

Nanodomains in early and later phases of FcεRI signalling

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Abstract

Our long-term efforts to elucidate receptor-mediated signalling in immune cells, particularly transmembrane signalling initiated by FcεRI, the receptor for 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 signalling components with antigen-cross-linked IgE–FcεRI and pointed to participation of ordered membrane domains in the signalling process. With a focus on this function, we have worked along with others to develop diverse and increasingly sophisticated tools to analyse the complexity of membrane structure that facilitates regulation and targeting of signalling events. The present chapter 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:

electrostatic association, exocytosis, IgE receptor (FcεRI), mast cell, phosphoinositide-dependent signalling, receptor immobilization and clustering, store-operated Ca²⁺ entry, 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.

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Cross-linking of IgE–FcεRI by multivalent ligands (antigen) in IgE-binding sites initiates transmembrane signalling 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 (linker for activation of T-cells), as well as PLC (phospholipase C)γ1 and γ2 [1]. Activation of these lipases by tyrosine phosphorylation results in hydrolysis of PI(4,5)P₂ (phosphatidylinositol 4,5-bisphosphate) to generate 2,3-diacylglycerol, which is the membrane-associated activator of protein kinase C, and I(1,4,5)P₃ (inositol 1,4,5-trisphosphate), which is the soluble activator of Ca²⁺ release from the ER (endoplasmic reticulum). I(1,4,5)P₃-mediated release of Ca²⁺ from ER stores triggers the process known as SOCE (store-operated Ca²⁺ entry), in which the ER Ca²⁺ sensor protein, STIM1, oligomerizes in response to Ca²⁺ depletion within the ER and couples to the CRAC (Ca²⁺ release-activated Ca²⁺) channel Orai1, resulting in sustained Ca²⁺ influx that is necessary for functional responses such as exocytosis and cytokine biosynthesis and secretion [2,3]. These signalling pathways lead to secretion of histamine and other cellular responses associated with allergic reactions.

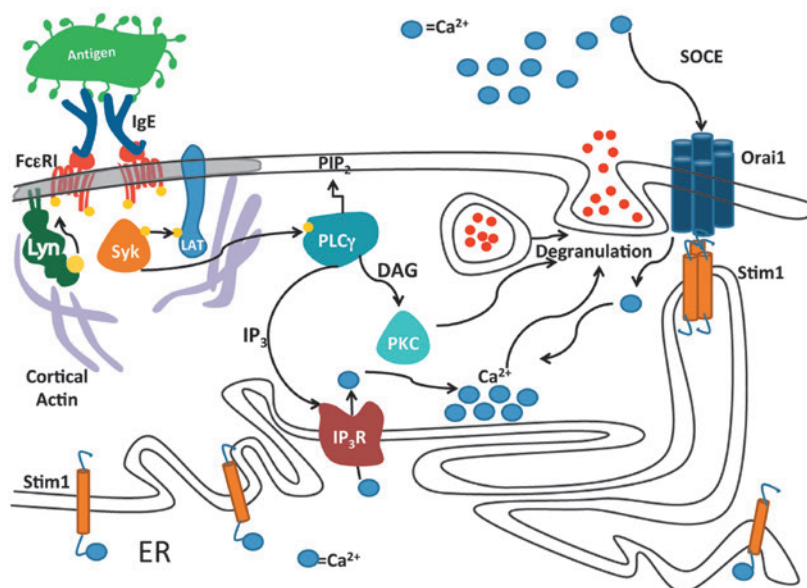


Figure 1. Schematic of intracellular signalling after antigen-mediated cross-linking of IgE–FcεRI on the surface of mast cells

High-affinity binding of antigen-specific IgE to FcεRI sensitizes these receptors to antigen-stimulated signalling that is initiated by a tyrosine phosphorylation cascade that 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 PLCγ. I(1,4,5)P₃ (IP3) produced by hydrolysis of PI(4,5)P₂ (PIP2) by PLCγ activates SOCE that depends on coupling between the ER Ca²⁺ sensor STIM1 and the plasma membrane Ca²⁺ channel Orai1 and is important for granule exocytosis. Activation of protein kinase C (PKC) by the lipid product of PI(4,5)P₂ hydrolysis, diacylglycerol (DAG), contributes to the exocytosis response.

Nanodomains formed by antigen clustering of IgE–FcεRI complexes

Early in our studies of cell signalling, using RBL-2H3 mast cells as a model system, we found using fluorescence microscopy that restricting IgE–FcεRI cross-linking to trimers caused much larger scale clustering of these receptors on the cell surface – the cell was somehow amplifying the original small cluster [4]. Our thought that this involves the plasma membrane structure was supported by later fluorescence microscopy experiments showing that antigen cross-linking of IgE–FcεRI causes certain types of lipids to co-redistribute with the clustered receptors [5]. This led us to a much larger realization: we had to consider how the plasma membrane structure contributes to cellular signalling. This is the subject of the present chapter.

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 signalling over the years, membrane lipids are receiving increasing attention. This interest is based in part on the impact of lipid phase behaviour, 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 whereupon 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 behaviour 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 L_o [liquid ordered; rich in high- T_m (melting temperature) lipids and cholesterol] and L_d (liquid disordered; rich in low- T_m lipids). A common term currently used to describe the L_o -like domains in biological membranes is ‘lipid rafts’, in which receptors connect effectively with other membrane and signalling 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 L_o -type or ‘ordered’ membrane domains include resistance to detergent solubility and dependence on cholesterol [6,7]. 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 towards understanding plasma membrane participation in cell signalling 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 [8–10]. In the present chapter, 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 (≥ 250 nm). To gain a nanoscale view, we first turned to scanning electron microscopy with IgE and selected signalling components labelled with gold particles by means of secondary antibodies. After overcoming multiple challenges to determine the appropriate analysis [11], we demonstrated that antigen-clustering of IgE–FcεRI on RBL mast cells causes co-redistribution of Lyn kinase and other signalling components on the inner plasma membrane [12]. Pair correlation analysis showed the average radius of these co-clusters to be 70–90 nm within 1 min 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 nanometre resolution in cell studies for many years. However, EM imaging has a number of limitations, including requirements for sample fixation and immunogold for specific labelling. 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 STED (stimulated emission depletion) microscopy [13], currently the most widely used are the localization microscopies, which are most readily built into standard fluorescence microscopes. These include PALM/fPALM (photo-activated localization microscopy/fluorescence PALM) [14,15] and STORM/dSTORM (stochastic optical reconstruction microscopy/direct STORM) [16], which typically also utilize TIRF (total internal reflectance fluorescence) optics (see also Chapter 8 in the present volume [16a]). 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 labelled cells by sequential activation and pinpointing 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 signalling events. Our collaborative studies with Sarah Veatch at the University of Michigan used fluorescently labelled IgE and dSTORM to image IgE–FcεRI on the cell surface, before and after addition of antigen [17]. 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

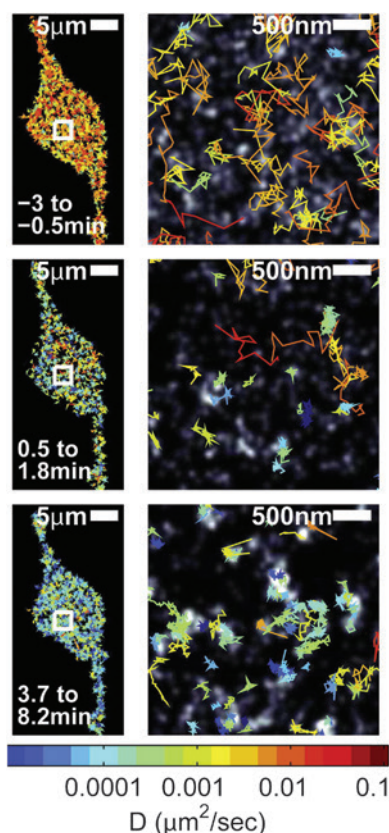


Figure 2. dSTORM imaging of fluorescently labelled IgE–FcεRI on the surface of RBL mast cells

Single-molecule trajectories persisting for at least 0.5 s are superimposed on a super-resolution image reconstructed from unstimulated data (upper panels), from data acquired within 1 min of antigen addition (middle panels) and from data acquired after several minutes of stimulation (lower panels). Track colouring indicates short time diffusion coefficients (D) for each track on a log scale from $10^{-5} \mu\text{m}^2/\text{s}$ (blue) to $1 \mu\text{m}^2/\text{s}$ (red). Images on the right are enlargements of the boxed regions in the images on the left. Figure 4(C) from Shelby et al. [17].

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, that is, clustering and mobility, can also be correlated to cell signalling events that are stimulated in parallel, and this study considered stimulated Ca^{2+} mobilization (see below). 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 signalling. During the second stage, beginning at about 1.5 min, receptors become tightly packed and confined. Although this stage probably represents multiple, stabilized interactions related to ongoing signalling, 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.

Functional evidence for PI(4,5) P_2 nanodomains relevant to Ca²⁺ signalling

PI(4,5) P_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 PI(4,5) P_2 clusters, which have a high negative charge density [19], and an unstructured segment of this protein [20]. In other cases, basic residues scattered throughout a wider sequence of a cytoplasmic segment have been identified as crucial for regulation by PI(4,5) P_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 PI(4,5) P_2 regulation thus far described, activation is the most common role for PI(4,5) P_2 association [18].

A previous study in our laboratory provided initial evidence for functionally distinct pools of PI(4,5) P_2 that influence Ca²⁺ signalling in response to IgE receptor activation. We found that PI(4)P5 K I γ (phosphatidylinositol 4-phosphate 5-kinase I γ) contributes to the synthesis of PI(4,5) P_2 that is hydrolysed by antigen-stimulated PLC γ to initiate SOCE. In contrast, another isoform, PI(4)P5 K I β , does not contribute to this pool, but confers negative regulation of SOCE in a mechanism that depends on its catalytic activity [21]. 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 [22]. We observed parallel inhibition of the stimulated association between oligomeric STIM1 and Orai1, as monitored by FRET between fluorescent protein-tagged constructs [22,23]. This stimulated FRET was also decreased by 10 μ M wortmannin, which inhibits several different phosphoinositide kinases important in Ca²⁺ signalling [24,25].

These results, taken together, suggest that cholesterol-dependent lipid order plays a role in the mechanism by which functionally distinct pools of PI(4,5) P_2 contribute to Ca²⁺ signalling, as indicated by our previous study. To test this hypothesis, we compared the effects of overexpression of PI(4)P5Ks and plasma membrane-targeted inositol 5-phosphatases, which hydrolyse PI(4,5) P_2 to PI(4)P, on the distributions of PI(4,5) P_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 [7,26]. Under these conditions, overexpression of PI(4)P5 K I β enhanced PI(4,5) P_2 levels in both detergent-resistant membranes and in detergent-soluble membranes, whereas PI(4)P5 K I γ enhanced PI(4,5) P_2 levels only in detergent-soluble membranes [22]. 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 PI(4,5) P_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 $\text{PI}(4,5)\text{P}_2$, and no significant decrease in the ordered membrane $\text{PI}(4,5)\text{P}_2$ pool. Importantly, overexpression of $\text{PI}(4)\text{P}_5\text{K I}\beta$ caused an increase in stimulated STIM1–Orai1 association, whereas overexpression of $\text{PI}(4)\text{P}_5\text{K I}\gamma$ 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 $\text{PI}(4,5)\text{P}_2$ 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 $\text{PI}(4,5)\text{P}_2$ 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 [22]. These results, taken together, can be accounted for by a model in which a higher ratio (>1) of $\text{PI}(4,5)\text{P}_2$ in ordered membrane domains to $\text{PI}(4,5)\text{P}_2$ in disordered membrane domains enhances stimulated SOCE, whereas a lower ratio (<1) inhibits stimulated SOCE. The structural basis for this $\text{PI}(4,5)\text{P}_2$ -dependent regulation of stimulated SOCE was revealed by mutational analysis: we found that the positive role of $\text{PI}(4,5)\text{P}_2$ in ordered membrane domains depends on the C-terminal polybasic tail of STIM1, whereas the negative role of $\text{PI}(4,5)\text{P}_2$ in disordered membrane domains depends on a sequence of basic amino acid residues in the N-terminal cytoplasmic segment of Orai1 [22].

As depicted in Figure 3, these results support a model in which two different pools of $\text{PI}(4,5)\text{P}_2$, 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 $\text{PI}(4,5)\text{P}_2$ in this milieu 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 [27] and undergoes a conformational change in its cytoplasmic segment ([28]; changes not shown in Figure 3) that together facilitate the binding of its C-terminal polybasic sequence to $\text{PI}(4,5)\text{P}_2$ in the cytoplasmic leaflet of the plasma membrane. For reasons that are not yet clear, this association occurs preferentially with $\text{PI}(4,5)\text{P}_2$ 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 $\text{PI}(4,5)\text{P}_2$ in ordered membrane domains to $\text{PI}(4,5)\text{P}_2$ in disordered membrane domains determines the probability by which STIM1–Orai1 coupling occurs, and changes in this ratio influence the Ca^{2+} influx response.

Structural evidence for $\text{PI}(4,5)\text{P}_2$ nanodomains relevant to Ca^{2+} signalling and exocytosis

Similar to the results of Calloway et al. [22], studies by Pike and colleagues identified a pool of $\text{PI}(4,5)\text{P}_2$ in detergent-resistant membrane domains from A431 cells and Neuro 2a cells that is

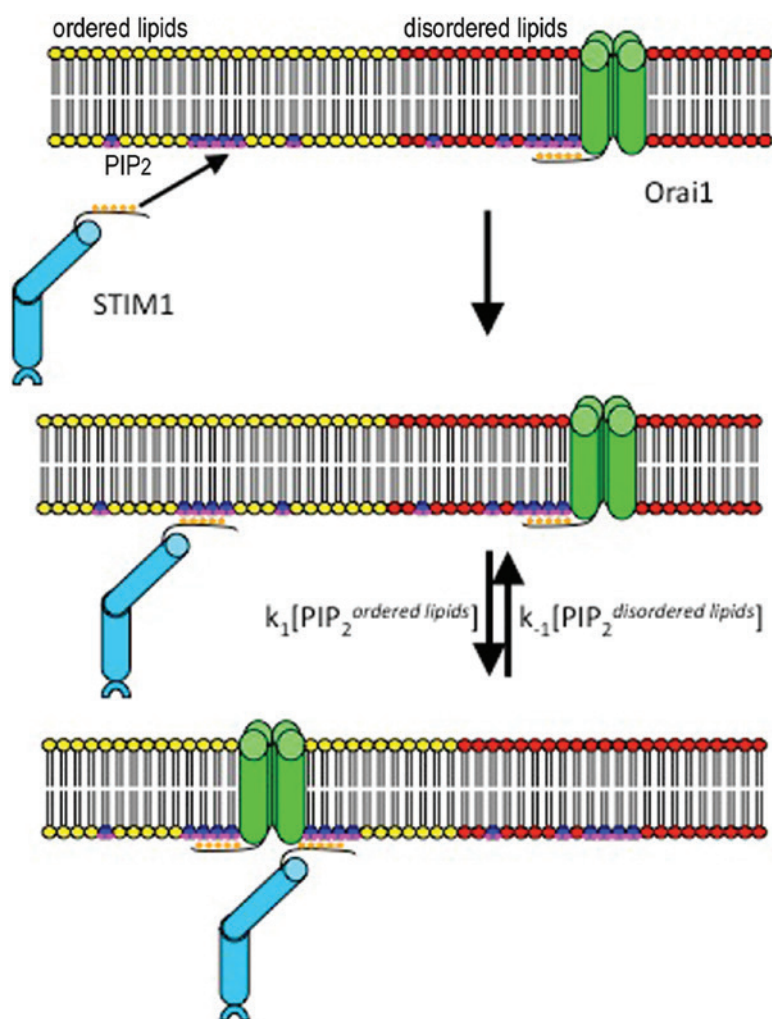


Figure 3. Minimal model for the regulation of STIM1–Orai1 coupling by PI(4,5)P₂ in ordered and disordered membrane domains

PIP₂, PI(4,5)P₂. Modified from [65].

differentially altered by receptor activation compared with that in disordered membranes [29,30]. One question raised by these biochemical identifications of a pool of PI(4,5)P₂ 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 PI(4,5)P₂ acyl chain composition in human fibroblast cells detected a PI(4,5)P₂ species with an acyl chain composition of C_{36:0} (two chains) as a significant component [31]. Interestingly, a biochemical study in plant cells, both from cultured cells and from tobacco leaves, provided evidence that more than half of the PI(4,5)P₂ in the cultured cells fractionates with detergent-resistant membranes, and the composition of PI(4,5)P₂ in these cells was found to be dominated by fatty acid species with saturated or monounsaturated acyl chains [32]. Furthermore, immunogold EM analysis of plasma

membrane-derived vesicles from these cells found evidence for a clustered distribution of PI(4,5) P_2 with an average diameter of ~50 nm.

In neither of the functional studies on PI(4,5) P_2 distributions in sucrose gradient-fractionated membranes from mammalian cells cited above was the distribution of PI(4,5) P_2 in the plasma membrane of intact cells characterized. Perhaps the most reliable way to specifically label PI(4,5) P_2 in live cells is by the use of the fluorescent protein-tagged PH (pleckstrin homology) domain from PLC δ 1 [33,34]. By confocal imaging, this protein typically labels the plasma membrane exclusively, and this labelling is usually uniformly distributed at the resolution of light microscopy, unless morphological features contribute to the appearance of non-uniformity [35,36]. In a recent study, we gained distributional information while investigating the effectiveness of inhibitors of PI-kinases in interfering with signalling responses downstream of Fc ϵ RI activation in RBL cells. In particular, we observed that resynthesis of PI(4,5) P_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 PLC δ 1 PH-EGFP at these sites on the timescale of several minutes at 37°C [25]. These PI(4,5) P_2 puncta were often as large as several micrometres in diameter, and they were stably localized over this time period. The puncta were co-labelled by fluorescent cholera toxin B, often used as a marker for detergent-resistant, ordered membrane domains, similar to previous observations [37]. At longer times, the distribution of PLC δ 1 PH-EGFP became more uniform at the plasma membrane in most cells. Interestingly, both PI-kinase inhibitors, phenyl arsine oxide and quercetin, inhibited the reappearance of PLC δ 1 PH-EGFP binding to these puncta, suggesting that new synthesis of PI(4,5) P_2 occurs in these membrane domains [25]. It is not yet clear whether these puncta represent physiologically relevant domains of new PI(4,5) P_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 resynthesis of PI(4,5) P_2 in these cells.

Taking advantage of the specificity of the PLC δ PH domain for PI(4,5) P_2 , Fujimoto and colleagues developed a freeze-fracture EM method to characterize the nanoscale distribution of PI(4,5) P_2 at the inner leaflet of the plasma membrane in cultured fibroblasts and smooth muscle cells ([38]; see also Chapter 7 in this volume [38a]). In addition to observing concentration of PI(4,5) P_2 both at coated pits and around the rims of caveolae, they also observed weak clustering of PI(4,5) P_2 throughout flatter regions of the plasma membrane with an average diameter of ~72 nm. The density of PI(4,5) P_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 PI(4,5) P_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 PI(4,5) P_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 PI(4,5) P_2 observed.

Several studies used fluorescence to examine the distribution of PI(4,5) P_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. [39] detected fluorescent puncta by confocal microscopy in cells expressing PLC δ 1 PH-EGFP or labelled with an anti-PI(4,5) P_2 mAb (monoclonal antibody). 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 PI(4,5) P_2 puncta play a role in stimulated exocytosis [39]. Interestingly, approximately half of the PI(4,5) P_2 puncta showed some co-localization with clusters of the GPI (glycosylphosphatidylinositol)-linked protein Thy-1, consistent with association of these with ordered membrane domains as described above. Van den Bogaart et al. [40] used a similar plasma membrane preparation to examine the distribution of PI(4,5) P_2 with nanoscale resolution STED microscopy. They found the organization of PI(4,5) P_2 labelled by either the PLC δ PH domain or by an anti-PI(4,5) P_2 mAb to be in clusters with average diameters of 73 and 87 nm, respectively. Like Aoyagi et al. [39], van den Bogaart et al. [40] also observed clusters of syntaxin-1 similar to those of PI(4,5) P_2 , and co-expression of the PI-phosphatase synaptojanin-1 reduced the size of these clusters by 3.7-fold. Furthermore, PI(4,5) P_2 in GUVs (giant unilamellar vesicles) caused binding and micrometre-scale clustering of the soluble C-terminal fragment of syntaxin-1, which contains PI(4,5) P_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 PI(4,5) P_2 . These results, obtained with high spatial resolution, provide evidence for the existence of plasma domains of PI(4,5) P_2 with functional implications in neuronal exocytosis.

The structural basis for the PI(4,5) P_2 nanodomains observed by van den Bogaart et al. [40] remains an open question. Although van den Bogaart et al. demonstrate that PI(4,5) P_2 -syntaxin-1 electrostatic association is sufficient to induce micrometre-scale clusters in GUVs, their data in plasma membrane sheets, and those of Aoyagi et al. [39], indicate that syntaxin-1 localizes to only a fraction (5–10%) of the observed PI(4,5) P_2 clusters. Super-resolution imaging with dSTORM by Wang and Richards [41] provided evidence that clusters of PI(3,4,5) P_3 (phosphoinositide 3,4,5-trisphosphate) are distinct from clusters of PI(4,5) P_2 , with average diameters of 103 nm for the latter. Although 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 [42]. 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 [43]. 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 [44]. BAR domain proteins are often implicated in cellular processes that require high degrees of membrane curvature, but some can stabilize planar membrane sheets [45]. The potential roles of this protein class in stabilizing phosphoinositide nanodomains in cells remain to be determined.

Segregation of PI(4,5) P_2 and PI(3,4,5) P_3 in micrometre-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 PI(4,5) P_2 at the cytoplasmic side of the forming phagosome relative to the region of the plasma membrane outside of the phagocytic cup [46]. Over the course of several minutes, the concentration of PI(4,5) P_2 declined and was replaced by

enrichment of PI(3,4,5) P_3 in the same region [47]. Subsequent to pinching off and complete internalization of the phagosome, PI(3,4,5) P_3 returned to levels similar to that in the non-phagocytosed plasma membrane. In this dramatic manifestation of plasma membrane lipid heterogeneity, McLaughlin and colleagues found that the PI(4,5) P_2 localized to the phagosomal cup is laterally segregated from PI(4,5) P_2 in the rest of the plasma membrane by a diffusion barrier: photobleaching of a fluorescent PI(4,5) P_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 [48]. 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 [49], and also at the midbody during mammalian cell cytokinesis [50].

In a recent siRNA screen for proteins that contribute to the regulation of STIM1–Orai1 coupling, Sharma et al. [51] 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 PI(4,5) P_2 in domains around STIM1–Orai1 puncta [51]. Septins contain a conserved polybasic sequence that appears to be involved in this organization, possibly similar to the association of PI(4,5) P_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 [52]. The details of the structural organization of septins with PI(4,5) P_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 PI(4,5) P_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 PI(4,5) P_2 and septins within the heterogeneous plasma membrane.

Conclusions and future directions

Studies summarized in the present review serve to illustrate how plasma membrane participation in cellular processes, strongly implicated in biochemical experiments, can now be examined at increasing spatial resolution approaching the level of molecular complexes. As described in the section ‘Nanodomains formed by antigen clustering of IgE–Fc ϵ RI complexes’, super-resolution fluorescence imaging now provides, with robust statistical analysis, not only ‘snapshots’ of receptor redistribution during stimulated signalling at the nanometre scale in fixed cells, but also, in live cells, the dynamics of individual receptor redistributions during this process. 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 with the onset of Ca²⁺ signalling indicates that the latter occurs only after the stage of small receptor clusters that are probably interacting with other membrane-associated components [17].

Key interactions involved in cell signalling initiated by clustered IgE–FcεRI remain to be investigated in detail, and two-colour super-resolution imaging offers exciting prospects [53]. For example, we expect that 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 [54], even though its occurrence has been established by genetic and biochemical methods [53]. Well-defined bivalent ligands, such as monoclonal anti-IgE [56] or paucivalent ligands based on DNA spacers [57,58] can be used to limit clustering of IgE–FcεRI on the cell surface, and this should allow clearer interpretation of resulting signalling events, for example, the relevance of receptor mobility and cluster properties. This information will be valuable in ongoing efforts to model early signalling events initiated by IgE–FcεRI and other multi-chain immune recognition receptors [59].

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 $\text{I}(1,4,5)\text{P}_3$, 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 $\text{PLC}\beta$ rather than $\text{PLC}\gamma$ to hydrolyse $\text{PI}(4,5)\text{P}_2$, subsequent events are quite similar, and both receptor classes activate STIM1–Orai1 coupling as a major pathway to SOCE [24,60]. As summarized in the section ‘Functional evidence for $\text{PI}(4,5)\text{P}_2$ nanodomains relevant to Ca^{2+} signalling’, there is now strong evidence that $\text{PI}(4,5)\text{P}_2$ 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 $\text{PI}(4,5)\text{P}_2$ in regulating STIM1–Orai1 coupling, and the ratio of $\text{PI}(4,5)\text{P}_2$ in ordered membrane domains to $\text{PI}(4,5)\text{P}_2$ in disordered membrane domains appears to be a key feature (Figure 3). With the advent of super-resolution methods to visualize nanoscale distributions of $\text{PI}(4,5)\text{P}_2$ at the plasma membrane, as described in the section ‘Structural evidence for $\text{PI}(4,5)\text{P}_2$ nanodomains relevant to Ca^{2+} signalling and exocytosis’, it should now be possible to investigate whether nanoscale clusters of $\text{PI}(4,5)\text{P}_2$ in ordered versus disordered membrane domains can be distinguished. The use of rapidly recruitable, rapamycin-dependent association of FK506 binding protein–inositol 5-phosphatase [21] with FKBP12–rapamycin-binding domains attached to order- versus disorder-preferring protein motifs [61] should permit rapid modulation of $\text{PI}(4,5)\text{P}_2$ pools in each of these domains, providing additional insight to spatial distribution. A prediction of our model (Figure 3) is that Orai1 clusters with $\text{PI}(4,5)\text{P}_2$ in disordered membrane domains in the absence of stimulation, and with $\text{PI}(4,5)\text{P}_2$ 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 $\text{PI}(4,5)\text{P}_2$ distributions [51].

The predicted role of $\text{PI}(4,5)\text{P}_2$ nanodomains in exocytosis discussed in ‘Structural evidence for $\text{PI}(4,5)\text{P}_2$ nanodomains relevant to Ca^{2+} signalling and exocytosis’ was recently evaluated by Honigsmann et al. [62], who provided evidence that the Ca^{2+} binding C2A/2B fragment of secretory vesicle-associated synaptotagmin-1 binds to $\text{PI}(4,5)\text{P}_2$ in syntaxin-1– $\text{PI}(4,5)\text{P}_2$ 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 (myristoylated alanine-rich C-kinase substrate) protein has been previously demonstrated [63], and electrostatic binding of this peptide to $\text{PI}(4,5)\text{P}_2$ at the plasma membrane has been implicated in this regulation. This 25-residue effector domain sequence contains three serine residues that, upon phosphorylation by protein kinase C, have been shown to result in dissociation from $\text{PI}(4,5)\text{P}_2$ -containing membranes [19,64]. Using super-resolution imaging methods, it should be possible to test whether this MARCKS effector domain peptide exhibits localized binding to $\text{PI}(4,5)\text{P}_2$ -syntaxin clusters, and whether dissociation occurs under conditions of secretory vesicle exocytosis. With the recent advances in super-resolution imaging highlighted in the present chapter, the stage is now set for a plethora of new insights into cell membrane biology questions, including the many that involve phosphoinositides in cell signalling.

Summary

- TIRF and super-resolution imaging reveal that cross-linking of IgE-Fc ϵ R1 complexes by multivalent antigen results in the time-dependent formation of nanoscale clusters that rapidly lose lateral mobility over several minutes and more slowly form larger clusters that continue to activate Ca^{2+} mobilization leading to granule exocytosis.
- Super-resolution imaging reveals nanoscale clusters of phosphoinositides, primarily $\text{PI}(4,5)\text{P}_2$, at the plasma membrane that appear to participate in exocytosis and other downstream signalling 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 $\text{PI}(4,5)\text{P}_2$ nanodomains are incompletely understood.

The present chapter reflects the contributions of many members of our research group and our collaborators over the years; their names are represented in cited publications. Our work was supported by the National Institutes of Health [NIAID (National Institute of Allergy and Infectious Diseases)] [grant numbers R01 AI018306 and R01 AI022499]. Figure 1 was contributed by Marcus M. Wilkes.

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