LAT and enzymes such as phospholipase Cγ (PLCγ). IP_3 produced by hydrolysis of PIP_2 by PLCγ activates store-operated Ca^{2+} entry (SOCE) that depends on coupling between the ER Ca^{2+} sensor STIM1 and the plasma membrane Ca^{2+} channel Orai1 and is important for granule exocytosis. Activation of protein kinase C (PKC) by the lipid product of PIP_2 hydrolysis, diacylglycerol (DAG), contributes to the exocytosis response.

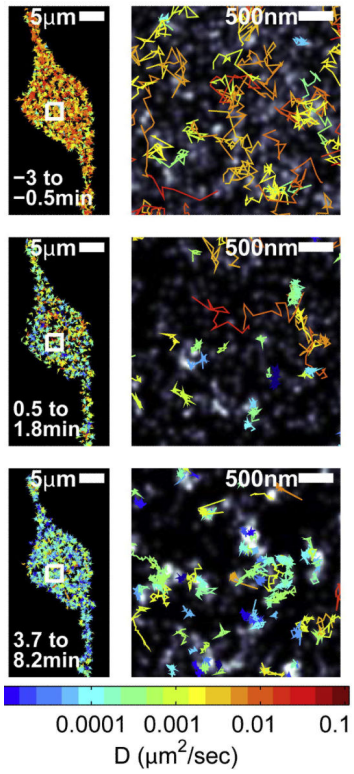

Figure 2.

dSTORM imaging of fluorescently labeled IgE-FcεRI on the surface of RBL mast cells. Single-molecule trajectories persisting for at least 0.5 s are superimposed on a superresolution image reconstructed from unstimulated data (*upper*), from data acquired within 1 min of antigen addition (*middle*), and from data acquired after several minutes of stimulation. Track coloring indicates D_S for each track on a log scale from $10^{-5}\mu m^2/s$ (*blue*) to 1 $\mu m^2/s$ (*red*). Images on the right are enlargements of the boxed regions in the images on the left. Figure 4C from Shelby et al [14].

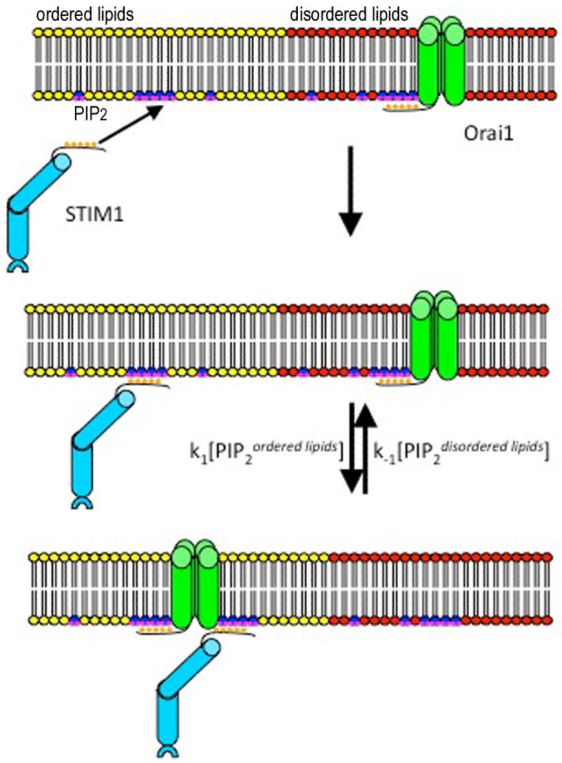

Figure 3.
Minimal model for the regulation of STIM1-Orai1 coupling by PIP_2 in ordered and disordered membrane domains. (Modified from [64]).