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Anoctamins/TMEM16 Proteins: Chloride channels flirting with lipids and extracellular vesicles

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Abstract

Anoctamin/TMEM16 proteins exhibit diverse functions in cells throughout the body and are implicated in several human diseases. Although the founding members ANO1 (TMEM16A) and ANO2 (TMEM16B) are Ca^{2+} -activated Cl^- channels, most ANO paralogs are Ca^{2+} -dependent phospholipid scramblases that serve as channels that facilitate the movement (“scrambling”) of phospholipids between leaflets of the membrane bilayer. Phospholipid scrambling significantly alters the physical properties of the membrane and its landscape and has vast downstream signaling consequences. In particular, phosphatidylserine exposed on the external leaflet of the plasma membrane functions as a ligand for receptors vital for cell-cell communication. A major consequence of Ca^{2+} -dependent scrambling is the release of extracellular vesicles that function as intercellular messengers by delivering signaling proteins and non-coding RNAs to alter target cell function. We discuss the physiological implications of Ca^{2+} -dependent phospholipid scrambling, the extracellular vesicles associated with this activity, and the roles of ANOs in these processes.

Keywords

Phospholipid scramblases; Exosomes; Ion channels; Calcium; Channelopathies; TMEM16F

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DISCLOSURE STATEMENT

The authors have no conflicts of interest.

FUTURE ISSUES

What is the substrate specificity of ANO PLSases for lipids?
Do ANO scramblases have accessory subunits?
What is the function of the ionic “leak” of ANO scramblases?
What is the function of PLSCR1 in ANO-mediated PLS?
How do Ca^{2+} -dependent and caspase-dependent PLS interact?
What is the precise mechanism of ANOs in EV production?

RELATED RESOURCES

Extracellular vesicle proteomic database.

http://student4.postech.ac.kr/evpedia2_xe/xe/

<http://www.microvesicles.org/>

Molecular dynamics simulation of membrane proteins in lipid bilayers.

<http://sbecb.bioch.ox.ac.uk/mempromtd>

Lipid Maps Structure database

<http://www.lipidmaps.org/data/structure/>

INTRODUCTION

The uncertainty that abounded in the field of Ca^{2+} -activated Cl^- channels (CaCCs) ten years ago was encapsulated in a review entitled: “Ca-activated Cl channels: (Un)known and (Un)loved?” (1, 2). The mood in the field changed dramatically in 2008 when it was shown unambiguously that ANO1 (TMEM16A) and ANO2 (TMEM16B) encode CaCCs (3–5). Since 2008, there have been nearly 400 papers published on ANO1/TMEM16A, and it has become apparent that these two founding members of the family have functions as diverse as the tissues in which they are expressed. Classical functions of ANO1 and ANO2 include roles in membrane excitability in the nervous system and cardiac muscle, olfactory transduction, smooth muscle tone in blood vessels and other tissues, gut myoepithelial cell contractility, and phototransduction. In addition, ANO1 functions have recently expanded to include unexpected roles in cancer, hearing, primary ciliogenesis, insulin secretion, inhibitory neurotransmission, and nociception. Unexpectedly, the field has been upended by the discovery that many, maybe most, ANO paralogs are not Cl^- channels but a re phospholipid scramblases, a type of lipid transporter.

Because the ANO literature has been well-reviewed (6–12), we have chosen to focus on one narrow aspect of the field. We believe that the key to understanding all of the ANOs, both Cl^- channels and lipid transporters, is dependent on understanding the relationships of ANOs with lipids. We will develop the idea that ANO-dependent phospholipid scrambling (PLS) alters the biophysical properties of the plasma membrane (PM) in a way that favors both membrane fusion and fission events that play important roles in cellular physiology.

MOST ANOCTAMINS ARE PHOSPHOLIPID SCRAMBLASES, NOT CHLORIDE CHANNELS

The ANO family is widely expressed in eukaryotes and many ANOs are linked to human disease (Figure 1). ANOs are most highly represented in the Chordates where each species typically has 10 ANO paralogs, although fish may have 12. Non-chordate species generally have fewer ANO genes, with fungi and plants often having only one or two, while *C. elegans* has two and *Drosophila* has five ANO genes (11, 13).

When ANO1 and ANO2 were first discovered to be CaCCs, it was expected that all ANOs would encode Cl^- channels because of their high sequence similarity. Comparing only transmembrane domains, the sequence identity of mouse ANO1 is 82% to ANO2, 51 – 59% to ANO3 – ANO7, 46% to ANO9, and 25% to ANO8 and ANO10. However, when expressed in HEK cells, most of the other ANOs unexpectedly either did not produce Cl^- currents and/or did not traffic to the PM (14, 15). The puzzle was solved in 2010 when the Nagata lab discovered that ANO6 is essential for Ca^{2+} -dependent PLS (Ca^{2+} -PLS) (16). Mutations in ANO6 are linked to a congenital bleeding disorder stemming from perturbed Ca^{2+} -PLS in both humans (Scott Syndrome) (16) and dogs (17). Disruption of *Ano6* in mouse phenocopies Scott Syndrome and perturbs Ca^{2+} -PLS in cells isolated from these animals (16, 18–20). Subsequently, it has been shown that ANOs -3, -4, -5, -6, -7, and -9 are also linked to Ca^{2+} -PLS (21, 22). If at first the proposal that some ANOs are scramblases was greeted with some reservation, the uncertainty was abandoned when fungal ANO homologs were purified and shown to mediate Ca^{2+} -PLS when reconstituted into liposomes (23, 24). These fungal homologs have ~25% sequence identity to human ANOs in their

transmembrane domains, their transmembrane topology closely matches that predicted for the human ANOs, and the residues that coordinate Ca^{2+} are highly conserved. Despite the fact that the founding members of the ANO family are Cl^- channels, to date only ANO1 and ANO2 have been shown unambiguously to be CaCCs.

PLSIS A UBIQUITOUS CELLULAR SIGNALING MECHANISM

Phospholipids are organized asymmetrically in the plasma membrane—Cell membranes are a 5 – 10 nm thick layer of proteins and amphipathic lipids. The lipids are highly polymorphic (thousands of structures), each having a hydrophilic head group attached to one or more hydrophobic hydrocarbon chains through a glycerol or a sphingoid backbone (Figure 2A–D). In our simplest conceptualization, membrane lipids are organized as two molecular monolayers (leaflets) with head groups facing the aqueous medium on either side and hydrocarbon chains forming the hydrophobic core of the membrane (25). This arrangement results in a large energy barrier for head groups to flip through the hydrocarbon layer between leaflets (15 – 50 kcal/mol). In model bilayers, lipid flip-flop occurs on a timescale of hours to days. Typically, the outer leaflet of the PM of eukaryotic cells is enriched in phosphatidylcholine (PtdCho) and sphingomyelin (SM) while the inner leaflet is enriched in phosphatidylserine (PtdSer), phosphatidylinositols (PtdIns), and phosphatidylethanolamine (PtdEtn). This lipid asymmetry comes about because ATP-dependent flippases actively transport certain lipids to the cytoplasmic leaflet while ATP-dependent floppases transport others to the outer leaflet (26–29) (Figure 2E). In addition, certain lipids can be trapped in one leaflet or the other by binding to other lipids or proteins.

Externalization of PtdSer and PtdEtn to the extracellular leaflet is regulated—

In opposition to ATP-dependent transporters that segregate lipids, phospholipid scramblases are thought to be lipid channels that lower the energy barrier for passive movement of lipids between leaflets (Figure 2F). Two major PLS pathways have been identified (30). One is activated by a process that involves caspase activation and XKR8 proteins (31). Most notably, caspase-dependent PLS marks apoptotic cells and cell fragments for phagocytosis (32–34). The other pathway involves elevation of cytosolic Ca^{2+} and the ANO scramblases (16, 21). These two PLS pathways are formally independent of one another because ANO6 disruption abolishes Ca^{2+} -PLS while leaving caspase-PLS intact (16). However, physiologically the two pathways likely interact because cytosolic $[\text{Ca}^{2+}]$ increases during apoptosis and some have demonstrated a Ca^{2+} requirement for caspase-dependent PLS (35–37).

Ca^{2+} -PLS is now recognized as a ubiquitous membrane signaling mechanism (30). There are two major consequences of PLS (Figure 2G). 1) Phospholipids that are normally sequestered in the cytoplasmic leaflet in unstimulated cells are exposed to the external environment. These externalized phospholipids, mainly PtdSer and PtdEtn, are recognized by soluble and cellular receptors that orchestrate various signaling cascades. 2) PLS changes the physical properties of the membrane by altering lipid packing, the lateral pressure between lipids, and membrane electrostatics, and by forming local non-bilayer phases. This has several important sequels that include changes in membrane curvature that favor membrane fusion

or fission events, effects on the function of integral membrane proteins, and the recruitment of new proteins to the membrane.

MECHANISMS OF PHOSPHOLIPID SCRAMBLING

Phospholipid scramblases are a type of channel for lipid head groups—Initial ideas about the mechanism behind phospholipid scrambling suggested that PLS was caused by Ca^{2+} -binding to membrane lipids like PIP_2 and did not invoke a scramblase enzyme, but in 1995 Williamson et al. demonstrated that Ca^{2+} -PLS required a specific protein (38). The first candidate scramblase was PLSCR1, which was able to elicit Ca^{2+} -PLS in reconstituted liposomes, but these experiments are now suspect because <8% lipids were scrambled over several hours, whereas the reconstituted ANOs can scramble a significant portion of the liposome lipids in minutes (23). Because knockouts of PLSCR1 and its paralogs have little effect on PLS, PLSCRs are unlikely to be scramblases, but they may play a role in regulating Ca^{2+} -PLS (30).

The structure of the fungal ANO nhTMEM16 has provided important insights into the mechanisms of PLS, but long before this structure was available, Pomorski and Mennon (29) imagined scramblases as proteins having a hydrophilic groove along their surface to provide a pathway so that lipid hydrophilic head groups could translocate between leaflets while the hydrocarbon tails remained in the hydrophobic phase of the bilayer (Figure 3A,B). The structure of nhTMEM16 has validated this model (24). Scramblases are essentially “ion channels” except that instead of being structured like the funnel of a classical ion channel, they are like a bobsled run with lipids being conducted with their head groups as sleds and their acyl chains as scarves blowing in the wind. The nhTMEM16 protein has 10 membrane helices with a hydrophilic furrow connecting the cytosolic and extracellular sides of the membrane. This furrow, bordered by helices 4 and 6, faces the lipid bilayer and is ideally-suited for lipid transport from one leaflet to the other. Mark Samson’s group has performed molecular dynamics simulations of nhTMEM16 in a PtdCho bilayer (<http://sbc.bioch.ox.ac.uk/memprotmd/beta/protein/pdbid/4WIS>) and has shown that lipid head groups are predicted to populate this furrow, as expected if the furrow was the phospholipid conduit across the membrane (Figure 3C). Homology models of ANO6 have a similar hydrophilic furrow (39) with one side of the furrow lined by the scramblase domain we recently identified in ANO6(40).

The mechanism of PLS activation by Ca^{2+} is likely to be the same for ANO scramblases as it is for ANO Cl^- channels – direct binding of Ca^{2+} to the ANO protein (19, 41–43). All ANOs share highly conserved acidic amino acids in TMDs 6, 7, and 8. These amino acids coordinate Ca^{2+} in the nhTMEM16 structure and to control its PLS activity (24). They also play a role in Ca^{2+} gating of ANO1 (42, 43) and regulate scramblase activity of afTMEM16 (23). Intriguingly, although mutations of the conserved Ca^{2+} binding residues nearly abolish PLS, reconstituted afTMEM16 and nhTMEM16 scramble lipids significantly in the absence of Ca^{2+} . This raises questions whether the purified scramblase may be missing a subunit.

Phospholipid scramblases are generally believed to be non-selective for lipids (30). While different rates of scrambling have been reported for different lipids, these differences generally are small and not always consistent. ANO6 has been shown to scramble PtdSer,

PtdCho, PtdEtn, and SM (16) with slightly different kinetics. Likewise, the reconstituted fungal ANOs exhibit little selectivity among different lipids tested and are capable of scrambling PtdCho, PtdSer, PtdEtn, glucosylceramide, and DOTAP (23, 24). However, quantitative comparisons between different lipids remains technically problematic and is a major issue that needs to be resolved, as is the question whether PLS depends on the length or saturation of the acyl chains.

Relationship of ion transport and phospholipid scrambling—Another important question concerns the relationship between PLS and ion transport in the ANO superfamily. While ANO6 and afTMEM16 scramblases exhibit ionic currents as well as PLS, the significance of these currents remains uncertain. Yang et al. (19) suggested that Ca^{2+} influx through ANO6 might play a role in regulating PLS by another unidentified protein that is the true scramblase, but this seems unlikely in light of the demonstration that the fungal ANOs are bona fide scramblases. It seems more likely that ion transport by ANO scramblases is an (inconsistent) byproduct of PLS (39). nhTMEM16 has no convincing ion channel activity, the ion channel activity of afTMEM16 depends on the lipid composition of the membrane and is non-selective for ions (23), and ANO6 conductance has variable ionic selectivity that activates concurrently with PLS (39, 40). To date, there is no evidence that ion transport by the scramblase has a physiological function. Cl^- transport does not seem to be required for PLS because Cl^- channel blockers or substitution of extracellular Cl^- with large organic anions has no effect on Ca^{2+} -PLS (44). PLS by reconstituted afTMEM16 is independent of the ions present and is unaffected by inhibiting ion transport (23). Whether ion transport by scramblases has a physiological function remains to be determined, however, one can imagine that if scramblases conduct Ca^{2+} , this would serve as a positive-feedback mechanism for PLS.

The idea that ion transport by scramblases is a byproduct of PLS suggests that the pathways of ion and lipid transport are the same or at least structurally overlap. If so, this implies that the Cl^- conduction pathway of ANO1 is structurally homologous to the lipid transport pathway of ANO scramblases (39). Mutagenesis of ANO1 has shown that residues involved in ion conduction and ionic selectivity are located in a region of the protein that is homologous to the hydrophilic furrow in nhTMEM16 (39). That ANO1 and ANO2 seem to form an out-group of the ANO superfamily (Figure 1A) is consistent with the idea that ANO Cl^- channels evolved from ancestral lipid scramblases. The bobsled run structure of the hydrophilic furrow may seem incompatible with ion transport because the permeant Cl^- ion would be exposed on one side to the lipid bilayer. However, we speculate that the membrane adjacent to ANO1 is a non-bilayer structure with phospholipid head groups oriented towards the furrow. We imagine that Cl^- ions are conducted across the membrane between the protein and lipid head groups. An alternative model entertained by Brunner et al. (24) is that the Cl^- channels and scramblases may differ in how they dimerize. In scramblases, the hydrophilic furrow is located on the surface of each monomer opposite to the dimerization surface so that each dimer contains two furrows. Brunner et al. suggest that in ANO1 Cl^- channels the dimer might be rearranged so that the interface is formed by the furrow surfaces to form a single enclosed Cl^- conduction pathway.

RECOGNITION OF ANIONIC PHOSPHOLIPIDS BY RECEPTORS

Externalized anionic phospholipids are recognized by soluble and cell-surface receptors—Multiple receptors for PtdSer have been identified (Figure 4). Cell surface receptors include stabilins, TIM1, TIM4, BAI1, and members of the CD300 family (34, 45). Soluble receptors include MFG-E8 that bridges the exposed PtdSer to $\alpha V\beta 5$ integrins and Protein-S and Gas6 which bridge PtdSer to TAM receptors. The physiological endpoint for PtdSer and PtdEtn externalization may depend to large extent on the receptors available to bind them. For example, different CD300 receptors can either suppress or support phagocytosis (46, 47). Furthermore, there is evidence that different receptors may mediate alternative steps in the phagocytic process (48). Exposure of PtdSer alone is not sufficient to mark a cell for phagocytosis because cells expressing a constitutively active form of ANO6 have a comparable amount of PtdSer externalized but are not engulfed by macrophages (49). However, these cells were capable of being phagocytosed when they were treated with the apoptotic Fas ligand.

Receptors recognize externalized anionic lipids by several different mechanisms. One mechanism is simply electrostatic: receptors having a stretch of cationic amino acids interact with the negative surface charge of membranes enriched in anionic phospholipids. In addition, certain proteins bind specifically (and sometimes stereospecifically) to PtdSer or PtdEtn head groups. PtdSer receptors are better understood than PtdEtn receptors (34, 45). Domains that have been identified to be important in PtdSer binding include Ca^{2+} -dependent C2 domains, Ca^{2+} -independent discoidin C2 domains, poly- γ -carboxylglutamic acid (Gla) domains, and WxND motifs. PtdEtn receptors are much less well characterized, but recently it has been shown that CD300a receptors bind PtdEtn preferentially to PtdSer via WxND motifs (46, 50). PtdEtn binding proteins 1–4 (PEBP1–4) are ubiquitously expressed, but their functions remain poorly understood.

Exposed anionic phospholipids function as a signaling platform—Externalized PtdSer not only serves as a ligand that brings two cells together (e.g.; phagocytosis), but may also serve as a scaffold to organize soluble proteins into signaling complexes. For example, when platelets are exposed to collagen and trace amounts of thrombin during vascular damage, cytosolic Ca^{2+} rises and stimulates ANO6-mediated Ca^{2+} -PLS. The externalized PtdSer serves as a surface for assembly of plasma-borne coagulation factors that stereospecifically bind to PtdSer to form the prothrombinase complex that generates the quantities of active thrombin necessary to catalyze coagulation. This binding surface increases the local concentration of the enzymes, cofactors, and substrates necessary for clotting and physically juxtaposes them. By constraining diffusion of the reactants, scaffolding further optimizes multi-step reactions resulting in a $>10^5$ -fold increase in the rate of thrombin formation (30).

PHOSPHOLIPID SCRAMBLIN REGULATES MEMBRANE CURVATURE

The externalization of PtdSer and PtdEtn also changes lipid packing in the membrane. As shown in Figure 2, membrane phospholipids are highly polymorphic, being composed of head group modules of different sizes and electrostatic charge and acyl chain modules of varying lengths and saturation. Intermolecular interactions between these polymorphic lipids

establishes a lateral pressure in the membrane (51, 52). Acyl chains repel one another because of entropic and steric interactions, while head groups repel one another electrostatically, entropically, and sterically (Figure 2G). The bilayer is held together by a strong attractive force at the interface between the head groups and the acyl chains created by the hydrophobic effect – essentially the large energetic cost of exposing the acyl chains to a hydrophilic environment. Some lipids in isolation (like PtdCho) energetically favor bilayer structures because the lateral pressures in the head group and acyl chain regions are balanced. Other lipids that are charged (like PtdSer) or have small (like PtdEtn) or large (like PtdIns) head groups relative to the acyl chains energetically prefer non-bilayer structures such as micelles or inverse hexagonal phases. When non-bilayer lipids are mixed with bilayer-promoting lipids, they cause strain in the monolayer due to an imbalance in lateral pressures. The membrane will then bend, depending on the sum of strains in the two monolayers (53). Thus, when the distribution of different lipids in the two monolayers changes during PLS, the lateral pressure in the membrane will be disrupted and the curvature of the membrane will very likely change. Proteins also play an important role in determining membrane curvature (53, 54), but do so in concert with membrane lipids.

The necessity of redistributing membrane lipids during formation of membrane vesicles may be appreciated by comparing the geometries of the PM of a 10 μm diameter cell (essentially flat) and a ~50 nm diameter synaptic vesicle (marked curvature). Assuming that the membrane is ~5 nm thick, the area of the outer surface of the vesicle is $4\pi r^2 = 7854 \text{ nm}^2$, while the area of the inner surface is 20% smaller, 6362 nm^2 . Although this difference could be accounted for by fewer lipids in the inner monolayer, this would come at a large energetic cost if all the lipids were essentially cylindrical in shape because the high curvature of the membrane would tend to expose the hydrophobic core of the membrane to the hydrophilic environment. The differences in the radius of curvature between monolayers would impose a significant lateral strain in the membrane. Changes in membrane curvature are essential for processes such as endocytosis or exocytosis (53, 55). This membrane trafficking is brought about partly by redistribution of lipids between bilayers and by insertion of membrane proteins that disrupt the packing of lipids in the bilayer to change the lateral pressure profile in the membrane.

Not only does membrane curvature affect lipids, it also affects the function of integral membrane proteins, because membrane proteins are coupled to the surrounding lipid bilayer by their hydrophobic membrane-spanning domains (51, 56). Conformational changes in proteins will be affected by the energetics of lipid-protein interactions which will be affected by the lateral pressure between lipids. In addition, the change in membrane electrostatics and curvature can affect the association of proteins with the membrane. For example, BAR-domain proteins that stabilize/induce membrane curvature are recruited to the membrane by specific membrane curvatures (54).

While more is known about PtdSer, PtdEtn plays a central role in recruiting proteins to membrane compartments, in vesicular/organellar fusion and fission and in the production of extracellular vesicles (57–62).

CHANGES IN MEMBRANE CURVATURE LEAD TO EXTRACELLULAR VESICLE PRODUCTION

The earliest descriptions of PLS were accompanied by observations that PLS is accompanied by the release of extracellular vesicles (EVs) into the medium (63–65). In fact, >25% of the procoagulant activity associated with activated platelets is actually elicited by platelet-derived EVs released during activation that expose PtdSer and assemble prothrombinase complex (65, 66). Because PLS alters membrane curvature and membrane curvature is an essential step in vesicle formation, a reasonable hypothesis is that PLS is necessary for and precedes EV membrane shedding by promoting budding and lowering the energy required for vesicle scission. EVs have now been shown to be released from most cell types and are found in every fluid in the human body examined thus far. EVs are typically loaded with precious cargo that includes both coding and non-coding RNAs and signaling proteins. There is growing evidence that EVs mediate diverse intercellular signaling functions(58, 60, 67–73).

EV nomenclature, definition, biogenesis: EVs are typically divided into two flavors, exosomes and ectosomes (also microparticles). A key distinction is that exosomes are released by exocytosis of multivesicular bodies whereas ectosomes are formed by outward budding of the PM. However, in practice, EV are often defined by their size, sedimentation properties, and the presence of certain molecular markers. Ectosomes are typically larger (50 nm – 1 μ m diameter) than exosomes (30–100 nm), but because isolation procedures are not standardized, and because there is significant overlap in size distributions and the presence of molecular markers, it is frequently difficult to evaluate the extent of cross-contamination in different EV preparations. Another problem is that while conventional fluorescence activated cell sorting is often used to separate exosomes and ectosomes, this technique cannot reliably separate particles in the 30 – 200 nm range which is near or below the diffraction limit of visible light.

Exosomes are expelled into the extracellular space by the Ca^{2+} -dependent exocytosis of multivesicular bodies (MVBs or multivesicular endosomes) (70). MVBs form from late endosomes by the inward budding of the membrane to form intraluminal vesicles (ILVs). ILVs are filled with various protein and nucleic acid cargo from the cytoplasm that have been positioned near sites of vesicle budding. Cargos may vary depending on cell type and physiological condition. ILV formation can occur by several mechanisms, but the best studied utilizes the ESCRT machinery (endosomal sorting complex required for transport), but ESCRT-independent mechanisms have also been described (reviewed in (68)). Exosome release is regulated by small GTPases and likely involves SNARE fusion machinery. Released exosomes are capable of eliciting a variety of changes target cells, suggesting that they play important intercellular signaling functions(reviewed (67)).

Ectosomes are, on average, larger than exosomes (100–1000 nm) and include apoptotic bodies. They are shed into the extracellular environment by blebbing from the PM itself, and can be released seconds after cell stimulation in contrast to exosomes that are released more slowly. Although ectosomes bud from the PM itself and do not rely on exocytosis for

release, many components of the ESCRT complex seem to be required for ectosome budding.

Topologically, neither ectosomes nor exosomes would be expected to have PtdSer externalized because the cytoplasmic leaflet of the membrane is oriented towards the lumen in both cases. However, many EVs typically have PtdSer and/or PtdEtn exposed on the extracellular surface, suggesting that scramblases are involved in the biogenesis or release of EVs. Platelet EVs have been reported to be diverse with regard to externalization of anionic phospholipids (57). Although many papers indicate that both exosomes and ectosomes (often identified on the basis of size alone) have externalized PtdSer, relatively few studies have carefully examined both exosomes and ectosomes at the same time. Two studies suggest that exosomes have much less externalized PtdSer than ectosomes (65, 74).

ANO6 regulates EV release associated with PLS: Although EV release and PLS occur concurrently, until recently it was unknown whether these two processes are mechanistically linked or simply occur in parallel. The first clue that Ca^{2+} -PLS regulates EV release came when investigators recognized that Scott Syndrome patients also exhibited defective EV release (75–77). Moreover, cells from ANO6 knockout mice exhibit both perturbed Ca^{2+} -PLS and EV release (78, 79). Although EV release from platelets is disrupted in both Scott's Syndrome patients and *Ano6* knockout mice, exocytosis appears unaffected because release of α -granules and dense granules is normal. Although the role ANO6 in MVB exocytosis and exosome release has not been directly investigated, the finding that Scott Syndrome platelets release of α -granules, which are a type of MVB that often contain some exosomes, suggests that ANO6 may be involved in the shedding of ectosomes but not in the exocytosis of MVBs and release of exosomes. Because ANO5 is closely related to ANO6, we suspect that ANO5 also plays a large role in EV formation in cell types, like muscle, that express it.

EVs have two major functions: scaffolding and communication: As discussed above, in blood clotting, platelet-derived EVs serve as catalytic surfaces for the assembly of the prothrombinase complex. Additionally, specialized EVs called matrix vesicles (MVs) are also essential in the mineralization of bone, where PtdSer plays an essential role in the scaffolding of factors required for mineral nucleation. MVs, 50–200 nm in diameter, bud from PM domains on osteoblasts and chondrocytes to mineralize collagen deposits and form bone (80). PtdSer plays an essential role in nucleating crystals of inorganic phosphate and calcium, in the form of hydroxyapatite necessary for mineralization, within the MV lumen. The role of PLS in MV biogenesis and function remain unclear. In MVs, PtdEtn is externalized but PtdSer remains luminal, likely anchored by PtdSer binding proteins required for hydroxyapatite nucleation (reviewed in (81)). Interestingly, *Ano6*^{-/-} mice exhibit major defects in bone mineralization during development, and osteoblasts from these mice exhibit perturbed Ca^{2+} -PLS (20). Because loss of ANO6 in Scott Syndrome causes impaired EV release, this suggests that MV biogenesis may be dependent on ANO6 and that defective MV production is to blame for defective bone mineralization.

In addition to their scaffolding function, platelet-derived EVs are capable of stimulating antigen-specific IgG production in B-cells, inducing angiogenesis, regulating endothelial cells, stimulating neutrophils, and can contribute to the invasiveness of certain types of

cancers (82–86). It has become increasingly clear that EVs perform a fundamental role in intercellular communication by fusing with target cells to deliver microRNAs, lipid, and protein cargo. Recent investigation has demonstrated a role for EV signaling in many cell types including EV regulation of myogenesis in myoblasts, sperm maturation, osteoclast precursor differentiation, and may regulate trophoblast fusion, suggesting the signaling role of EVs is a general cellular phenomenon (62, 69, 87, 88).

ROLE OF PLS IN MEMBRANE FUSION

Although most eukaryotic cells are mononucleated, there are several notable exceptions: skeletal muscle, osteoclasts, syncytiotrophoblasts, and giant multinucleated cells that form by the fusion of mononucleated cells to make a multinucleated syncytium. Cell-cell fusion involves multiple steps, numerous transcription factors, adhesion/migration proteins, and signaling proteins, but cellular fusion process exhibits many common themes in diverse cell types (89, 90). One common theme is the reorganization of the lipid component of the PM. Many aspects of bilayer structure and lipid physical properties are thought to contribute to membrane fusion including lipid packing, changes in membrane fluidity, formation of local non-bilayer phases, and alterations in bilayer curvature.

Myoblast fusion involves PtdSer exposure: Almost 20 years ago, van den Eijnde and coworkers observed exposure of PtdSer on the outer leaflet of the PM in differentiating muscle *in vivo* (91). At first, this was interpreted as indicative of apoptosis, however, subsequent observations suggest a more sophisticated scenario. PtdSer is exposed on the surface of myoblasts around the same time that they begin to fuse to form myotubes. PtdSer exposure is transient, localizes to sites of cell-cell contact, and diminishes as myotubes form (92). The idea that PtdSer exposure is required for fusion is provided by observations that prolonged exposure to Annexin-V or a PtdSer-antibody during myoblast fusion significantly inhibits the myotube formation (92, 93) and that liposomes of PtdSer, but not PtdCho, stimulate myoblast fusion (93). Recently it has been reported that the PtdSer receptors BAI1 and BAI3 play roles in myoblast fusion (94, 95). For example, BAI1 overexpression increases myoblast fusion through the ELMO/Dock180/Rac1 pathway. These investigators suggest that PtdSer exposure occurs in a subset of apoptotic myoblasts that promote fusion, but do not actually fuse themselves, because myoblast fusion is impaired by caspase inhibitors (95). While this model is intriguing, the effect of caspase inhibitors conflicts with earlier studies (92).

SIDEBAR

Annexins are an important class of Ca^{2+} - and PtdSer-binding proteins that have opposing functions. For example, while Annexin-V blocks myoblast fusion, Annexin-I clearly promotes myoblast fusion (145, 146). This illustrates an important aspect of PtdSer exposure, namely, that different PtdSer receptors promote different outcomes. Annexins bind PtdSer in a Ca^{2+} -dependent manner with Ca^{2+} bridging annexin carbonyl and carboxyl groups and PtdSer phosphoryl groups (147). Annexin-V and Annexin-I, however, differ in important ways. Annexin-I forms a symmetrical bivalent heterotetramer with two Annexin phospholipid binding domains connected by an S100

dimer (148). In contrast, Annexin-V does not form a bivalent structure because it has a short N-terminus. In the presence of membranes Annexin-V self-assembles into a trimers that form a 2-dimensional crystal lattice. Consistent with these structural data, Annexin-I aggregates PtdSer vesicles or chromaffin granules, whereas Annexin-V does not (149, 150). This also explains why Annexin-V plays an anti-coagulant role in the blood: the 2-D crystal of Annexin-V essentially masks PtdSer that may be externalized at low levels on cells. Annexin-V also inhibits phagocytosis of apoptotic cells while Annexin-I supports phagocytosis.

ANO5 KO mice suggest that PLS may be important in muscle regeneration: Myoblast fusion is important not only for muscle development during embryogenesis but also for muscle regeneration after damage. Adult muscle tissue is populated with mononucleated muscle progenitor cells (satellite cells) that play a role throughout adulthood to regenerate injured muscle fibers (96). Recently, it has been shown that mutations in ANO5 are linked to a spectrum of myopathies that might be explained by defective membrane repair and muscle regeneration (97–100). These disorders range from severe muscle weakness and atrophy to exercise-induced muscle pain associated with elevated serum creatine kinase indicative of “leaky” muscle cells. Dominant ANO5 mutations cause a different disorder, gnathodiaphyseal dysplasia (GDD1), a skeletal syndrome characterized by bone fragility and thickening (101). ANO5 is likely to be a phospholipid scramblase because it is 65% identical to ANO6 in the transmembrane domains and it contains a scramblase domain nearly identical to the one we described in ANO6 (22, 40). Knockout of ANO5 in mice phenocopies human limb girdle muscular dystrophy type 2L (LGMD2L) (102). *Ano5*-deficient mice exhibit delayed regeneration of muscle after cardiotoxin injury. This is at least partly explained by a defect in the ability of myoblasts to fuse, because, for myoblasts in culture, the fusion index (percentage of nuclei in cells with >1 nucleus) was 35% less for ANO5^{-/-} myoblasts than for wild type. In addition, the number of nuclei per myotube was greatly reduced in *Ano5*^{-/-} cells. (102). Because humans with ANO5 myopathies and *Ano5* knockout mice have no obvious muscle phenotypes at birth, this suggests that ANO5 may not play a significant role in myoblast fusion during embryogenesis but may be more important in satellite cell fusion in the adult. Two other groups have reported that *Ano5* knockout mice have no muscle phenotype (22, 103). This may be related to genetic background or to the way in which the knockouts were constructed. The Xu et al. (104) and Gyobu et al. (22) mice may be true nulls, with knockout constructs inserted in exons 1 or 2, while the Griffin et al. (102) mice may express residual truncated transcript, as stop codons are inserted after exon 8. This may mimic more closely the LGMD2L founder mutation (c. 190dupA) in exon5 that is predicted to cause a frameshift and premature stop.

Membrane repair involves PtdSer: Membrane damage occurs during ordinary activity and cells have evolved complex repair mechanisms to deal with it. Membrane repair by active resealing requires extracellular Ca²⁺ and is thought to involve formation of a “patch” at the site of injury (105–108). After membrane damage, increases in intracellular Ca²⁺ trigger endocytosis of damaged PM that relies on ESCRT function, similar to EV biogenesis (106, 107, 109, 110). These endocytosed vesicles then fuse with lysosome-like organelles that are

transported to the site of damage and fuse with the PM by an exocytosis-like process to patch the lesion (106, 111, 112). Membrane repair is complex and involves a myriad of proteins, but a common feature is the requirement for C2 domain-containing proteins that bind Ca^{2+} and PtdSer to induce a positive curvature strain that destabilizes the membrane to promote fusion (103, 113–116). In non-muscle cells, fusion of repair vesicles is mediated by the C2-domain-containing synaptotagmin-7 and SNARE proteins (109, 117). In muscle, dysferlin, a C2-domain protein serves the same purpose as a Ca^{2+} sensor and/or a fusogenic protein. Dysferlin is a member of the ferlin family that is involved in a range of membrane fusion events. Dysferlin along with other Ca^{2+} - and PtdSer-binding proteins including annexins A1, A2, and A6 promote membrane repair via Ca^{2+} -dependent fusion of vesicles with the sarcolemma (118–121). When membrane damage occurs, dysferlin-containing PM is endocytosed. These endosomes fuse with other endo-lysosomal organelles to form larger vesicles that undergo a process similar to exocytosis to patch the lesion. The damage-induced recruitment of dysferlin concentrates PtdSer at the lesion, forming a PtdSer-rich platform for assembly of other components.

Role of Ano5 in membrane repair: In many muscular dystrophies, the cellular lesions are caused by a breach of sarcolemmal integrity. In the most common muscular dystrophies (e.g.; Duchenne) mutations in the dystrophin-glycoprotein complex connecting the extracellular matrix and the actin cytoskeleton render the PM more susceptible to mechanical damage (122). In contrast, dysferlin mutations associated with dysferlinopathies affect the ability of the muscle to repair normal physiological damage (119, 123). ANO5-linked muscular dystrophies share features with dysferlinopathies at the histological, fine-structural, and clinical levels (97, 124, 125). Mutations in both dysferlin and ANO5 are associated with elevated serum creatine kinase levels, weakness in both proximal and distal musculature, disruptions of the sarcolemma, defects in sarcolemmal repair, and amyloid deposits (126). This similarity of ANO5-myopathies to dysferlinopathies has led to the suggestion that ANO5, like dysferlin, participates in membrane repair processes. This idea is supported by observations that muscles from ANO5-myopathy patients have sarcolemmal lesions at the electron-microscope level (97) and that fibroblasts from MMD3 patients fail to repair wounded membranes (97, 125). Repair of muscle fibers damaged by a laser pulse is defective in ANO5 knockout mice, suggesting that ANO5 plays a role in membrane fusion (102).

Sperm-egg interaction: Sperm-egg fusion is one of the iconic examples of cell-cell fusion, but the role of PtdSer externalization in fertilization is unclear. Mammalian spermatozoa are unable to fertilize the egg immediately after leaving the male reproductive tract; they must first undergo capacitation to become capable of executing the acrosome reaction and to develop the high motility necessary for fertilization. Capacitation involves a number of physiological changes, one of which includes changes in membrane lipids such as loss of cholesterol and exposure of PtdSer (127, 128). The acrosome reaction involves the exocytosis of the acrosome, a large lysosome-like organelle. This exocytotic event may produce significant alterations in the composition of the PM. Fertilization requires the PtdSer binding protein MFG-E8 (129). As with other cell-cell fusion processes, one might expect that MFG-E8 to bridge PtdSer on the sperm to a receptor on the egg. In addition, the

exocytosis of the acrosome releases swarms of EVs formed by the fusion of the PM and the acrosomal membrane (69, 130). Recently it was shown that *Ano5* knockout mice exhibit reduced male fertility and sperm motility, but otherwise the sperm appeared normal. It remains unclear whether *Ano5* functions as a scramblase in sperm, because the *Ano5* knockout sperm underwent capacitation normally including externalization of PtdSer(22).

Osteoclasts and multinucleated giant cells: Fusion of cells of the monocyte/macrophage lineage is required to form osteoclasts that are involved in bone reabsorption and to generate multinucleated giant cells that are found in a variety of inflammatory conditions (131). In these cells it has been observed that PtdSer is locally externalized and that fusion is blocked by Annexin-V or by PtdSer-containing liposomes (132). Although ANO5 and ANO6 mutations affect bone development (20, 101), it remains unknown whether osteoclast function is affected.

Syncytiotrophoblast fusion depends on PtdSer externalization: During implantation of an embryo in the uterus, trophoblast cells surrounding the inner cell mass of the blastocyst fuse to form a multinucleated syncytium called the syncytiotrophoblast. PtdSer externalization is associated with this fusion event (133, 134). The finding that antibodies to PtdSer inhibit fusion supports the essential role for PLS in trophoblast fusion (135). However, it remains unsettled which scramblase pathway is activated and whether PLS is mediated by a scramblase or an ATP-dependent transporter (136). Interestingly, in anti-phospholipid syndrome, a disease linked to increased risk of thrombosis and recurrent pregnancy loss, the serum of patients frequently contains antibodies to phosphatidylserine and Annexin-V (137, 138). Although there is no evidence that the ANOs play a role in syncytiotrophoblast fusion, ANO6 is expressed in human placenta (www.bioGPS.org).

LOOSE ENDS: IST2 and ER-PM junctions

The yeast *S. cerevisiae* has one ANO homolog called IST2. The encoded protein, Ist2p, plays a key role in the formation of specific contact sites between the endoplasmic reticulum (ER) and the PM that are important for communication between these two cellular compartments. Deletion of IST2 in yeast results in separation of the cortical ER and PM (139, 140) and overexpression of IST2 in mammalian cells induces formation of ER-PM contact sites (141). Ist2p brings the ER and PM membranes to within 30 nm of one another because its C-terminus binds to the PM while the transmembrane domains are embedded in the ER membrane (142). The C-terminus binds to the PM because it contains an amphipathic α -helix and a cluster of basic amino acids called the cortical sorting signal (CSS) that associates with PtdIns(4,5)P₂ in the PM (143). IST2 is most closely related to Chordate ANO10 (39% similar) and nhTMEM16 and afTMEM16 (44 % similar). However, the finding that reconstituted Ist2p does not support PLS (23) raises questions concerning exactly how it fits into the ANO story.

Intriguingly, ER-PM junctions are involved in lipid and cholesterol trafficking (144). The main site of phospholipid, ceramide, and cholesterol biosynthesis in the cell is the ER (25). From the site of synthesis, lipids are transported to the plasma membrane by both vesicular and non-vesicular routes. One of the non-vesicular routes involves ER-PM junctions where

lipids are exchanged between membranes by various mechanisms including diffusion of lipids (either free or bound to transfer proteins) across the 10 – 30 nm gap between the ER and PM, exchange of lipids during direct membrane contact, and transient bilayer hemifusion. If Ist2p is not a lipid scramblase, this raises an intriguing possibility that one role of ANOs may be to bring membranes into apposition to facilitate membrane fusion, fission, or lipid transfer. If so, perhaps PLS is a more intricate process than an enzyme that facilitates free lipid diffusion between membrane leaflets, and instead involves the exchange of lipids between membranes that promotes membrane scrambling at these sites of membrane mixing. Considering that PLS can be induced by a variety of chemicals and peptides as well as proteins, PLS caused by ANOs in reconstituted systems might simply be a consequence of a protein-lipid interaction that is more complex in the physiological context. In other words, is PLS the trigger for production of EVs or is PLS the consequence of vesicular/lipid trafficking?

CONCLUSION

The realization that many ANOs function as Ca^{2+} -dependent phospholipid scramblases has reinvigorated both the fields of phospholipid transport and Cl^- channel physiology. Now that the proteins responsible for phospholipid scrambling have been identified, a host of questions have become accessible to investigation. Major issues that will be addressed in the coming years include: 1) Is direct Ca^{2+} -binding essential/sufficient for ANO-PLS or are there other activators or regulatory factors involved? The pertinence of this question is emphasized by the finding that ANO6-associated ionic currents activate very quickly in excised patches but very slowly in whole-cell recordings (19, 40) and that there is significant scrambling activity of the fungal ANOs in the absence of Ca^{2+} (23, 24). Are subunits of an ANO6 complex lost in excised patches and reconstituted systems? 2) What are the roles of ANOs and PLS in the production of EVs? Are these parallel processes or linked mechanisms, and if the latter, is PLS essential for all vesicle release or just for ectosome release? 3) Does membrane trafficking play a role in the actual mechanism of scrambling itself in cells? 4) Do any of the ANOs exhibit specificity among different phospholipid head groups or acyl chains? 5) Are Ca^{2+} - and caspase-PLS truly separate processes? Does caspase-dependent PLS operate in the absence of all ANOs? 6) What is the structure of the ANO1 Cl^- channel pore and is there a role for lipids (39)? The tank is full, let's begin!

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DEFINITIONS

afTMEM16 *Aspergillus fumigatus* TMEM16

ANO anoctamin or TMEM16

Ca^{2+} -PLS Ca^{2+} -dependent phospholipid scrambling

CaCC	Ca ²⁺ -activated Cl ⁻ channel
ER	endoplasmic reticulum
EV	extracellular vesicle (both exosomes and ectosomes)
ILV	intraluminal vesicle
MV	matrix vesicle
nhTMEM16	<i>Nectria haematococcus</i> TMEM16
PLS	phospholipid scrambling
PM	plasma membrane
PtdCho	phosphatidylcholine
PtdEtn	phosphatidylethanolamine
PtdIns	phosphatidylinositol
PtdSer	phosphatidylserine
SM	sphingomyelin
TMDs	transmembrane domains

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SUMMARY POINTS

- Most anoctamin/TMEM16 proteins are scramblases; the Cl channel members probably evolved from scramblases.
- ANO-mediated phospholipid scrambling elicits major physical and chemical alterations in the membrane and is associated with the release of extracellular vesicles that play crucial roles in cell signaling.
- The major cellular effect of scrambling is the exposure of phosphatidylserine that functions as a ligand for numerous receptors that function in cell-cell communication.
- Phospholipid scrambling is a major mechanism of cell-cell communication in tissues as diverse as skeletal muscle and gametes.
- Membrane trafficking is an integral component of PLS.

Figure 1. Anoctamins are diverse and cause human disease

A. Anoctamin family tree. Branches are colored according to Phylum. Chordate ANO1 – 10 are colored in two shades of green to differentiate between adjacent families. Individual ANOs of note are labelled: *C. elegans* anoh-1 and anoh-2; *Drosophila* SUBDUED, AXS, CG10352, CG6938, CG15270; fungal nhTMEM16 and afTMEM16, and yeast IST2. The tree was generated as follows: 4131 anoctamin sequences were obtained from Uniprot. After removing duplicates, fragments, sequences lacking the initial methionine, sequences <600 amino acids, and sequences having sequencing or assembling errors, 1066 sequences remained. One species was chosen to represent each phylogenetic order, leaving 392 sequences. These were aligned using MUSCLE with 16 iterations. The N- and C-termini were then deleted to leave only the predicted TMD1 – TMD10 with 50 – 100 flanking amino acids. Phylogenetic trees were constructed using a neighbor-joining Kimura protein algorithm in CLC Main workbench 7.7. **B.** An illustration of organ systems in which anoctamins have been associated with disease. Red: human diseases resulting from ANO mutations. Blue: abnormalities resulting from disruption of the ANO gene in murine models.

Figure 2. Phospholipids have different shapes that determine membrane curvature, which is altered during PLS

A. Structural formula of PtdCho, illustrating that phospholipids are constructed of three different modules: the hydrophilic head group, a linker (either phosphoglycerol –shown here - or sphingoid base), and one or more hydrophobic fatty acid chains. **B.** Space-filling models of major phospholipids head groups to illustrate variations in size: Ethanolamine (Etn), Choline (Cho), Serine (Ser), and Inositol (Ins). **C.** Structure of phosphatidic acid, composed of a phosphoglycerol linker and two hydrophobic acyl chains. Hydrocarbon chains differ in length and saturation. Unsaturated double bonds are kinked and occupy more space than saturated chains. PA 18:0/18:0 shows two saturated 18-carbon chains linked to phosphoglycerol. PA 20:4/18:0 shows one unsaturated 20-carbon chain (left, with 4 double bonds) and one saturated 18-carbon chain (right). These images are somewhat misleading, because in the membrane the hydrocarbon tails are quite flexible and they assume a wide variety of conformations. **D.** Examples of different glycerol phospholipids illustrating that their shapes vary from cylindrical (open rectangle) to cone-shaped (brown wedge) and

inverted cone-shaped (blue wedges). **E.** ABC transporters and P4-ATPases establish phospholipid asymmetry in the plasma membrane in an ATP-dependent manner. The model membrane here is composed of PtdCho (head group N colored blue) and PtdSer (head group N colored red). **F.** Elevation of cytosolic Ca^{2+} or activation of caspases activates phospholipid scramblase enzymes that passively allow lipids to equilibrate across the bilayer. In addition, there is evidence that phospholipid scrambling under some circumstances may also be facilitated by an inhibition of the ATPases. **G.** The consequences of PLS include receptor binding and changes in membrane biophysics. The top panel shows a lipid bilayer with lipid species distributed asymmetrically and the bottom panel shows the membrane after PLS has occurred. Interaction between adjacent phospholipids is shown by the graph on the right and the background shading. Attraction is colored green and repulsion is colored with longer wavelengths proportional to the repulsion. Before PLS, the head groups and hydrophobic tails repel equally and the bilayer is held together mainly by the hydrophobic effect at the interface between the head groups and the tails. After PLS, intermolecular interaction between lipids becomes unbalanced between the head groups and the tails. This alters the biophysical properties of the membrane by changing the electrostatics and lipid packing to produce stress curvature that leads to production of extracellular vesicles. Extracellular vesicles play important roles in physiological responses by serving as scaffolds for signaling complexes and as vehicles for delivering signaling machinery including both mRNA and non-coding RNAs such as microRNAs, and various signaling proteins. When anionic lipids are externalized, they can be recognized by soluble and cellular receptors.

Figure 3. The Passive Diffusion Model of ANO-scramblase Function

A. Classical ion channels are represented in cartoon form as funnel shaped structures where the ion conductance pathway is completely surrounded by protein. **B.** Phospholipid scramblases are envisioned as membrane proteins that have a slot that faces the lipid phase of the membrane. The slot is hydrophilic so that lipid head groups can move through it while the hydrophobic tails remain in the hydrophobic phase of the lipid. **C.** Crystal structure of nhTMEM16 showing a molecular dynamics simulation of PtdCho moving through the hydrophilic furrow. Head group nitrogen atoms are colored blue. nhTMEM16 surface is colored to indicate hydrophobicity (cyan = hydrophilic; magenta = hydrophobic). Note the deformation of the lipid bilayer by nhTME16. Arrows indicate the hydrophilic furrow.

Figure 4. PtdSer Receptors and the Role of PLS in Cell-Cell Signaling

A. PtdSer binding proteins. Soluble proteins are on top and membrane bound receptors are below. Regions that bind PtdSer in a Ca^{2+} -dependent manner are colored at the red end of the spectrum, yellow-red, as follows: Orange= Immunoglobulin-like, Magenta= Epidermal Growth Factor-like, Yellow = Glutamic Acid-rich γ -carboxyglutamic Acid, Red= C2-like, Purple = thrombospondin. Other domains shown: White = Transmembrane, and Blue= Protein Specific Domains. Lipid preference indicates the preference of the receptor to bind PtdSer or PtdEtn. Engulfment indicates whether the receptor has been shown to promote or inhibit phagocytic engulfment. **B.** Consequences of PLS. An unstimulated cell on the left has an asymmetric plasma membrane (blue = PtdCho and SM; red = PtdSer and PtdEtn) and contains multivesicular bodies (MVBs). PLS scrambles the plasma membrane and stimulates release of exosomes by exocytosis of MVBs and ectosomes by budding of the plasma membrane. Externalized PtdSer and PtdEtn on the plasma membrane and on released

EVs are recognized by soluble receptors and by cell bound receptors that bind directly or indirectly to the externalized phospholipid. EVs deliver cargo by binding or/and fusion to target cells.

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