

Cells on the MEND: exploring the role of lipidic forces in membrane trafficking

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The massive endocytosis (MEND) phenomenon

In this issue, the Journal publishes the last two of three articles by Donald W. Hilgemann and colleagues (see [Fine et al.](#) and [Hilgemann and Fine](#) in this issue; see [Lariccia et al.](#) in the January 2011 issue). The authors have used whole cell patch clamp and fluorescence microscopy in an impressive effort to investigate a previously unidentified, and surprising, process of MEND that can occur in a wide variety of cell types, including baby hamster kidney (BHK) and HEK293 cells and cardiac myocytes. MEND can be elicited by a wide variety of experimental maneuvers—including manipulations that alter lipid bilayer properties and domain organization—and the process can truly be massive, with internalization of as much as 75% of a cell's plasma membrane. Ruffles, abundant in BHK and HEK293 cells before MEND, are absent afterward. But ruffles are not required for MEND, which occurs also in myocytes, even though they are not ruffled. For myocytes, perhaps the invaginations are internalized. The size of the internalized vesicles is not extraordinary: <100 nm in diameter. But the MEND vesicles are somewhat unusual in that they do not readily acidify.

Summary of findings

In view of the novelty of the discovered phenomenon and the amount of experiments and results presented in the three papers, we will first summarize some of the significant findings and then discuss some of their possible mechanisms and ramifications.

The three articles characterize the newly discovered phenomenon of MEND and demonstrate that it proceeds through pathways different from those of classical endocytosis. Pharmacological evidence suggests that neither clathrin nor reorganization of the actin cytoskeleton is needed to bend plasma membranes into endocytotic vesicles, and that dynamins are not the pinches needed for fission. Importantly, there is selectivity among the membrane regions that are internalized through MEND, with strong evidence for a preferential internalization of liquid-ordered (l_o) domains. There also is selectivity among the membrane proteins being

internalized: Na^+/K^+ -ATPase is preferentially internalized, whereas the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is not, and shows a small preference to be retained in the plasma membrane.

MEND can be activated in several ways: through large transient Ca^{2+} fluxes across the plasma membrane, by adding amphiphiles to the extracellular solution, or by exposing the cells to sphingomyelinase C (leading to the production of ceramide). Experimental results show that these pathways are functionally coupled and that the controls for the onset of MEND are complex and do not follow the expected patterns (Doherty and McMahon, 2009). Normally, protein binding (e.g., endophilin and amphiphysin, acting through BAR domains) to the plasma membrane sculpts the lipid bilayer around the curvature of the protein network, and the invaginated membrane that buds into cytosol is pinched off by other proteins (e.g., dynamin), completing the formation of the endocytotic vesicle. In MEND, however, the lipids appear to have a more autonomous action in the formation of the curved endosome. The plasma membrane is apparently replenished by normal routes of exocytosis.

Calcium-induced MEND

In the experiments reported in the first of the three articles (Lariccia et al., 2011), MEND was induced by large Ca^{2+} transients mediated by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger operating in reverse. By overexpressing the exchanger, the authors were able to investigate endocytosis that results from much larger Ca^{2+} influxes than would occur naturally. A combination of membrane capacitance and fluorescence microscopy experiments show that MEND internalizes (and does not shed) membrane. Ca^{2+} -induced MEND requires high cytosolic ATP (>5 mM), and the influx of Ca^{2+} into BHK and HEK293 cells results in exocytosis followed by MEND. (Some properties of MEND depend on cell type: in cardiac myocytes, large Ca^{2+} transients induce MEND but without prior exocytosis.) Except at very high cytosolic Ca^{2+} concentrations (>10 μM) or polyamines (e.g., 1 mM spermidine),

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MEND does not occur when cytosolic ATP levels are low, but it can be triggered by the introduction of ATP subsequent to Ca^{2+} -induced exocytosis.

Cholesterol-dependent MEND

Hydrolysis of ATP leads to the generation of phosphatidylinositol-bis 4,5-phosphate (PIP_2), and a strong case is made that it is the production of PIP_2 , and not the activation of conventional protein kinases, that underlies the ATP dependence of MEND. Having excluded, by pharmacological means, the cells' canonical endocytotic mechanisms as causative of MEND, the authors turned to the role of membrane lipids. They found that MEND depends on membrane cholesterol, becoming Ca^{2+} independent after experimental maneuvers that increase membrane cholesterol—and is inhibited after maneuvers that deplete membrane cholesterol. Bacterial sphingomyelinase (SMase), which hydrolyzes sphingomyelin to ceramide and phosphocholine, induces Ca^{2+} - and ATP-independent MEND. But Ca^{2+} -induced MEND will proceed in the presence of SMase inhibitors. It is not clear if the SMase-dependent MEND is a result of ceramide production or sphingomyelin depletion. In contrast to SMase, neither phospholipase C (whether intracellular or extracellular) nor phospholipase A_2 (extracellular) induced MEND; yet, in a seeming contradiction, MEND is inhibited by the (Ca^{2+} -insensitive) phospholipase A_2 inhibitor bromoenol lactone, which does not inhibit the group iV cytoplasmic, Ca^{2+} -sensitive phospholipase A_2 s. Therefore, the lipid manipulation—and Ca^{2+} -induced MEND can occur independently of each other, but the two pathways do couple: maneuvers that alter membrane cholesterol content modulate Ca^{2+} -induced MEND and—as shown in the third article in the series (Hilgemann and Fine, 2011)—the amphiphile-induced MEND is facilitated by a preceding Ca^{2+} transient. The authors suggest that the coupling of the two pathways may be a consequence of Ca^{2+} transients affecting the physical state of the bilayer portion of the cell membrane—and vice versa.

Amphiphile-induced MEND

To further explore which lipid bilayer properties are used by MEND, the authors used a wide variety of maneuvers that are known to alter lipid bilayer properties. In the first of the two articles in this issue, Fine et al. (2011) use an assortment of amphiphiles to explore the possible role of lipidic forces. They discovered a striking difference between the Ca^{2+} -induced and the amphiphile-induced MEND: the initial Ca^{2+} influx in the former leads to exocytosis, which is followed by MEND. The amphiphile-induced MEND can occur without preceding exocytosis; exocytosis occurs subsequent to MEND, replenishing the plasma membrane. The amphiphile-triggered pathway leading to MEND is “sided”: amphiphiles initiate their effects when added to the extracellular

solution but not to the intracellular solution—acting from within the outer, but not the inner, cytosolic leaflet of cell membranes. (Some of the amphiphiles used, such as Triton X-100, are membrane permeable and would be expected to equilibrate between the two leaflets—but might bind to cytosolic proteins.)

Most amphiphiles induce MEND only at rather high concentrations (relative to their critical micellar concentrations [CMCs]), suggesting that they act by lipid-based physicochemical mechanisms rather than by inducing signaling pathways. There is some specificity of type, as shown by the fact that many, but not all, of the amphiphiles tested can trigger MEND. Those that do include: the nonionic detergents Triton X-100 and NP-40, lysophosphatidylcholine (LPC), and an eclectic variety of drugs that include tamoxifen and phospholipase inhibitors. Those that do not cause MEND, or that merely cause membrane “disruption,” include β -octylglucoside, CHAPS, and other cholic acid-related detergents, as well as compounds such as sphingosine. Membrane disruption usually occurs at concentrations close to the CMC; β -octylglucoside is an exception, which oddly causes membrane disruption at concentrations much lower than the CMC. Perhaps β -octylglucoside disrupts membranes by initiating a signaling cascade, rather than through a detergent lytic action. The ionic detergent SDS provides an example of an amphiphile whose addition to cell membranes does not cause MEND. But, curiously, its removal immediately results in MEND. Amphiphile-triggered MEND is not coupled to G protein cycling and can proceed without activating the other major MEND pathway, which is initiated by Ca transients and ATP hydrolysis.

Low-ordered lipid domains preferentially involved in MEND

The physical state of lipids in the portions of the plasma membrane that are internalized by MEND is explored in the third article in the series (Hilgemann and Fine, 2011). In this study, the authors added membrane-permeable ions and ion carriers, and measured their displacement currents before and after inducing MEND. Somewhat unexpectedly, the displacement currents were relatively unaltered, despite the great reduction of the plasma membrane area during MEND. This demonstrates that plasma membranes are not uniformly internalized in MEND, but rather that certain domains are the sources for endosomes.

Assuming that membrane-permeable ions more readily traverse liquid-disordered (l_d) than l_o domains, the authors conclude that l_o domains are preferentially internalized. As part of these studies, Hilgemann and coworkers investigated the internalization of several lipophilic fluorescent probes that occur as a result of MEND. Most probes were retained in the surface membrane, indicating that they preferentially interact with

I_d domains. An exception is the common fluorescence reporter FM 4-64: it did not segregate within plasma membranes, and its decreased fluorescence accurately reported the extent of internalization. As an important corollary to the principal findings of MEND, this third paper shows that FM 4-64 is a reliable and ideal probe for monitoring endocytosis.

Mechanisms of MEND

The density of experiments in these studies is enormous, and a large array of processes may be contributing to MEND. So, what are some of the mechanisms that may underlie the wealth of phenomena that have been uncovered? The authors suggest that the central mechanism underlying MEND is the merger of nanoscopic I_o domains into larger domains of 100-nm or so diameters, which then pinch off to enter the cytosol. To evaluate this suggestion, it is useful to consider the physical chemistry of membrane merger that is tacitly used by the authors, including the concept and principles of line tension.

Line tension-dependent merger of low-ordered domains

The physical meaning of the term line tension can be understood by considering the analogy between two-dimensional membrane domains (such as “rafts”) and three-dimensional phases, such as spheres of decane in water. The line tension of a membrane domain is the one-dimensional equivalent of the two-dimensional surface tension of a decane sphere. Each is the force (per unit length for line tension and per unit area for surface tension) pulling a molecule at the surface into the interior of the object. The decane spheres will tend to merge because the merger will reduce the total surface area of the decane spheres, minimizing the aggregate surface energy. Likewise, the membrane domains will merge to reduce their aggregate boundary energy. But that is not all. There is an entropic penalty for merger for the simple reason that the entropy of one large domain (or sphere) is less than that of many small domains (or spheres). Entropy is independent of line tension (or surface tension), and there is therefore a critical line (or surface) tension, such that domains will merge for tensions greater than the critical value but will remain separate for tensions below critical. Careful measurements of line tensions of domains in lipid bilayer membranes show that the line tension can be greater or less than the critical value, depending on lipid composition (Esposito et al., 2007; Tian et al., 2007).

Amphiphile reduction of area compression

Domain merger, with selective endocytosis of I_o -merged domains, certainly could account for the authors' finding that displacement currents induced by the membrane-permeable ions are not reduced by MEND. But amphiphiles can have consequences in addition to

altering line tension and causing membrane budding, and the authors' suggestion that MEND is induced simply by domain merger may be too sweeping a generalization. An additional mechanism for amphiphile-induced MEND follows from the fact that water-soluble amphiphiles reduce the area compression (Evans et al., 1995) and bending (Ly and Longo, 2004) moduli of lipid bilayers. These reductions occur because these amphiphiles are buffered by the aqueous phase and readily enter or leave the membrane. The additional degree of freedom afforded the membrane by the partitioning of amphiphiles between water and bilayer should reduce the energy necessary to change membrane shape, thereby allowing membrane reorganizations that would be energetically precluded in the absence of the amphiphile. The energy reduction could be profound: high concentrations of LPC, for example, can reduce the area compression modulus of a bilayer by more than a factor of two, thereby reducing the energy of membrane deformations by the same factor (Zhelev, 1998). Such reductions could result in much larger membrane undulations and/or infoldings than normally occurs, allowing gesticulations of plasma membranes that would otherwise be energetically prohibitive.

Lipid biochemistry and signaling cascades

In some cases, biochemistry may be more important than physical chemistry in the initiation and/or progression of MEND. For the amphiphilic LPC induction of MEND, the addition of LPC to the outer leaflet should cause outward budding of domains, rather than the inward budding required for endocytosis. Ceramide, with only a hydroxyl as head group, is expected to rapidly flip-flop between the two leaflets (as does cholesterol and nonionic detergents such as Triton X-100), and asymmetries resulting from the abundance in one leaflet over the other should quickly dissipate—unless the amphiphile binds to cytosolic proteins, leading to a net removal of the amphiphile from the inner leaflet. Whether such buffering by cytosolic proteins could be important would be determined by the relative rate constants for inter-leaflet flip-flop and for desorption and protein binding. Therefore, ceramide may not promote inward budding of membranes solely for physical chemical reasons. LPC and ceramides, for example, are well-known signaling molecules and thus could promote MEND through mechanisms not directly related to membrane mechanics. Possibly, they trigger signaling cascades that promote MEND, maybe acting in concert with physicochemical mechanisms, as these molecules are present at quite high concentrations. It is thus important that LPC acts only at relatively high concentrations, which would tend to exclude the canonical signaling mechanisms, and that amphiphiles that are not specific signaling molecules have also been shown to promote MEND. But even in this case, caution is

called for: amphiphiles partition at high concentrations in membranes, raising the possibility that they might activate or modulate signaling cascades, as is known to occur for several detergents (Strupp et al., 2000), although the detergent activation of the signaling cascade could be a result of changes in bilayer elastic properties.

Physical forces could promote MEND through multiple means. The finding that the removal, but not the addition, of SDS promotes MEND may provide an example. If SDS were to preferentially reside at the boundaries of l_o domains, line tension would be lowered for the same reason the surface tension of a three-dimensional object is reduced by adsorption, as quantitatively given by the Gibbs isotherm. An additional physical chemical mechanism by which SDS could reduce line tension is by reducing any difference in height between a domain and its surrounding phase. Exposure of hydrophobic surfaces at the boundary to water is energetically prohibitive, and so lipids at the boundary deform to prevent this exposure. An agent that preferentially localizes to the domain boundary, such as SDS, could reduce this energy of deformation. In either case, the SDS would alter the energetics of domain formation. Whether SDS adsorbs to domain boundaries or compensates for height mismatch (or both), its presence would lower line tension. Its removal would increase the line tension, which in turn could promote merger of domains to sizes needed to form endocytotic vesicles.

The authors make a strong case for the existence of non-canonical endocytotic pathways involving lipidic forces, as evident in MEND. But many non-clathrin-mediated endocytotic pathways have been previously characterized, and it is possible that some of these pathways share mechanistic commonalities with those underlying MEND. One such non-clathrin-mediated pathway is the rapid endocytosis observed in calf (but not adult bovine) chromaffin cells (Artalejo et al., 1995). Others occur in viral entry into endosomes—in fact, a slew of non-classical pathways are observed in viral entry (Mercer et al., 2010). If the primary, or even secondary, uptake pathways are blocked, virus will find another. Perhaps entry of some viruses through normally non-preferred endocytic pathways, including those underlying MEND, come into prominent play as alternatives when cells are stressed.

Questions of the native lipidic environment in which membrane proteins function

In addition to questions of mechanism, these studies raise new functional questions and provide an important set of experimental tools for sorting membrane proteins. For example, based on the relative internalization of membrane and proteins ($\text{Na}^+/\text{Ca}^{2+}$ exchanger and Na^+/K^+ -ATPase), the authors conclude that the $\text{Na}^+/\text{Ca}^{2+}$ exchanger resides in l_o and l_d domains, with little preference for either domain, whereas the Na^+/K^+ -ATPase

resides mostly in l_o domains. One would expect that the greater lipid order in the l_o domains would inhibit the motions required for ion transporters and pumps to catalyze regulated stoichiometric movement of ions across the membrane. Are these ion transporters localized to the domain boundaries rather than embedded within the interior of an l_o domain?

More generally, the experimental tools that Hilgemann and colleagues have developed and put to good use demonstrate that there still is much to understand about membrane trafficking and endocytosis. In this context, MEND provides a powerful criterion for sorting the wide gamut of electrically (and otherwise functionally) active membrane proteins with respect to the lipid domains in which they normally function.

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