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REVIEW

Beyond pore formation: reorganization of the plasma membrane induced by pore-forming proteins

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Abstract

Pore-forming proteins (PFPs) are a heterogeneous group of proteins that are expressed and secreted by a wide range of organisms. PFPs are produced as soluble monomers that bind to a receptor molecule in the host cell membrane. They then assemble into oligomers that are incorporated into the lipid membrane to form transmembrane pores. Such pore formation alters the permeability of the plasma membrane and is one of the most common mechanisms used by PFPs to destroy target cells. Interestingly, PFPs can also indirectly manipulate diverse cellular functions. In recent years, increasing evidence indicates that the interaction of PFPs with lipid membranes is not only limited to pore-induced membrane permeabilization but is also strongly associated with extensive plasma membrane reorganization. This includes lateral rearrangement and deformation of the lipid membrane, which can lead to the disruption of target cell function and finally death. Conversely, these modifications also constitute an essential component of the membrane repair system that protects cells from the lethal consequences of pore formation. Here, we provide an overview of the current knowledge on the changes in lipid membrane organization caused by PFPs from different organisms.

Keywords Lipid distribution · Membrane organization · Plasma membrane · Pore-forming proteins

Introduction

The plasma membrane of mammalian cells reveals heterogeneous distribution of lipids, which manifests in the existence of nano-domains, so-called lipid rafts [1]. Lipid rafts are defined as nanoscale, fluctuating, lateral assemblies of proteins and lipids in membranes that are generated through lipid–lipid interactions; these lead to the formation of liquid-ordered (Lo) lipid domains that are segregated from the bulk liquid-disordered (Ld) environment [2].

Due to both the presence of proteins and their heterogeneous distribution, the plasma membrane plays a key role in many cellular processes, including the transport of ions and molecules into and out of the cell, cell migration and adhesion, signal transduction, and the catalysis of specific reactions necessary for proper cell function [3–10]. Furthermore, components of the plasma membrane are targets for various pathogens, including viruses, fungi, and bacteria [11–14], as well as toxic molecules produced by a wide range of organisms [15–21]. Of particular note are pore-forming proteins (PFPs). Their binding to lipid bilayers leads to pore formation, which disrupts the integrity of the host cell membrane, resulting in deregulated ion homeostasis, cellular dysfunction, and consequently cell death.

Membrane disruption through pore formation is a common mechanism employed by PFPs (i.e., bacterial toxins) to kill target cells. However, some PFPs use pores as transporters of catalytically active toxin subunits or virulence factors into the cytosol of host cells [22–24]. In addition to pore formation, ample evidence indicates that PFPs induce structural and organizational changes in the lipid membrane, affecting its curvature and lipid arrangement [25–32]. In this review, we provide an overview of lipid membrane reorganization that is associated with the interaction of PFPs with the cell membrane and that directly affects target cell function. Proper membrane structure, composition, and organization ensure proper cell functioning, whereas disrupted membrane integrity and morphology lead to membrane dysfunction and ultimately cell death [33, 34]. Conversely, PFP-induced plasma membrane reorganization plays an essential role

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in the membrane repair systems that protect cells from the lethal consequences of PFP-induced membrane perforation. This suggests that both the loss of lipid membrane integrity and membrane reorganization should be considered when investigating the cytotoxic activity of PFPs and developing new therapeutic agents against the harmful effects of PFPs on target cell membranes.

The plasma membrane as a target of PFPs

PFPs are a large group of proteins produced by a variety of organisms from all kingdoms of life, including prokaryotes and eukaryotes. PFPs produced by many pathogenic bacteria serve as important virulence factors for bacterial pathogenesis and constitute the best characterized and largest class of PFPs, namely pore-forming toxins (PFTs) [35]. Conversely, PFPs expressed by invertebrates (e.g., sea anemones and earthworms) are employed as components of the innate immune system to protect their cells against microbial attack and help fight off pathogens [36–38]. PFPs also refer to proteins produced and secreted by the immune cells of vertebrates, including members of the membrane attack complex/perforin (MACPF) superfamily (perforin and the complement membrane attack complex (MAC)), gasdermins, and βγ-crystallin and trefoil factor complex (βγ-CAT), which form pores structurally similar to those produced by bacterial α-PFPs or β-PFPs [39–43]. Mammalian PFPs are described as components of the immune system that play important roles in the defense against pathogens and elimination of infected or cancerous host cells [44–46]. The MACPF superfamily is the largest mammalian family of PFPs and is involved in the defense against bacterial and viral infections as well as in tumor surveillance [46–48]. The common feature of all the proteins belonging to the MACPF family is the presence of the MACPF domain. The structure of the MACPF domain strikingly discloses folds related to the pore-forming domain of the bacterial cholesterol-dependent cytolysin (CDC) protein family, suggesting that MACPFs and CDCs share a similar mechanism of pore formation [49, 50].

Despite the fact that PFPs are produced by a wide spectrum of organisms, many PFPs share similar pathways of pore formation, which lead to membrane disruption and cell lysis [51, 52]. Nonetheless, structural analyses of PFP-induced pores revealed differences in the secondary structures of the pore regions that penetrate the host cell's plasma membrane [31]. Therefore, PFPs have been classified into two groups, α-PFPs and β-PFPs, depending on the secondary structure of the membrane-inserted region. While α-PFPs form pores by incorporating α-helices into the lipid membrane, β-PFPs form transmembrane β-barrel pores by inserting β-strands into the lipid membrane [37, 53]. In turn, according to the mechanism of pore formation PFPs can be classified as a barrel-stave or toroidal protein–lipid pore. Although both models are functionally similar, the structure and membrane interactions are fundamentally different [54, 55].

A general model of PFP-induced pore formation includes the following three steps: (1) binding of the water-soluble PFP monomer to the plasma membrane, (2) assembly and oligomerization, and (3) incorporation of the PFP oligomer into the lipid bilayer (Fig. 1A). The first step of membrane binding is common to both α-PFPs and β-PFPs and relies on the recruitment of soluble PFP monomers to the host membrane through the interaction of the PFP lipid-binding domain with components of the plasma membrane that act as specific receptors. To date, several molecules in the plasma membrane have been identified as cellular receptors for PFPs. Among them, lipid molecules or lipid derivatives constitute a group of essential plasma membrane compounds recognized by a variety of PFPs. However, some PFPs use glycosylphosphatidyl-inositol-anchored proteins (i.e., N-linked glycans or glycan cores) and gangliosides as receptors [41, 56–60].

In addition to specific receptor-mediated interactions, PFPs can also bind regions of the plasma membrane characterized by specific physicochemical properties. Specifically, negatively charged phospholipids (e.g., phosphatidylserine, cardiolipin, and phosphatidic acid), lipid organization, and membrane fluidity may all stimulate PFP binding [61–64]. Furthermore, these properties of the plasma membrane influence the subsequent stages of pore formation [65].

The initial interaction with the plasma membrane forces PFPs to assemble within specific regions of the plasma membrane and triggers the oligomerization process. Depending on the class of PFPs, oligomerization takes place either on the membrane surface (β-PFPs) or even within the lipid bilayer (some α-PFPs). However, recent evidence indicates that both PFP classes also contain members that exploit non-classical mechanisms of pore formation [65–69]. PFP oligomerization on the membrane surface leads to the formation of non-lytic oligomeric intermediates, called "prepores". For β-PFPs, these fully assembled oligomeric complexes undergo large-scale structural rearrangements to transform the prepore into a functional pore, with a characteristic β-barrel spanning the membrane [70, 71]. Conversely, α-PFP oligomerization usually occurs simultaneously with the insertion of α-helical segments into the lipid membrane, leading to the formation of partially or completely assembled active pores [72, 73]. The oligomerization and membrane penetration of both PFP classes largely depend on the properties of the membrane (i.e., fluidity, thickness, curvature, and the strength of the interfacial hydrogen bonding network); as such, lipid composition affects not only PFP binding to the membrane but also the

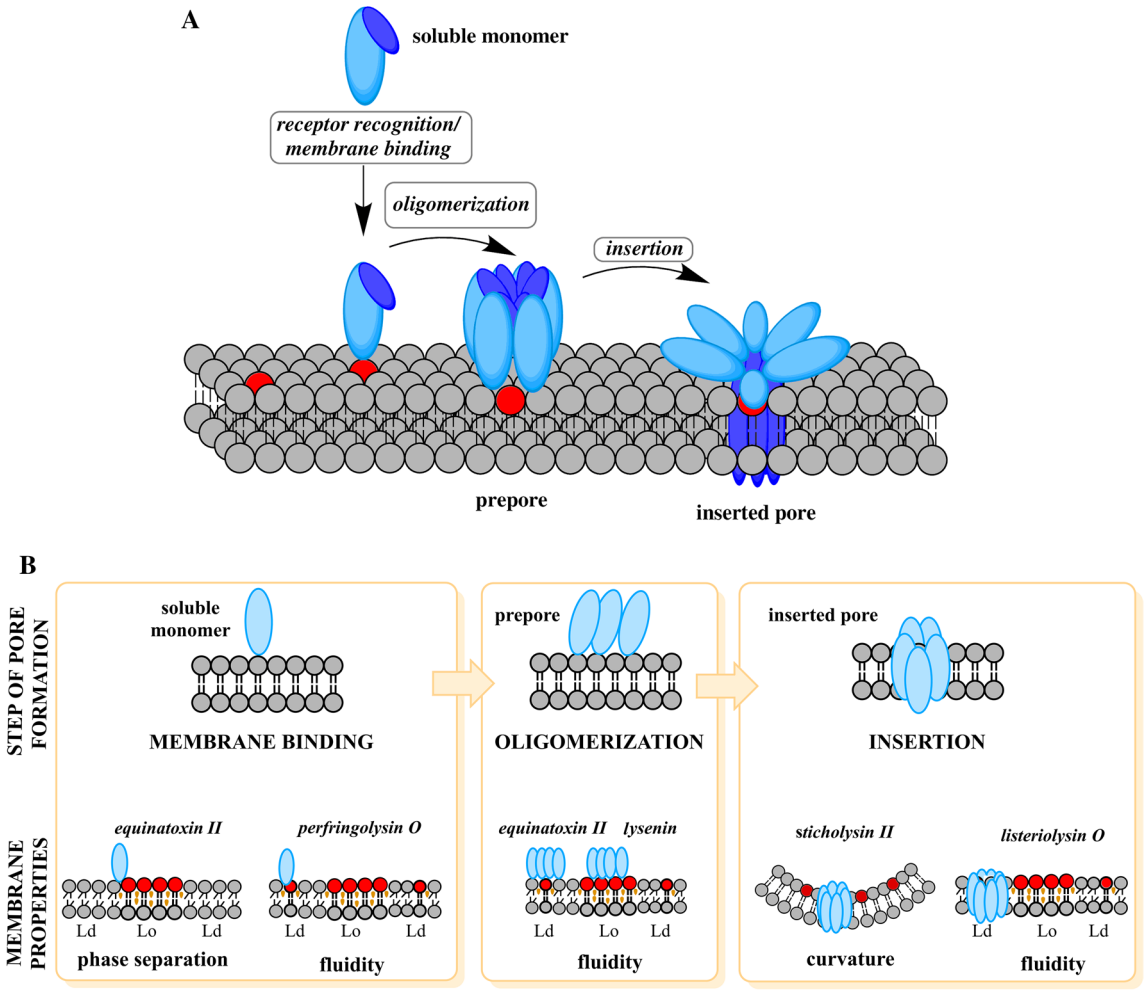

Fig. 1 The effects of membrane properties on pore formation by pore-forming proteins (PFPs). **A** General mechanism of pore formation by PFPs. The water-soluble PFP monomer (*blue*) recognizes a receptor molecule (*red circle*) and binds to the cell membrane. At this stage, PFP molecules undergo substantial conformational changes, allowing the insertion of the pore-forming domains (*dark blue*) of the fully assembled oligomer into the lipid membrane and the formation of a transmembrane pore. **B** Schematic diagram showing the impact of membrane properties on different steps of the pore formation by selected PFPs. The monomer of PFP (*blue*) recognizes receptor molecule (*red circle*) and binds to the cell membrane. Binding of PFPs to the plasma membrane mainly depends on phase separation (i.e., the Lo vs Ld phase) and membrane fluidity. Upon binding to the membrane, PFP monomers in many cases oligomerize to form a non-lytic prepore. This process is affected by membrane fluidity. The transition from the prepore to the transmembrane channel is dependent on membrane fluidity and is enhanced by membrane curvature

further steps of pore formation [29, 65, 74–80] (Fig. 1B). It has been clearly demonstrated that the lipid composition (e.g., the presence of sphingomyelin or cholesterol) in the plasma membrane promotes the assembly or stabilizes the oligomeric structures of α-PFPs, enhancing pore formation [75, 76, 81, 82]. This effect has also been observed for β-PFPs, demonstrating that bilayer fluidity affects the shape and properties of transmembrane pores (Fig. 1B) [20, 83, 84].

As a result, pore formation causes perturbations in the order and dynamics of the surrounding lipids, the loss of membrane integrity, ion imbalance, and increases in the non-selective passage of molecules. The release of cellular content is accompanied by uncontrolled water influx into the cell that causes high cytoplasmic osmotic pressure, cell swelling, and membrane rupture, leading to cell lysis [40, 57, 85, 86]. While ion imbalance (e.g., calcium influx) caused by pore formation may lead to cell death, it can also promote processes that contribute to plasma membrane remodeling, including blebbing, and shedding [87]. In many cases, these mechanisms trigger the cellular repair system in response to pore-induced plasma membrane damage and are required for cell survival during PFP-mediated pathogen invasion. On the other hand, potassium release through the pores activates signaling events, including the mitogen-activated protein kinase (MAPK) pathway that promote cell

survival and recovery of the plasma membrane integrity [85, 88, 89].

Plasma membrane rearrangements induced by PFPs

The interaction of PFPs with lipid membranes disrupts the integrity of plasma membranes, resulting in membrane rupture and cell lysis. However, growing evidence shows that PFP activity is not limited only to direct membrane permeabilization due to transmembrane pores but also to biophysical alterations and modifications of membrane properties during the early stages of pore formation. It has been demonstrated that already the binding of PFP monomers to the membrane alters the physical properties of the membrane, e.g., generating membrane tension by membrane bending [29, 32, 90–92]. This promotes further structural reorganization of membrane-bound protein, leading to pore formation. Thus, preliminary PFP-induced membrane modulation initiates further changes within the plasma membrane that facilitate the insertion of PFP into the lipid bilayer and membrane permeabilization.

The MAC is a perfect example of PFPs for which the subsequent stages of pore formation depend on the initial protein-induced alterations to the membrane. The MAC is a multiprotein complex composed of five complement proteins (C5b, C6, C7, C8, and C9) that contain the MACPF domain (except C5b), assemble and form pores in the plasma membrane of pathogens or targeted cells, leading to osmolysis. Formation of MAC pores is initiated by assembly of C5b and C6 fragments on target membranes. The C5b6 complex binds C7 to form the lipophilic precursor C5b7 that anchors to the membrane and binds C8, resulting in the formation of the precursor assembly C5b8 that partially penetrates the lipid bilayer. Finally, multiple copies of C9 protein associate with the C5b8 complex and polymerize to form the complete MAC pore [93]. In contrast to other MACPF domain-containing proteins (e.g., perforin), which form a closed ring, the MAC pore is an irregular β-barrel with a 'split-washer' configuration [43, 93–95]. Structural studies based on cryo-EM analyses revealed that the MAC may perturb biophysical properties of the membrane during the stage of precursor assembly, reorganizing the bilayer and decreasing the activation energy required to bend the bilayer. However, a detailed analysis of giant unilamellar vesicle (GUV) fluctuations revealed changes in the intrinsic properties of lipid membranes even before precursor assembly. Already at the stage of ionic association of the soluble C5b6 complex with the lipid bilayer, the amount of energy required to change the mean curvature of a lipid bilayer (i.e., the bending modulus) is reduced, whereas the tension is not affected. This effect was maintained even upon partial insertion of C5b7 β-hairpins into the outer leaflet of the GUV bilayer. An increase in the bending module and membrane stiffness was observed during the formation of the membrane-inserted C5b8 complex. Thus, it was proposed that membrane rigidification observed after C5b8 β-hairpin insertion across the bilayer is the consequence of the mechanical strain of bilayer distortion caused by partially inserted β-hairpins of the assembly precursor [94]. In addition, cryo-EM analysis revealed flexibility of the MAC pore structure, suggesting that rotation of pore β-hairpins within the bilayer impacts local curvature of the membrane and provides an additional level of membrane destabilization that causes MAC-induced lipid bilayer rupture and contributes to the lytic activity of β-PFPs [43].

Biophysical perturbation of the plasma membrane induced by MAC oligomerization clearly demonstrates that PFPs enable the modification of membrane properties at early stages of pore formation. However, for many members of PFPs, the interaction with the plasma membrane involves extensive reorganization of the plasma membrane. Depending on the type of PFP-induced re-modeling of the plasma membrane of mammalian cells, two types of changes can be distinguished: (1) lateral lipid rearrangement, including phase mixing and domain coalescence, and (2) membrane deformation (Fig. 2). It is worth noting that in many PFPs, lateral lipid rearrangement constitutes an initial and necessary step for plasma membrane deformation.

Pore formation

A common feature of PFPs is the formation of nanoscale pores in the plasma membrane from which lipids are excluded (i.e., barrel-stave pores) or polar phospholipid head groups together with PFP monomers are involved in the building of pore walls (toroidal protein–lipid pores) [31, 96–98]. Besides different strategies of pore formation, the transmembrane pores of PFPs differ in their architectural features, oligomer stoichiometries, sizes, and degrees of PFP protomer penetration into the lipid bilayer. Such variations are observed both between and within different PFP classes (i.e., α-PFPs and β-PFPs). Structural studies show that the diameters of PFP-induced pores range from 0.5 nm to 30 nm [52] and are correlated with the oligomer stoichiometry, i.e., the number of protomers forming individual pores. For example, perfringolysin O (PFO) from *Clostridium perfringens* and α-hemolysin from *Staphylococcus aureus* are β-PFPs, which form membrane-spanning β-barrels composed of different numbers of protomers, resulting in different pore sizes (25–30 nm and 1.5–3 nm, respectively) [99, 100]. Interestingly, the same toxin may produce pores of various sizes; this is relatively common among CDCs, such as PFO or listeriolysin O (LLO), which are produced by the pathogenic bacterium *Listeria monocytogenes* during

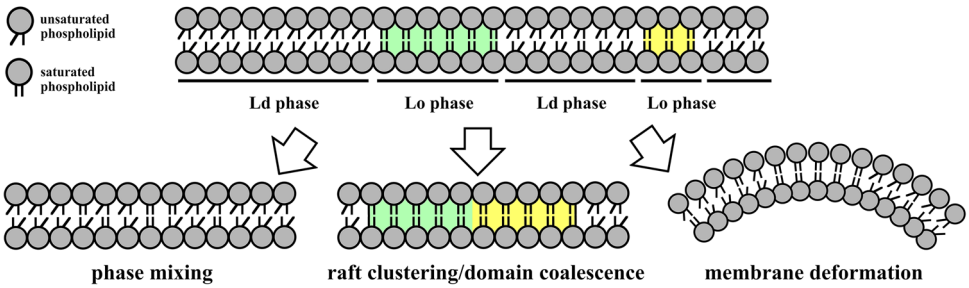

Fig. 2 The different possible plasma membrane rearrangements induced by PFPs. Most cell membranes contain a mixture of phospholipids with saturated (*black, straight*) and unsaturated (*black, bent*) lipid acyl chains. PFP interactions with the receptors in the plasma membrane induce changes in membrane organization and structure, such as phase mixing, domain (*green and yellow shading*) coalescence/aggregation, and membrane deformation

its intracellular life cycle [78, 101–103]. The formation of mature LLO pores enables bacteria to escape from phagolysosomes into the cytosol of host cells [104]. LLO displays a four-domain structure that is common to CDCs [105, 106]. Pore formation begins with the interaction of the receptor-binding domain (D4) with the cholesterol-containing membrane. After binding to cholesterol, LLO self-assembles into oligomeric, ring-shaped prepores, which then undergo drastic conformational changes within the four domains, leading to the insertion of the transmembrane β-sheet into the lipid bilayer and the formation of large β-barrel pores [105, 106].

Recent work suggests that the length of transmembrane segments represent critical factor that affects the interactions between transmembrane regions of adjacent monomers and size of pores formed by β-barrel toxins [78]. Ring-shaped pores are the most commonly observed PFP pores capable of perforating lipid membranes. However, some PFPs perforate the membrane in the form of arc- and slit-shaped oligomeric assemblies, suggesting that these oligomeric intermediates can form functional pores [31, 105, 107–109]. Differences in pore size and architecture also result from other factors, including membrane lipid composition, temperature, pH, and toxin concentration and incubation time [84, 110, 111]. This suggests that pore formation is a dynamic process that can be modified by various factors that alter the biophysical properties and organization of lipid membranes.

The assembly of PFP subunits into well-defined, barrel-stave structures, in which a continuous interface between the core of the bilayer and the channel lumen is provided by the protein, is not the only possibility for pore formation. Some PFPs involve both protein molecules and polar phospholipid head groups to form toroidal protein-lipid pores [112]. This type of pore is widespread among α-PFTs including actinoporins, colicins, Bax apoptotic regulators, and β-PFTs, such as MACPF/CDC [31, 113–115]. In the toroidal model of pore formation, the membrane-inserted PFP domain induces local defects in the lipid bilayer by bending into a torus-like structure. As a result, toroidal pores are characterized by a positive lipid curvature out of the membrane plane and a negative curvature in the membrane plane around the pore [116]. In addition, the lipid reorientation that accompanies the formation of toroidal pores facilitates the flip-flop movement of lipids between the two leaflets [117]. This phenomenon was observed for the α-PFP sticholysin II, which induced the relocation of the negatively curved lipid phosphatidylethanolamine from the membrane into the pore ring [29, 77], and for the β-PFP perforin [118].

Perforin is one of the best characterized MACPF members. This multi-domain protein is produced and secreted by cytotoxic lymphocytes, aiding the intracellular delivery of granzymes to target cells and promoting apoptotic death [50]. Previous structural studies have shown that upon binding to lipid membranes, perforin causes a series of structural rearrangements that lead to the oligomerization and formation of heterogeneous pores that allow the diffusion of granzyme molecules into the cytosol of the target cell [119]. Studies on mammalian cells showed that the interaction of perforin with the plasma membrane induced a flip-flop movement of phosphatidylserine from the inner to the outer leaflet of the membrane, allowing the translocation of granzyme through the membrane [118, 120]. Structural analysis of oligomeric perforin structures revealed that the observed movement of anionic phospholipids to the external leaflet is due to the formation of toroidal proteo-lipidic structures composed of arc-shaped perforin oligomers and plasma membrane lipids [118, 121]. This suggests that the perforin-mediated flip-flop of lipids is the viable pathway for granzyme translocation and that perforin-lipid oligomers act as a gateway for granzyme into target cells.

Lateral lipid rearrangement

The interaction of PFPs with plasma membranes affects lipid membrane rearrangement. Cells normally use lateral lipid rearrangement and lateral assembly of lipid/protein complexes within the plasma membrane to function properly and

maintain cellular homeostasis [122]. Different membrane components are compartmentalized into domains, e.g., SM/cholesterol-rich domains in the plasma membrane (i.e., lipid rafts, Lo phase) or cardiolipin-enriched domains in the mitochondrial membrane [123, 124]. These domains differ from the surrounding lipid environment (the Ld phase) in their local composition, lateral organization, and dynamics. Consequently, the plasma membrane exhibits lateral lipid heterogeneity, which is important for numerous cellular processes, such as signal transduction and membrane trafficking [6–8].

A growing body of evidence shows that some PFPs initially bind at the Lo/Ld-phase boundary but eventually accumulate in either the Lo or Ld phase [82, 125, 126]. This points to the fact that PFP-induced reorganization of the lipid bilayer is accompanied by three types of lateral lipid rearrangements: lipid mixing, lipid domain coalescence, and lipid domain fragmentation [27, 28, 101, 127]. Changes in lateral lipid organization are observed during oligomerization of both α-PFPs and β-PFPs, and in many cases, facilitate their pore formation.

Phase separation supports the accumulation of actinoporins in specific regions of the plasma membrane and promotes their pore-forming activity [27, 125, 128]. However, the coexistence of lipid phases, which leads to different distributions of SM in the plasma membrane, also influences the mechanism of action of other PFP families [56].

Sticholysins (StnI and StnII) are produced by the sea anemone *Stichodactyla helianthus* and are highly hemolytic α-PFPs that belong to the actinoporin family. Sticholysin-induced pore formation is initiated by SM-dependent protein binding to the lipid membrane and is enhanced by lipid-phase separation in the target membrane. Upon membrane binding, sticholysins oligomerize and insert their amphipathic α-helix into the lipid membrane, resulting in pores consisting of 8–9 protomers [129, 130]. Although the initial binding of sticholysin to the membrane occurs at the Lo/Ld-phase boundary, oligomerization and pore formation are observed in the cholesterol-poor Ld phase, instead of the SM-rich Lo domains, and are enhanced by the presence of unsaturated phospholipids [27, 79]. Atomic force microscopy (AFM) studies on SM/dioleoylphosphatidylcholine/cholesterol bilayers showed that the initial binding of sticholysins to the lipid membrane simultaneously smoothed and lowered the height of the SM/cholesterol-rich Lo domains and disrupted the regular shape of the lipid domain boundaries [27]. This phenomenon was attributed to sticholysin-induced destabilization of membrane-phase boundaries and transitions, which promote phase mixing and thus favors the formation of disordered membrane regions that are more suitable for the insertion of sticholysin into the lipid bilayer (Fig. 3A).

Equinatoxin II (EqtII, produced by *Actina equina*) is another member of the sea anemone actinoporin family that also induces drastic lipid reorganization after binding to the plasma membrane [28]. Similarly to StnI and StnII, EqtII preferentially binds to Lo/Ld-phase boundaries, which leads to membrane reorganization and subsequent pore formation in the Ld phase [82, 125, 131]. However, in vivo studies showed that shortly upon binding to the plasma membrane, EqtII promotes the formation and stabilization of microscopic lipid domains [28]. Subsequently, this lipid perturbation leads to lipid-phase separation, enabling the insertion of the N-terminal α-helix of EqtII into the membrane and pore formation [132]. Long incubation times and high concentrations of EqtII promote bleb formation in the plasma membrane. It has been suggested that the formation of pores causes plasma membrane to detach from the actin cytoskeleton, which subsequently elicits membrane blebbing [28]. The mechanism of EqtII action shows that both pore-induced disturbances of plasma membrane integrity and disruptions in lateral lipid organization generate membrane injury, leading to swelling, collapse, and ultimately cell death.

Lysenin from the earthworm *Eisenia foetida* belongs to the aerolysin family of small β-PFPs that bind to SM in the plasma membrane [19, 20, 133, 134]. Upon binding to SM-containing membranes, lysenin assembles into oligomeric structures composed of nine monomers and undergoes a series of structural changes that lead to the formation of 3 nm diameter pores [134–136]. Interestingly, in contrast to EqtII, lysenin preferentially binds to the membrane and forms pores in the presence of SM clusters, indicating that its pore-forming activity depends on the distribution of SM [20, 62]. Early studies showed that a single lysenin molecule binds to several SM molecules in the plasma membrane, thereby inducing SM to cluster within lipid rafts [137]. It was also demonstrated that lysenin oligomerization at the plasma membrane of monocytes leads to the coalescence of lipid rafts, which triggers the clustering of FcγIIA transmembrane receptors in the lipid rafts and receptor activation [127]. Further AFM studies showed that the formation of lysenin pores disrupts interactions between lipid molecules in the SM-rich Lo domains and that this subsequently induces the translocation of excess SM and cholesterol from the Lo domains into the Ld phase and thus phase mixing (Fig. 3B) [30, 138]. These results suggest that the excluded SM and cholesterol molecules form small clusters that facilitate the binding and oligomerization of subsequent lysenin molecules [30].

The reorganization of lipid rafts is not induced by only SM-binding toxins but also by PFPs that recognize the second main raft component, cholesterol. Lipid raft aggregation, which is a result of cholesterol clustering, has also been demonstrated for CDCs such as LLO. In 2005, Gekara et al. demonstrated that the oligomerization of LLO monomers at the plasma membrane of J774 cells induced the clustering of raft-associated molecules (i.e., GM1, CD14, CD16, CD24,

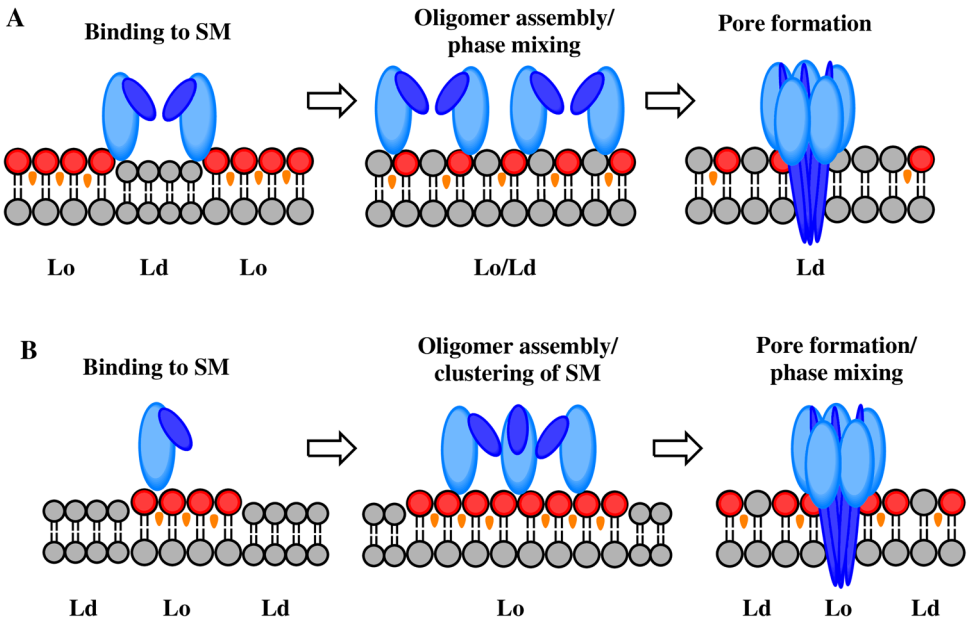

Fig. 3 Membrane reorganization induced by SM-binding PFPs at various steps of pore formation. **A** The binding of StnII (an α-PFP) to SM (*red circle*) at the Lo/Ld-phase boundary initiates protein oligomerization and is accompanied by the redistribution of SM and cholesterol (*orange*) from the Lo phase to the Ld phase, i.e., phase mixing. The formation of disordered regions in the membrane promotes StnII insertion into the lipid bilayer and pore formation. **B** Lysenin (a β-PFP) binds to SM in the Lo phase and assembles into a prepore oligomeric structure. The oligomerization of lysenin induces local clustering of SM in the plasma membrane. The transition of the prepore complex into a membrane-inserted pore induces the translocation of excess SM and cholesterol from the Lo domains to the Ld phase and thus phase mixing. The lipid-binding and membrane-inserted domains of PFPs are colored in light and dark blue, respectively

and Lyn kinase). Consequently, LLO oligomerization triggered tyrosine phosphorylation of Lyn kinase and induced a signaling cascade in target host cells via co-aggregation of raft-associated receptors, kinases, and adaptor proteins [101]. These results suggest that Lyn phosphorylation and raft-associated molecule clustering are induced by LLO oligomerization at the plasma membrane. Detailed analyses of LLO interactions with the lipid bilayers enabled the characterization of the dynamic states of membrane lipids, including cholesterol at different steps of LLO pore formation.

As AFM observations have shown, LLO preferentially binds to the SM/cholesterol-poor non-raft Ld phase of supported lipid bilayers where it oligomerizes and forms pores [139]. This causes an increase in lipid diffusivities within the Ld phase, which leads to the formation and significant growth of gel-like nano-domains in the Ld phase [139, 140]. This large-scale reorganization of the bilayer is probably a result of LLO pore–pore coalescence and depends on the concentration and location of cholesterol in the individual leaflets of the lipid bilayer [139, 141]. All-atom molecular dynamics simulations showed that LLO oligomerization increases the local density of cholesterol near the membrane-binding LLO domain. This consequently leads to marked differences in the mobility of lipids and cholesterol, especially in the extracellular leaflet of the plasma membrane. In addition, lipid mobility is increased in cholesterol-depleted regions [142]. These data confirm that LLO insertion into the membrane increases lipid disorder and stimulates lipid reorientations, forming a truncated toroid that stabilizes the membrane-inserted state. Interestingly, LLO binding to the membrane decreased the mobility of cholesterol and other lipids in the immediate vicinity of the LLO–membrane interaction, especially in the extracellular membrane leaflet. It is hypothesized that cholesterol segregation in the membrane may induce variations in lipid mobility that facilitate the co-aggregation of lipid rafts during LLO oligomerization [101]. Decreased cholesterol mobility in the direct vicinity of pores was also observed for other CDCs, such as pneumolysin (PLY) secreted by *Streptococcus pneumonia* [143], as well as for α-PFPs, such as cytolysin A (ClyA) produced by *Escherichia coli* [75, 144].

However, in contrast to LLO, the interaction of ClyA with the Lo phase fluidizes the Lo domains, causing lipid-phase mixing and eliminating membrane heterogeneities. Membrane perturbations may enhance the local concentration of SM/cholesterol around ClyA pores, leading to cholesterol clustering. Furthermore, it should be emphasized that cholesterol does not only serve as a membrane receptor for ClyA binding, as is the case with CDCs, but also stimulates ClyA pore formation by stabilizing the oligomer structure

within the Lo phase in the plasma membrane. The stabilization of intermediate structures is possible due to the specific interactions between cholesterol and the cholesterol recognition amino acid consensus residues (the CRAC motif) in the N-terminal helix of ClyA [75]. This finding reveals that cholesterol plays a critical role not only for ClyA binding but also for stabilizing the membrane-inserted structures of protomers and oligomeric intermediates during pore formation, thereby inducing plasma membrane rearrangements.

Membrane deformation

During pore formation, PFPs and lipid membrane components are engaged in a dynamic interplay that relies on cooperation and re-modeling. On the one hand, the interaction of water-soluble PFPs with the lipid membrane elicits conformational changes in PFP structure that are required for PFP insertion into the membrane and pore formation. On the other hand, the interaction of water-soluble PFPs with the lipid membrane also modifies the lipid bilayer structure. Membrane re-modeling is a consequence of the rearrangements that accompany protein assembly and pore formation. Among the various PFP-induced membrane perturbations, membrane deformation warrants special attention. This type of membrane modification was characterized by an altered membrane curvature and was first described for PLY.

PLY is a member of the CDC family and shares similar structural domains and pore-forming mechanisms with LLO [145–147]. Upon binding to cholesterol-containing membranes, PLY assembles and forms prepores, which then undergo conformational changes. Consequently, they penetrate the membrane to form mature, large β-barrel pores, which deform the membrane (inset in Fig. 4) [143]. Early structural studies based on nuclear magnetic resonance (NMR) spectroscopy revealed that PLY oligomerization and pore formation redistribute lipid components in the bilayer [146]. This suggested that the effect of PLY on the lipid bilayer is a complex process including the formation of pores, extraction of lipids into free oligomeric complexes, aggregation and fusion of lipid vesicles, and destabilization of membranes that generates small vesicles [146]. Further studies demonstrated that PLY activity is not limited to pore-induced membrane permeabilization but also involves several membrane modifications, such as membrane blebbing, fusion, and aggregation.

Membrane blebbing

Cryo-EM analysis of membrane-bound PLY revealed that PLY protomers in the pore complex interact with the lipid membrane at a greater angle than in the prepore complex. This structural rearrangement during the prepore-to-pore transition causes the collapse of the D2 domain; this brings the D3 domain to the membrane and allows its insertion into the lipid bilayer. Conformational changes within the D2 domain also induce the tilting of the D4 domain, bending and breaking the membrane around the prepore complex [143, 147]. The bending of the membrane allows the pore to open when the β-hairpins of the D3 domain are inserted into the membrane. Another study showed that the insertion of PLY pores into the bilayers of multi-lamellar giant vesicles induces membrane enlargement, wrinkling, and peeling [148]. PLY pore formation in the cholesterol-containing bilayer of multilamellar vesicles enlarges the outer layer and generates large membrane blebs that contain an aqueous cavity between lipid bilayers. The formation of surplus membrane area and membrane deformation (i.e., the development of an inter-layer cavity and membrane wrinkling) may be the result of the accumulation of lipids that remains in the membrane after discrimination from the interior of pores (Fig. 4).

Both membrane enlargement and wrinkling promote the back-to-back pore association that induces further membrane deformation. This leads to the rupture of the outer membrane and layer-by-layer detachment (peeling). Such back-to-back associations were also observed for PLY prepores in microvesicles released from HEK293 cell membranes [149]. This process exposes underlying layers to more PLY monomers and subsequent pore formation, resulting in a repetitive process that triggers the layer-by-layer peeling of multi-lamellar giant vesicles. Such PLY-induced peeling of multi-lamellar membranes reveals the broad functionality of PLY and may be significant for the cytotoxicity of *S. pneumoniae*. High PLY concentrations may overcome plasma membrane repair mechanisms and induce plasma membrane peeling. Thus, other cholesterol-containing cellular compartments (e.g., endosomes, the Golgi, or trafficking vesicles) may become targets of PLY attack. Another way via which underlying membranes are exposed to PLY attack is by diffusion of PLY monomers into the cytosol through pores inserted into the plasma membrane. According to the model proposed by Drücker et al., prolonged PLY incubation times enable the formation of pores in the outer bilayer that serve as entry gates into the inter-membrane lumen for PLY monomers [148]. PLY passively diffuses through the pores and then binds to the underlying membrane layer as well as to the inner leaflet of the outer membrane. Consequently, the formation of new pores in both layers enables back-to-back pore interactions that connect adjacent aggregated PLY bilayers and stabilize membrane wrinkles and tubules. Nevertheless, even in this case, PLY insertion into the inner membrane induces membrane perforation.

The formation of a large membrane bleb with an aqueous cavity between the layers of multi-lamellar vesicles is an initial and common step for both PLY-induced layer-by-layer peeling and back-to-back pore aggregation. Several studies on cellular models have demonstrated membrane

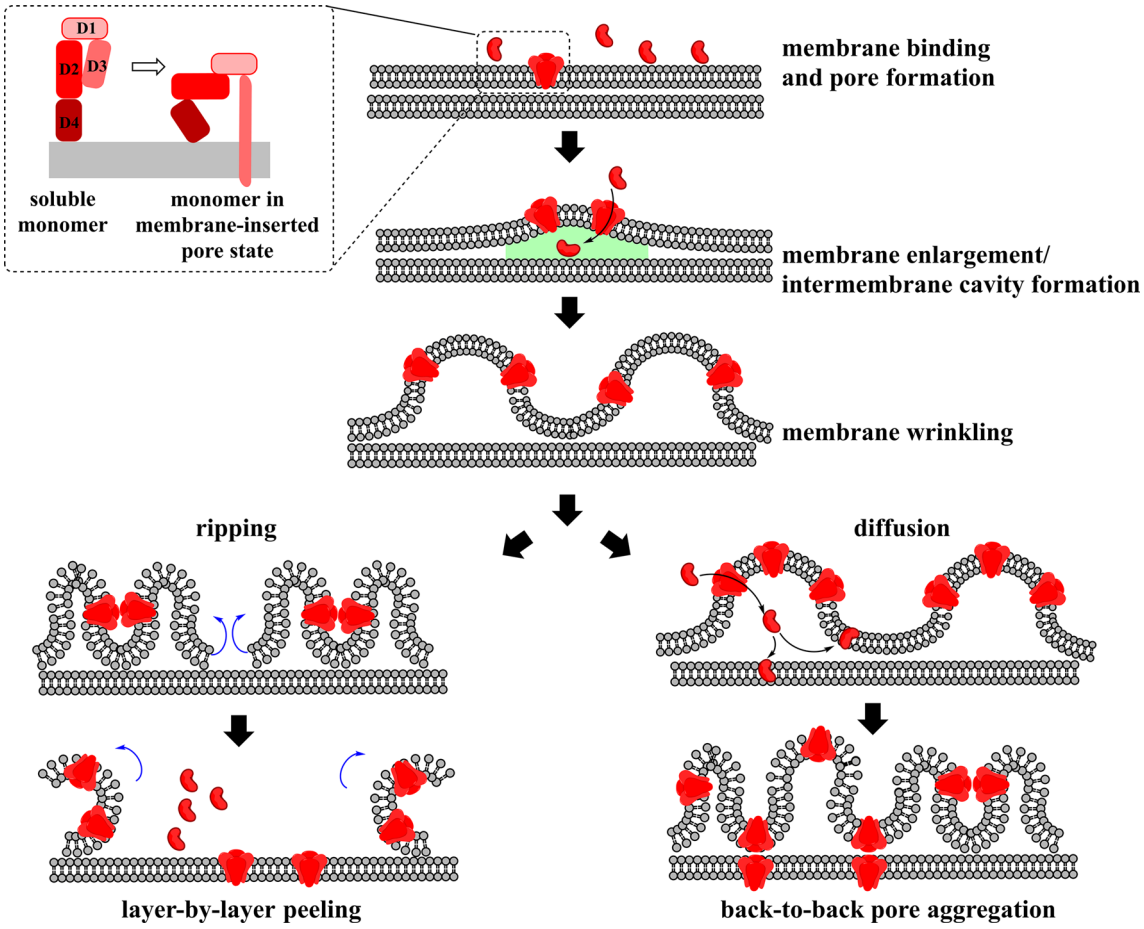

Fig. 4 Pneumolysin (PLY)-induced lipid membrane deformation. After binding to multi-lamellar vesicles, PLY (*red*) oligomerizes and forms pores in the outer bilayer of the vesicles. The pores result in an inter-membrane cavity and promote membrane enlargement and wrinkling. The PLY pores in the outer membrane allow monomers to enter the inter-membrane cavity (*green*) and interact with the underlying membrane. Membrane enlargement and wrinkling stimulate pore–pore interactions that induce strain on the surface and further membrane deformation. As a result, the outer membrane rips open and exposes the underlying layer to PLY binding; this is repeated layer by layer. Alternatively, the pores in the outer membrane enable PLY monomers to traverse the multi-lamellar layers and bind to the next underlying membrane layer, as well as the outer layer from the inner side. The formation of new pores on both layers enables back-to-back pore interactions that connect adjacent bilayers and stabilize the aggregated PLY membranes (adapted from [148]). The inset (*dashed lines*) displays a magnified view of the PLY domains and their conformational changes during pore formation

blebbing induced by other CDCs (e.g., PFO, streptolysin O- SLO, LLO, and intermedilysin), but also β PFP *Vibrio cholerae* cytolysin and α-PFPs (e.g., EqtII and StnII)[28, 85, 150–153]. However, the mechanism and role of this process during pathogen invasion have not yet been thoroughly explored.

Plasma membrane blebbing is regulated by the cytoskeleton and forms transient and dynamic membrane protrusions in a wide variety of cell types and in response to numerous mechanical and chemical stimuli. Blebbing plays an essential role in several physiological processes, including cytokinesis, cell spreading, and locomotion. However, it is mostly related to apoptosis and protective mechanisms against membrane injury [151, 154].

The PFP-induced loss of membrane integrity triggers protective mechanisms in eukaryotic cells [87]. The ability of cells to initiate membrane repair in response to PFP-induced membrane damage strongly depends on the cell type, PFP concentration and incubation time, and pore properties (e.g., stoichiometry and size) [155, 156]. Several studies demonstrated that plasma membrane repair occurs faster for membrane wounds induced by large transmembrane pores (30–50 nm in diameter) compared to small pores (~2 nm in diameter) [155–161] and that the mechanism underlying fast, PFP-mediated membrane repair by blebs depends on Ca^{2+} influx [151, 162]. Large pores are more permeable to calcium and thus trigger rapid calcium-dependent repair mechanisms more efficiently than small pores. This

explains why cells recuperate more slowly after an attack by *S. aureus* alpha-toxin or aerolysin than cells treated with SLO or LLO [163–167]. The formation of large pores in the plasma membrane leads to a rapid and massive increase in cytosolic Ca^{2+} concentrations, thus affecting cellular signaling and viability.

One of the cellular responses to disrupted intracellular calcium homeostasis is remodeling of the actin cytoskeleton. Such alterations to the cytoskeleton are initiated by the disrupted interactions between actin and actin-binding proteins. Disconnecting the cytoskeleton from the plasma membrane detaches the plasma membrane from the cell, decreases membrane tension, and forms blebs [168]. PFP-induced membrane blebbing protects cells from plasma membrane injury by increasing the cell surface area and creating confined spaces. An important role in this process is played by annexins, cytosolic calcium sensors that translocate to PFP-induced membrane lesions according to their different calcium sensitivities and seal damaged membrane regions from the cell body [158, 169]. Furthermore, the surplus of plasma membrane supplies material to shed damaged membrane regions and remove PFP pores (Fig. 5) [87, 151, 153, 170]. It is believed that shedding of PFP-loaded membrane vesicles (ectocytosis) is the primary method by which cells defend themselves against various PFPs, including CDCs and the MAC [150, 152, 156, 166, 171–173]. It was observed that excreted ectosomes surround the cell, suggesting that the ectosome cloud protects the host's cells from subsequent waves of PFP attacks [174].

The final outcome of cellular protection against Ca^{2+} intoxication depends not only on the type of PFP but also on PFP concentration and cell type [166, 175]. Romero et al. indicated that the three CDCs SLO, PFO, and ILY at sublytic concentrations cause the shedding of PFP-containing vesicles from various cells (e.g., fibroblasts, epithelial cells, and immune cells) [150]. Furthermore, using mutants with impaired pore-forming activity, it was shown that oligomerization is required and sufficient for membrane shedding, indicating that Ca^{2+} influx is not required for CDC pore clearance. Nevertheless, enhanced vesicle shedding was observed upon pore formation [150]. In addition, it was found that macrophages were tenfold more resistant to the SLO, PFO, and ILY and released smaller vesicles in comparison to fibroblasts and epithelial cells [150]. Further

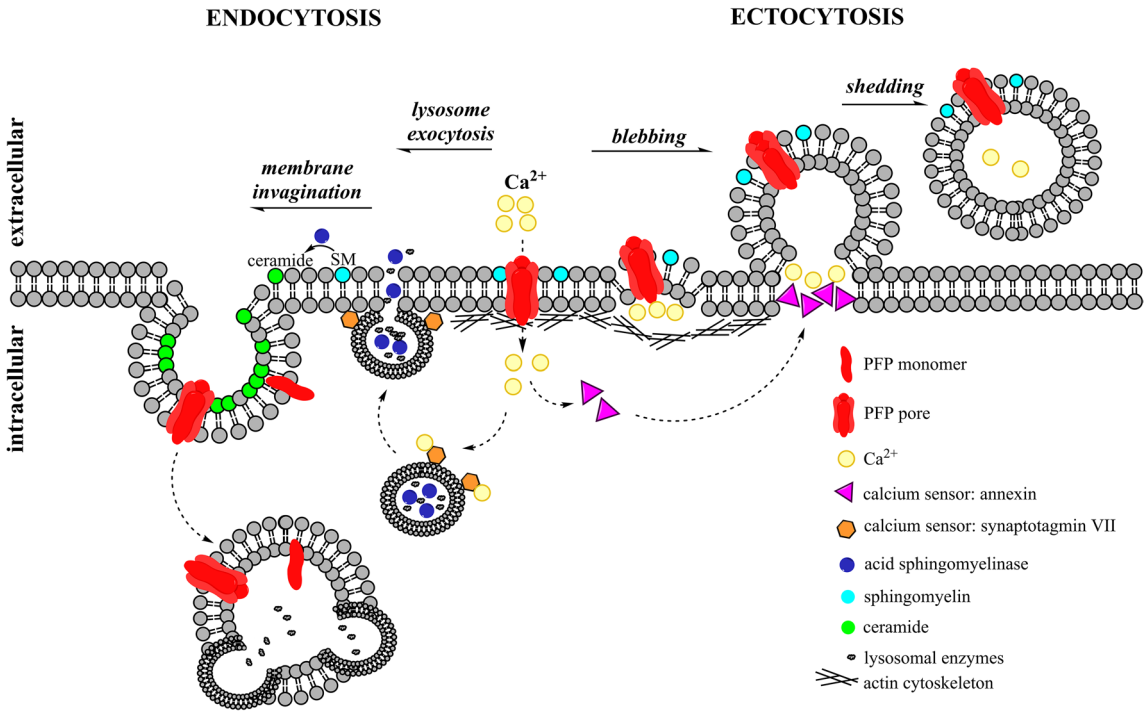

Fig. 5 PFP-induced, calcium-dependent membrane repair pathways accompanied by membrane deformation: endocytosis and ectocytosis. PFP pores allow the influx of Ca^{2+} ions from the extracellular environment into the cytosol. The interactions between the lysosomal calcium sensor synaptotagmin VII and Ca^{2+} ions stimulate the fusion of lysosomes with the plasma membrane and the release of acid sphingomyelinase, which hydrolyses sphingomyelin to ceramide, into the extracellular space. The formation of ceramide-rich domains facilitates membrane invagination and endocytosis of PFP pores and membrane-bound monomers. Finally, PFP pores are possibly degraded upon endosomal/lysosomal fusion. Nevertheless, intracellular increases in Ca^{2+} ions also trigger the detachment of the plasma membrane from the actin cytoskeleton, which promotes plasma membrane blebbing and exocytosis. Furthermore, Ca^{2+} ions recruit the calcium sensor annexin to the neck of blebbed membrane to separate damaged membrane from the cytosol. The PFP pores are released with damaged membrane via micro-vesicle shedding

studies on other CDCs confirmed that Ca^{2+} influx induces pore clearance by membrane vesicle shedding and decreases the lytic activity of PLY and LLO [171]. The rapid and efficient resealing of membrane injuries clearly explains why PLY is non-lytic in the presence of PLY pores in the target membrane, described by Wolfmeier et al. [149, 158]. As expected, reduced calcium concentrations increased the lytic activity of PLY due to reduced vesicle shedding and increased membrane load. On the contrary, reduced calcium concentrations did not increase the lytic capacity of LLO, indicating that LLO activity is less dependent on calcium than PLY. Furthermore, LLO pores were not shed by membrane micro-vesicles. The above data suggest that even though PFPs belong to the same family and share common molecular mechanisms of pore formation, they exhibit different calcium-dependent activities and trigger different mechanisms of pore elimination.

Membrane invagination

An alternative for eliminating PFP pores from the plasma membrane may be endocytosis (Fig. 5) [176–179]. This mechanism was proposed for *S. aureus* α-toxin [156], perforin [180], *V. cholera* cytolysin [181], and CDCs [156, 178]. Interestingly, endocytic removal of membrane pores and shedding of pore-containing membranes can both occur, and their relative contribution towards toxin removal depends on the cell type [153, 156, 172, 178, 179, 182, 183]. However, it is believed that PFP pore clearance via endocytosis of membrane lesions may occur after membrane repair by vesicle shedding to remove inactivated and monomeric toxins from the cell surface [150]. According to this model, PFP clearance and membrane repair are initiated by pore formation, which enables Ca^{2+} influx into the intracellular space. Increased cytosolic calcium concentrations lead to calcium-dependent interactions between the lysosomal calcium sensor synaptotagmin VII and plasma membrane SNARE proteins that stimulate the fusion of lysosomes with the plasma membrane. This results in the secretion of the lipid hydrolytic enzyme acid sphingomyelinase into the extracellular medium, which converts membrane sphingomyelin into ceramide [184]. The formation of ceramide-rich domains induces the outer membrane leaflets to condense and form an inverted non-lamellar phase, a process that causes membrane invagination and endocytosis of PFP pores [156, 182, 185]. Endocytosed PFPs are sorted into multivesicular bodies and, upon fusion with lysosomes, undergo degradation. However, due to the fact that the internalization of active pores has never been directly visualized, it is believed that pore elimination via the endocytic pathway is limited to monomers or oligomers [150, 156, 178, 180, 186]. Nevertheless, as suggested for the *V. cholera* cytolysin, the degradation of endocytosed α-hemolysin-like PFT pores might occur via an alternative pathway, autophagy [187]. In this case, the internalization of active PFP pores may damage endosomes, which are then recognized by the autophagy machinery.

Biophysical studies combined with imaging analysis that focused on the interaction of perforin with different lipid membrane systems showed that perforin promotes membrane invaginations and remodeling [32]. Interestingly, membrane deformations were found in living human cells prior to perforin-induced pore formation, suggesting that perforin-induced membrane unfolding may trigger the formation of endocytic vesicles that take up granzyme into the target cell. This evidence indicates that the interaction of perforin with the target membrane is also involved in other cellular events besides pore formation. These may include facilitating endocytosis, which is an essential process that protects host cells from pathogen attack.

Intracellular membranes as targets for PFPs

In addition to the pore-forming activity aimed at the plasma membrane, PFPs can perforate intracellular membranes and destroy intracellular organelles or indirectly modify their properties [188]. For instance, different PFPs can directly target mitochondria and affect mitochondrial permeability, morphology, and functioning [189, 190]. Bcl-2-associated X protein (BAX) and BCL2-antagonist/killer 1 (BAK) form pores that permeabilize the mitochondrial outer membrane during apoptosis. Under physiological conditions, BAX and BAK exist as inactive, monomeric proteins. Upon the induction of apoptosis, both proteins are accumulated and inserted into the mitochondrial membrane, after which they undergo conformational rearrangements and oligomerization, forming pores that release pro-apoptotic factors [191]. Current studies suggest that active BAX and BAK form toroidal pores. Pores formed by BAX are affected by the physical properties of the membrane and the presence of lipids with intrinsic monolayer curvature [192, 193]. As shown by X-ray diffraction and conductance experiments, the formation of membrane pores with lipid molecules in the lumen is caused by a fragment of helix α5 [54, 194]. Interestingly, pore stability depends not only on BAX/BAK molecules but also on the mechanical properties of the membrane [195]. According to the toroidal pore model, insertion of BAX α-helices into the cytosolic leaflet of the mitochondrial outer membrane generates membrane tension. It is suggested that as a result of protein accumulation or oligomerization, locally enhanced tension reorganizes lipids out of the bilayer structure, which enables pore opening [196]. To avoid exposing the hydrophobic acyl chains to the water environment, lipids reassemble into a torus around the pore. Consequently, the two membrane monolayers form a continuous surface at the

pore edge with negative curvature in the plane of the membrane and positive curvature in the plane perpendicular to the membrane. The bending of the lipids at the pore rim has an energetic cost that is directly proportional to the length of the pore, giving rise to line tension that acts as the driving force for pore closure. As a result, toroidal pores are metastable structures whose lifetime is governed by the balance between membrane tension and line tension [77, 197].

The gasdermin (GSDM) family is a newly discovered class of PFPs essential for the highly inflammatory pathway of pyroptosis [198]. However, due to their specificity to negatively charged lipids (e.g., mitochondrion-specific lipid cardiolipin), they also interact with the mitochondrial membrane [199]. Cardiolipin is present on the inner membrane of the mitochondria, however, upon mitochondrial stress, cardiolipin is exposed on mitochondrial surface and form a binding platform for signaling molecules [200]. Intense research during recent years has provided insight into the structure of GSDMs. Most of GSDMs display a two-domain architecture formed by an N-terminal (GSDM-N) and a C-terminal (GSDM-C) domain, separated by a linker region. The crystal structures of full-length GSDMA3 [64] and GSDMD [201] revealed that the GSDM-N is inhibited by inter-domain interactions with juxtaposed regions of the GSDM-C. For many GSDMs, caspase-mediated proteolytic processing induces the dissociation of the GSDM-N from its auto-inhibitory C-domain [201, 202]. Although GSDMs are structurally unique PFPs, their pores resemble the transmembrane β-barrel channels formed by MACPF/CDCs [66, 91, 116]. Thus, certain features of MACPF/CDC pores can be extrapolated to gasdermin pores. Results obtained for different MACPF/CDCs suggests that oligomer insertion into the lipid membrane results in the flow of lipids from the pore rim back to the bilayer [107, 116]. Lipids return from the semi-toroidal pore edges to the bilayer structure, rather than being extruded into the solution during oligomer insertion [116]. Based on this, GSDMs may follow a similar mechanism of lipid clearance that involves the evolution of intermediate protein-lipid semi-toroidal structures to a fully protein-lined pore.

Besides mitochondrial membrane, PFP interacts with other intracellular membranes and its activity may also indirectly affect the functioning of various organelles. PFP-mediated perturbations of calcium homeostasis due to calcium release from intracellular calcium stores, and, possibly, from direct damage of endoplasmic reticulum (ER) compartments, leads to ER stress [87, 203]. To date, it has been demonstrated that PFPs (e.g., LLO and aerolysin) can trigger ER expansion, fission, and vacuolation [204, 205]. As shown for LLO, changes accompanying ER vacuolation range from mild fission to the disruption of the entire ER network. However, lipid rearrangement within the ER requires further elucidation. In the case of aerolysin, the ER undergoes dramatic fission and vacuolation, whereas the post-ER compartments and Golgi apparatus remain unaffected [205]. In addition, aerolysin-induced efflux of intracellular potassium triggers the activation of caspase-1, which activates the central regulators of membrane biogenesis. This in turn promotes cell survival, possibly by facilitating membrane repair [206].

PFPs can also affect lysosomal compartments. The CDCs LLO, PFO, and PLY permeabilize lysosomal membranes and release lysosomal content, such as cathepsin proteases [207]. One possible mechanism of LLO-induced lysosome permeabilization is via endocytosed CDCs pores that are trafficked through the cell and fuse with lysosomal membranes. Another explanation is that toxin monomers traverse the pores formed at the plasma membrane to reach intracellular targets such as lysosomes. However, pores in the plasma membrane are known to trigger several signaling cascades, of which one may indirectly lead to lysosome permeabilization [208].

In summary, PFP-induced organelle damage is linked to membrane permeabilization, ion imbalance, cellular metabolism, and cell death. Nevertheless, in different model systems, cells have been shown to recover from such stress via different processes that may involve putative calcium sequestration mechanisms, increased membrane synthesis and lipid metabolism, and recycling of damage compartments.

Conclusion

More than a decade of extensive studies focused on the effect of pore formation on lipid membrane properties have provided strong evidence that the interaction between PFPs and lipid bilayers is not limited only to membrane perforation but is indeed much more complex. As such, studies focusing on the mechanism of pore formation and its effects on cellular function should be analyzed in a broader context (Table 1).

Structurally and taxonomically distinct PFPs employ different strategies that affect plasma membrane properties and functions of the target cell. However, PFP interactions with the lipid membrane always lead to conformational changes of the protein that promote its oligomerization and incorporation into the lipid bilayer; these changes strongly depend on the biophysical properties of the membrane (e.g., fluidity, lipid composition, and curvature). Furthermore, PFP-associated structural rearrangements extensively alter the biophysical properties and lipid organization in the membrane, i.e., lateral lipid reorganization (phase mixing and lipid raft aggregation) and membrane deformation. Interestingly, PFP-induced membrane modifications elicit responses with opposite effects with respect to the viability of the target cell. While some PFP-induced membrane modifications increase the accessibility of the rearranged membrane to subsequent PFP molecules, which leads to pore formation

Table 1 Examples of PFP-induced lipid reorganization in the plasma membrane and their biological relevance

Type of membrane lipid rearrangement	Final membrane alteration	Cellular effect	Biological relevance	Step of pore formation	Representative PFP
Phase mixing	Mixing of liquid-ordered and liquid-disordered phases	Clustering of FcγIIA/ induction of FcγIIA signaling pathway	Enhanced immune response/phagocytosis	Oligomer	Lysenin *Eisenia foetida* [127]
Lipid domain coalescence	Lipid raft clustering	Clustering of raft-associated molecules/ induction of CD14/CD24 signaling pathway	Induced pro-inflammatory cytokines and chemokines in macrophages that facilitate *L. monocytogenes* spreading by recruiting more potential host cells	Oligomer	Listeriolysin O *Listeria monocytogenes* [217]
Membrane deformation	Membrane shedding	Macrophage polarization	Enhanced response to Gram-positive bacterial ligands	Pore	Pneumolysin *Streptococcus pneumoniae* [215]
Membrane deformation	Membrane blebbing	Plasma membrane dysfunction	Inhibited endocytosis	Pore	Equinatoxin II *Actinia equina* [28]
Membrane deformation	Membrane invagination	Antigen presentation	Dissemination of infectious agent	Monomer, oligomer	α-hemolysin *Staphylococcus aureus* [156]

and cell death, other modifications are involved in cellular protective mechanisms aimed at removing pore-containing damaged membranes, thus reducing the lethal consequences of pore formation. While pore-induced disturbances in cellular ion balances (e.g., Ca^{2+} influx) activate the membrane repair machinery, they simultaneously stimulate intracellular signaling cascades that induce programmed cell death or cell survival pathways [35, 88, 209–211]. In addition, PFP-induced membrane damage leads to decrease of intracellular K^+ concentration, which activates signaling pathways aimed at promoting host cell survival, including restoring plasma membrane integrity and ion homeostasis. Activation of p38 mitogen-activated protein kinase (MAPK) pathway was found for α-hemolysin, SLO, PLY, *Bacillus anthracis* anthrolysin O, and *Bacillus thuringiensis* Cry5B [88]. In turn, the JNK/MAPK and ERK/MAPK signaling pathways have been shown to be activated by PLY, LLO, and aerolysin [155, 212, 213]. Interestingly, PFP-induced intracellular decrease in potassium level leads to the activation of signaling pathways that trigger inflammation and modulate

the immune response [206, 214]. A recent study demonstrated that PLY-carrying micro-vesicles formed during the membrane repair process display immunomodulatory effects that lead to macrophage polarization and enhanced responses to Gram-positive bacterial ligands [215].

Furthermore, there is growing evidence that PFP-mediated membrane remodeling is involved in vesicular transport (endocytosis and exocytosis) to repair a membrane injury or remove a membrane pores [156, 177, 178, 216]. This indicates that the binding of PFP to the plasma membrane has an indirect effect on processes that are critical for the proper functioning of cells. For example, reorganization of lipid composition was already detected at the step of actinoporin binding and oligomerization [27, 28]. This suggests that membrane modifications, and thus perturbations of cellular processes, can even occur in the absence of mature, membrane-inserted pores. This in turn calls for vigilance in the development of new therapies aimed at inhibiting the final step of toxic pore formation.

Furthermore, the constantly increasing number of newly discovered PFPs involved in the innate immune response indicates that our attention should not only be focused on pore-induced membrane permeabilization. We must focus on more than just the mechanism of pore formation in the plasma membrane and investigate all aspects and consequences of membrane rearrangement at the cellular level, including the uptake of pathogens, activation of inflammatory pathways, and release of inflammatory mediators.

Despite decades of intensive studies, the functional consequences of the interaction of PFPs with lipid membranes remain unclear. Many aspects remain to be elucidated, such as the interplay between the membrane repair system and the number and size of pores, the additional physiological functions of PFPs in immune cells, and the consequences of damaged membranes in other cellular compartments.

Additionally, interpretation of the results for the changes in lipid membrane organization caused by PFPs requires special attention should be made with care. To date, the effects

of PFPs on lipid membrane properties, such as dynamics, order, and structure, were analyzed both on cellular and artificial membranes. It is well known that the plasma membrane is a dynamic structure composed of lipids and proteins that is constantly remodeled and deformed to achieve important cellular functions (e.g., endocytosis/exocytosis, trafficking, motility, cytokinesis, and processes during pathogen infection). Many proteins are involved in actin dynamics and membrane organization. In addition, lipid ratios and lipid accessibilities may differ among different cells and depend on extracellular and intracellular conditions. In comparison to cellular membranes, artificial membranes, such as liposomes, supported lipid bilayers, and lipid monolayers, exhibit different stabilities, lipid motions, curvatures, and compositions. Furthermore, experimental constraints also limit the use of artificial membranes as plasma membrane models. For these reasons, it is worth highlighting that data obtained on artificial membranes do not fully reflect the properties of cellular membranes. To this end, it is believed that the results of PFP-induced membrane alternation studies on model membranes should be verified on natural membranes. Relatively recently, it was demonstrated that a promising approach to obtain membranes with lipid and protein compositions similar to those of cells is the formation of giant unilamellar vesicles (GUVs) by cell blebbing, a result of the detachment of the plasma membrane from the cortical cytoskeleton [218]. This method generates lipid vesicles with the exact composition and asymmetry (in terms of lipids and membrane proteins) as the native plasma membrane [123, 219, 220]. This method was already successfully used to isolate PLY-containing lipid vesicles from HEK293 cells and seems promising to extend the range of cells and PFPs that can be investigated [215, 218].

In conclusion, the characterization of PFP-induced membrane rearrangements has broadened our understanding of PFP activity at the molecular and cellular levels. PFPs are often used as model tools and lipid-binding probes in other fields of biology [221–224]. For this reason, a thorough understanding of the differences in membrane behavior in the presence of PFP is also extremely important for the interpretation of any experimental results. However, further studies are needed to explore other PFP-induced membrane modifications as well as to uncover the importance of PFPs in host–pathogen interactions and host cell function.

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Availability of data and material Not applicable.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

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