Massive endocytosis driven by lipidic forces originating in the outer plasmalemmal monolayer: a new approach to membrane recycling and lipid domains

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The roles that lipids play in endocytosis are the subject of debate. Using electrical and imaging methods, we describe massive endocytosis (MEND) in baby hamster kidney (BHK) and HEK293 cells when the outer plasma membrane monolayer is perturbed by the nonionic detergents, Triton X-100 (TX100) and NP-40. Some alkane detergents, the amphipathic drugs, edelfosine and tamoxifen, and the phospholipase inhibitor, U73122, are also effective. Uptake of the membrane tracer, FM 4–64, into vesicles and loss of reversible FM 4–64 binding confirm that 40–75% of the cell surface is internalized. Ongoing MEND stops in 2–4 s when amphipaths are removed, and amphipaths are without effect from the cytoplasmic side. Thus, expansion of the outer monolayer is critical. As found for Ca-activated MEND, vesicles formed are <100 nm in diameter, membrane ruffles are lost, and β-cyclodextrin treatments are inhibitory. However, amphipath-activated MEND does not require Ca transients, adenosine triphosphate (ATP) hydrolysis, G protein cycling, dynamins, or actin cytoskeleton remodeling. With elevated cytoplasmic ATP (>5 mM), MEND can reverse completely and be repeated multiple times in BHK and HEK293 cells, but not cardiac myocytes. Reversal is blocked by *N*-ethylmaleimide and a nitric oxide donor, nitroprusside. Constitutively expressed Na/Ca exchangers internalize roughly in proportion to surface membrane, whereas Na/K pump activities decrease over-proportionally. Sodium dodecyl sulfate and dodecylglucoside do not cause MEND during their application, but MEND occurs rapidly when they are removed. As monitored capacitively, the binding of these detergents decreases with MEND, whereas TX100 binding does not decrease. In summary, nonionic detergents can fractionate the plasma membrane in vivo, and vesicles formed connect immediately to physiological membrane-trafficking mechanisms. We suggest that lateral and transbilayer inhomogeneities of the plasma membrane provide potential energies that, when unbridled by triggers, can drive endocytosis by lipidic forces.

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INTRODUCTION

Numerous recent studies document the plasticity of endocytic processes in eukaryotic cells. Neurons, like yeast (Geli and Riezman, 1998), continue to carry out essential membrane cycling after the disruption of classical players of "scaffold-mediated" endocytosis. Besides clathrin deletion (Sato et al., 2009), the deletion of adapter protein 2 (Kim and Ryan, 2009) and multiple dynamins (Ferguson et al., 2007) follows a similar pattern. Although it is possible that membrane internalization is then supported by nonclassical scaffolding proteins, an alternative explanation is that classical endocytic proteins function primarily to bring control and specificity to a core mechanism that continues to operate in their absence.

The existence, diversity, and activity of clathrin-independent endocytic mechanisms in mammalian cells are abundantly documented (Mayor and Pagano, 2007; Ivanov, 2008; Doherty and McMahon, 2009; Donaldson et al., 2009). Multiple forms of clathrin-independent endocytosis depend on cholesterol and sphingomyelins (Lajoie and Nabi, 2007; Mayor and Pagano, 2007; Sandvig et al., 2008; Doherty and McMahon, 2009; Romer et al., 2010). These dependencies may reflect, at least in part, the formation of domains in the outer monolayer by these membrane components (Rajendran and Simons, 2005; Lingwood and Simons, 2010). However, the mechanisms by which lipid domains, membrane proteins (e.g., integrins), and membrane-associated proteins interact to drive clathrin-independent endocytosis remain poorly established (Mayor and Pagano, 2007).

In this light, we have attempted over several years to develop new experimental models to study endocytic

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Abbreviations used in this paper: AMP-PNP, adenylyl imidodiphosphate; BHK, baby hamster kidney; BMCD, β-methylcyclodextrin; C6TPP, hexyltriphenylphosphonium; DAB, diaminobenzidine; DDG, dodecylglucoside; DDM, dodecylmaltoside; HRP, horseradish peroxidase; LPC, lysophosphatidylcholine; MEND, massive endocytosis; NEM, *N*-ethylmaleimide; SMase, sphingomyelinase; TX100, Triton X-100.

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processes that do not rely on adapters. In a companion paper in the January 2011 issue (Lariccia et al.), we have described multiple forms of Ca-dependent endocytosis that seem to involve no classical endocytic protein. In a second companion paper (see Hilgemann and Fine in this issue), these processes are demonstrated to rely on, and internalize, ordered membrane domains that do not bind well many electrogenic and fluorescent membrane probes. As an initial model of lipid-driven endocytosis in intact cells, we have demonstrated that the generation of ceramide by bacterial sphingomyelinases (SMases) can drive more than one half of the cell surface to internalize within seconds. Although perhaps not a physiological mechanism, these experiments demonstrate a profound potential for lipidic forces, related to lipid domains, to drive endocytosis. Lipid domains, whenever they occur in membranes, will experience "line tension" to the surrounding membrane that tends to minimize their perimeters (Garcia-Saez et al., 2007; Yanagisawa et al., 2007; Vind-Kezunovic et al., 2008). Besides line tension, related to mismatch of lipid dimensions at a boundary, the shapes of phospholipids within domains, their tendency to tilt in a concerted manner, and asymmetries across monolayers will come into play in intact cells.

At the theoretical level, it is increasingly possible to simulate bilayer behaviors that are relevant to endocytosis. Relevant to the influence of detergents, small excess areas in a monolayer promote membrane "buckling" that in turn causes coalescence of lipid domains with phase separations occurring between membrane "caps" and "valleys" (Minami and Yamada, 2007; Yanagisawa et al., 2007). Further, it has been suggested from simulations that ordered membrane domains can develop chirality-dependent "collective tilt," leading to spontaneous budding and fission of vesicles when domain diameters reach 50–100 nm (Sarasij et al., 2007). Even in clathrin-driven endocytosis, it has been suggested that phase separations of lipids may drive the final steps of fission (Liu et al., 2006). Clearly, there is now a great need to bridge insights from the theoretical, liposome, and cellular studies of domains in relation to endocytic processes in real cells.

Moving beyond biochemical membrane modifications, we show in this study that common membrane perturbants, including multiple detergents and other amphipathic compounds (i.e., compounds with both hydrophilic and hydrophobic elements), can drive massive endocytic processes that are even larger and faster than SMase-activated massive endocytosis (MEND). The modification of membrane structures and forces by low concentrations of detergents has been the subject of much careful biophysical analysis over several decades (Heerklotz, 2008). Besides affecting membrane tension, nonionic detergents, such as Triton X-100 (TX100), can cause domain formation in artificial membranes composed of complex phospholipid mixtures, with detergent segregating to the fluid domains (Heerklotz et al., 2003). On the one hand, this outcome clearly shows that lipid raft fractions isolated in biochemical experiments can be generated artificially by the amphipaths used to isolate them. On the other hand, this outcome suggests that detergents can be used in much more subtle ways to probe complex biophysical behaviors of biological membranes. Most importantly with respect to this article, nonionic detergents can cause domain formation in complex giant lipid vesicles that is followed by the budding and fission of small vesicles with a suggested involvement of line tension as a primary driving force (Staneva et al., 2005; Hamada et al., 2007).

Using cell capacitance (C_m) recording and optical methods to monitor changes of membrane area and conductance, we describe here the largest and fastest endocytic responses described in any cell type to date. These amphipath-activated MEND responses can reverse via ATP-dependent trafficking mechanisms that are inhibited by oxidizing agents. However, their occurrence does not require nucleotides, Ca transients, an intact actin cytoskeleton, or the activity of dynamins. Although triggered here by nonphysiological perturbants, namely detergents, amphipath-activated MEND responses evidently rely on preexisting membrane domains and asymmetries.

MATERIALS AND METHODS

All methods and cell lines used were as in our companion paper (Lariccia et al., 2011), using the modified HEK293 cell line, T-REx-293 (Invitrogen), to express NCX1. NP-40 detergent was from CalBiochem. The fixable membrane tracer, FM 1-43FX, was from Invitrogen.

Solutions and materials

Solutions used minimized all currents other than NCX1 current. Free Mg of all cytoplasmic solutions was 0.4 mM. Standard extracellular solution contained (in mM): 120 LiOH, 4 $MgCl_2$ or 2 $MgCl_2$ plus 2 $CaCl_2$, 20 TEA-OH, 10 HEPES, and 0.5 EGTA, pH 7.0 with aspartate. The standard cytoplasmic solution contained (in mM): 80 LiOH, 20 TEAOH, 15 HEPES, 40 NaOH, 0.5 $MgCl_2$, 0.5 EGTA, and 0.25 $CaCl_2$, set to pH 7.0 with aspartate. Unless indicated otherwise, 0.2 mM GTP was used in nucleotide-containing solutions. Modified cytoplasmic solution contained (in mM): 60 KOH, 50 NaOH, 15 TEAOH, 15 HEPES, 0.5 $MgCl_2$, 1.0 EGTA, and 0.2 $CaCl_2$, set to pH 7.0 with aspartate. The lysophosphatidylcholine (LPC) used was a preparation from bovine brain (Sigma-Aldrich) that is reported to contain predominantly palmitic, stearic, and oleic acids.

Fluorescence imaging of fixed cells

Trypsinized HEK293 cells were allowed to attach to glass-bottom dishes for 1 h in growth medium. They were washed with extracellular solution, incubated with and without NP-40 (150 µM) in the presence of 10 µM FM 1-43FX for 1 min, washed again three times to remove excess dye, fixed with 2.5% glutaraldehyde in 0.1 M of cacodylate buffer for 20 min, and finally washed with buffer and treated with 50 mM glycine for 5 min to minimize background. Cells were imaged on a microscope (IX-90; Olympus) with a camera (Coolsnap ES21; Photometrics).

NP-40 experiments for SEM and TEM

Trypsinized HEK293 cells were allowed to attach on poly-L-lysine–coated coverslips for 1 h in growth medium. NP-40–treated and untreated groups were washed with extracellular solution before incubation with 10 mg/ml horseradish peroxidase (HRP) with and without NP-40 (150 µM) for 1 min. The cells were then washed and fixed with 2.5% glutaraldehyde in 0.1 M of cacodylate buffer for 1 h. After washing with 175 mM Tris-Cl buffer, the cells were incubated in 0.02% H_2O_2 and 0.1% diaminobenzidine (DAB) in buffer for 20 min. The cells were washed and placed in 1% OsO_4 for 30 min and washed with 0.1 M of cacodylate buffer.

TEM

One set of coverslips for TEM imaging (control and NP-40 treated) was further placed in 2% uranyl actetate for 15 min. They were then dehydrated in an ascending ethanol series and infiltrated with Embed812 (Electron Microscopy Sciences). Coverslips were mounted on BEEM capsules and polymerized overnight at 60°C. Separation of coverslips was done by plunging the capsules into liquid nitrogen. Thin (70–80-nm) sections were prepared using an Ultramicrotome EM UC6 (Leica) and mounted on 200 mesh copper grids. Sections were post-stained with 2% uranyl actetate for 15 min and lead citrate for 5 min. Cells were imaged in a Tecnai G2 Spirit Biotwin (FEI Company) TEM.

SEM

Samples for SEM were also dehydrated using ethanol followed by transition into pure hexamethyldisilazane. Cells were air dried and immediately coated with a gold layer using a sputter coater (108; Cressington). Coverslips were mounted on Al stubs using conductive carbon tape and imaged with an XL30 ESEM (FEI Company).

Online supplemental material

Figs. S1 and S2 show that β-cyclodextrin treatments only partially block detergent-activated MEND, whereby β-methylcyclodextrin (BMCD) treatment can itself cause large decreases of C_m from both membrane sides. Fig. S3 illustrates that benzyl alcohol, commonly used as a "membrane fluidizer," does not block, but rather promotes and accelerates TX100-activated MEND. Fig. S4 documents that dodecylmaltoside (DDM) induces MEND by a pattern that is intermediate between TX100 and SDS. Fig. S5 shows that that C6TPP induces MEND by the SDS pattern when used in concentrations of 5–10 millimolars. Fig. S6 shows that amphipaths, which are effective MEND inducers in fibroblasts, are also effective in cardiac myocytes. Fig. S7 documents that MEND occurring upon the removal of SDS reverses quickly upon the reapplication of SDS. Finally, Fig. S8 shows that many detergents disrupt patch clamp experiments without inducing MEND. Figs. S1–S8 are available at http://www.jgp.org/cgi/content/full/jgp.201010469/DC1.

RESULTS

MEND induced by nonionic detergents

As outlined in the Introduction, low concentrations of nonionic detergents can cause phospholipid-phase separations in complex artificial membranes (Heerklotz et al., 2003) that can in turn promote membrane vesiculation (Staneva et al., 2005). Figs. 1 and 2 present the effects of sublytic concentrations of TX100 and NP-40 (<250 µM) in baby hamster kidney (BHK) cells. Fig. 1 shows the complete electrical parameters of a cell during extracellular application of 150 µM TX100 for 15 s. C_m rises very briefly and then plummets by >50% with a maximal decline rate of 13% per second. Thus, the response is at least as large and fast as SMase-induced MEND described in our companion paper (Lariccia et al., 2011). The small rise of C_m that precedes the fall probably reflects expansion and thinning of the membrane by detergent insertion. Other cell electrical parameters are nearly unaffected by the detergent, and this was similar for the other amphipaths described subsequently.

The structurally related nonionic detergent, NP-40, causes MEND responses that are indistinguishable from those of TX100 (>500 observations), and we have used these detergents interchangeably over the last 3 yr. Fig. 2 describes MEND responses induced by these detergents in more detail. Fig. 2 A shows averaged C_m data for four TX100 concentrations from which we determined, as indicated by gray lines, the average maximal rates of C_m decline in percentage per second. The results are described in Fig. 2 B by a power function ($k \cdot [TX100]^n$) with an exponent, *n*, of 3.8. Thus, detergents promote MEND precipitously at a critical concentration that is less than the critical micelle concentration. Often, an adjustment of the detergent concentration by 10% caused or prevented MEND that involved >50% of the cell surface in a few seconds.

Responses to NP-40 are shown in Fig. 2 (C–E). The standard cytoplasmic solution in Fig. 2 C is without ATP or GTP. In Fig. 2 D, it contains the nonhydrolyzable ATP analogue, adenylyl imidodiphosphate (AMP-PNP; 2 mM), with no ATP or GTP. Therewith, these results illustrate that detergent-activated MEND does not require nucleotide-hydrolyzing enzymes. As illustrated in Fig. 2 C, we attempted extensively to determine the

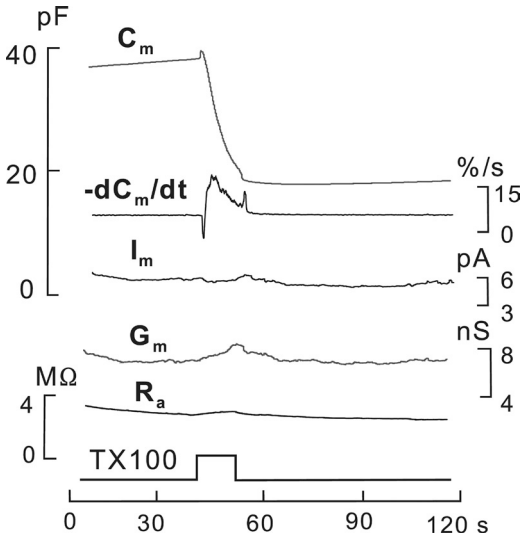

Figure 1. Activation of MEND in a BHK cell by extracellular application of 120 µM TX100 for 10 s. Membrane area, i.e., C_m, decreases at a maximal rate of 13% per second, and the endocytic response stops abruptly upon the removal of detergent. During MEND, there is a barely detectable transient increase of cell conductance (G_m). Other cell electrical parameters do not change.

size of vesicles formed during MEND via capacitance recording at high resolution, but C_m steps were not routinely observed. To optimize the resolution of capacitance steps, we used relatively small cells at room temperature with sinusoidal voltage perturbation (Wang and Hilgemann, 2008). In brief, we occasionally observed clear steps of 15–50 fF, but this was not routine. Fig. 2 C shows an optimized record using 2-kHz sinusoidal voltage perturbation (R_a < 2 MΩ). Five perturbation cycles were averaged per point displayed. There is a clear increase of RMS noise variance upon applying detergent, and noise variance decays in parallel with C_m. However, when exponential functions were subtracted from declining C_m signals, the residual signals still did not allow clear resolution of C_m steps to a resolution of ∼10 fF (Fig. 2 C, inset). The ratio of signal variance to declining C_m was <0.2 fF, suggesting that vesicles are not >80 nm in diameter (1 pF ≈ 100 μm^2) upon fission.

Fig. 2 D illustrates the rapidity with which C_m changes stop when detergent is removed from the extracellular solution. In this experiment, NP-40 was applied initially for 2 s and removed for 10 s; then, it was reapplied for 10 s, removed for 60 s, and reapplied for 10 s. As shown in the inset of Fig. 2 D at higher resolution, the initial MEND response stops at an intermediate C_m level within ∼2 s when detergent is removed. Because much longer times are required to clear the cytoplasm, especially when compounds bind to cellular components, this observation suggests that detergent is acting from the extracellular monolayer. Further support for this conclusion is presented subsequently. Upon reapplying detergent, a smaller and slower response is evoked. The fall of C_m continues briefly upon the removal of detergent until C_m reaches 48% of its initial value. Thereafter, NP-40 causes rapidly reversible, small increases of C_m that probably reflect detergent binding/dissociation kinetics with no further endocytosis being possible. We point

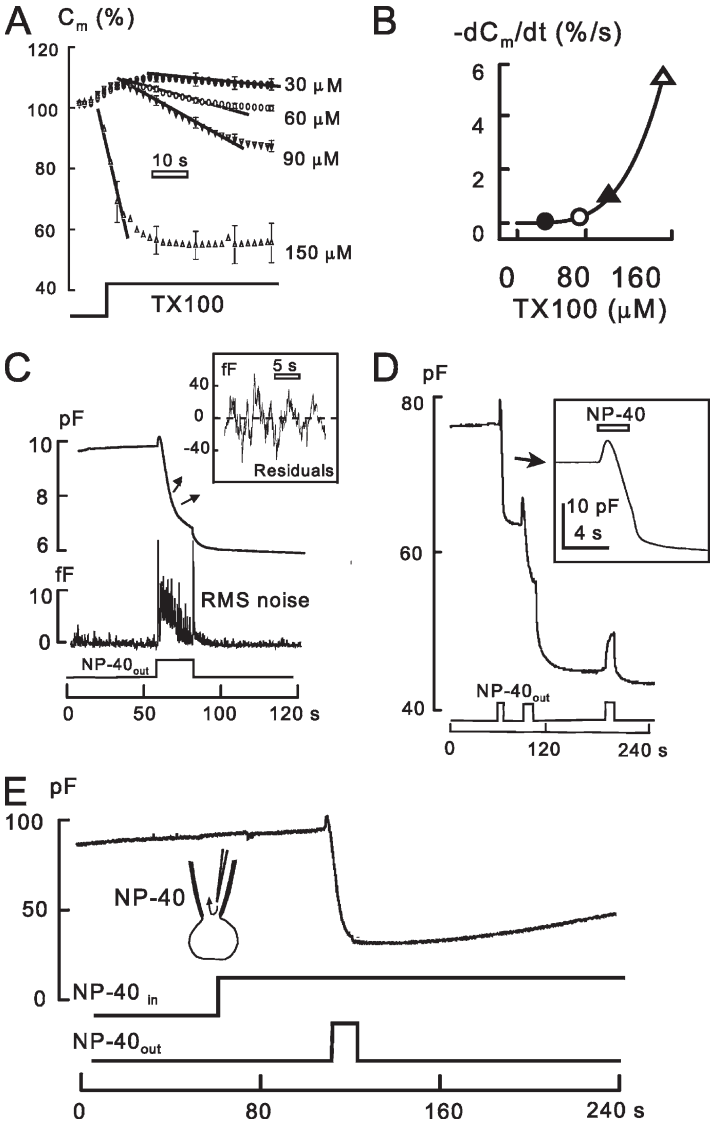

Figure 2. Salient characteristics of TX100 and NP-40–induced MEND. In A and B, the pipette solution contained 2 mM ATP and 0.2 mM GTP. C is without nucleotides, D is with 2 mM AMP-PNP and no other nucleotides, and E is with 2 mM ATP and 0.2 mM GTP. (A) Average MEND responses of BHK cells upon the application of four different concentrations of TX100 (n = 5–7). (B) Concentration dependence of the maximal rate of change of C_m by applying TX100 in A. The response rate increases with roughly the fourth power of the concentration, given by solid line. (C) MEND in a small BHK cell at 22°C. Variance of RMS noise of C_m increases immediately upon the application of NP-40 and decreases during the decline of C_m. The inset shows the residual signal after subtracting an exponential function from the declining C_m signal. (D) MEND induced by 120 µM NP-40. Upon the application of NP-40, MEND is preceded by a rapid rise of cell C_m, and MEND stops within 2–3 s upon removing detergent. After a second MEND response, detergent causes small increases of C_m that reverse almost as quickly as solutions can be changed. (E) Rapid pipette perfusion of 150 µM NP-40 into a BHK cell by pipette perfusion. As in four additional experiments, NP-40 has no effect from the cytoplasmic side, nor does intracellular detergent have any evident influence on the MEND response to extracellular detergent.

out additionally that the initial increase of C_m upon applying NP-40 is very similar before and after MEND. As described later in more detail, this suggests that binding of NP-40 is almost unaffected by MEND.

Fig. 2 E illustrates the lack of effect of detergent (140 µM NP-40) when perfused into the cytoplasm of cells, representative of six similar experiments. As shown further, the presence of cytoplasmic detergent does not block or change in a conspicuous way the subsequent C_m response to 140 µM of extracellular NP-40. Similar results were also obtained for other detergents, as described subsequently in this article and in our companion paper (Hilgemann and Fine, 2011). Even when perfused into cells at five times higher concentrations than needed to cause endocytosis from outside, cytoplasmic detergents were ineffective in inducing MEND or membrane shedding. Although some detergents cross the membrane rather rapidly (Heerklotz, 2008), it appears that MEND is caused specifically by detergent interaction with the extracellular leaflet of the plasma membrane.

To test whether nonionic detergents are causing endocytosis or membrane shedding, or both, we performed experiments with the membrane tracer dye, FM 4–64, illustrated in Fig. 3. In these experiments, we used 2 mM ATP and 0.2 mM GTP in cytoplasmic solutions. The continuous fluorescence signal in Fig. 3 A gives total cell fluorescence for an experiment in which dye was applied and washed off before the application of TX100. The FM dye (5 µM) washes off multiple times by ~90% within seconds of its removal. With the brief application of 120 µM TX100, C_m falls by 66%. Thereafter, fluorescence labeling of the cell is reduced by 73% when dye is applied and removed in similar fashion. Thus, the decrease of the plasmalemma area accessed by FM 4–64 is in good agreement with the electrophysiological measurements. However, this result does not indicate whether membrane has been internalized or shedded to the outside. To address directionality, the continuous fluorescence signal in Fig. 3 B gives total cell fluorescence for an experiment in which dye was applied and washed off multiple times, but in which 120 µM TX100 was applied during the application of FM dye. In this case, dye cannot be washed off after inducing endocytosis. TX100-induced endocytosis amounts to 65% of the cell membrane by capacitance recording. The fraction of fluorescence that forms a ring close to the cell surface, and cannot be washed off, amounts to 70% of the initial total cell fluorescence. Thus, all of the

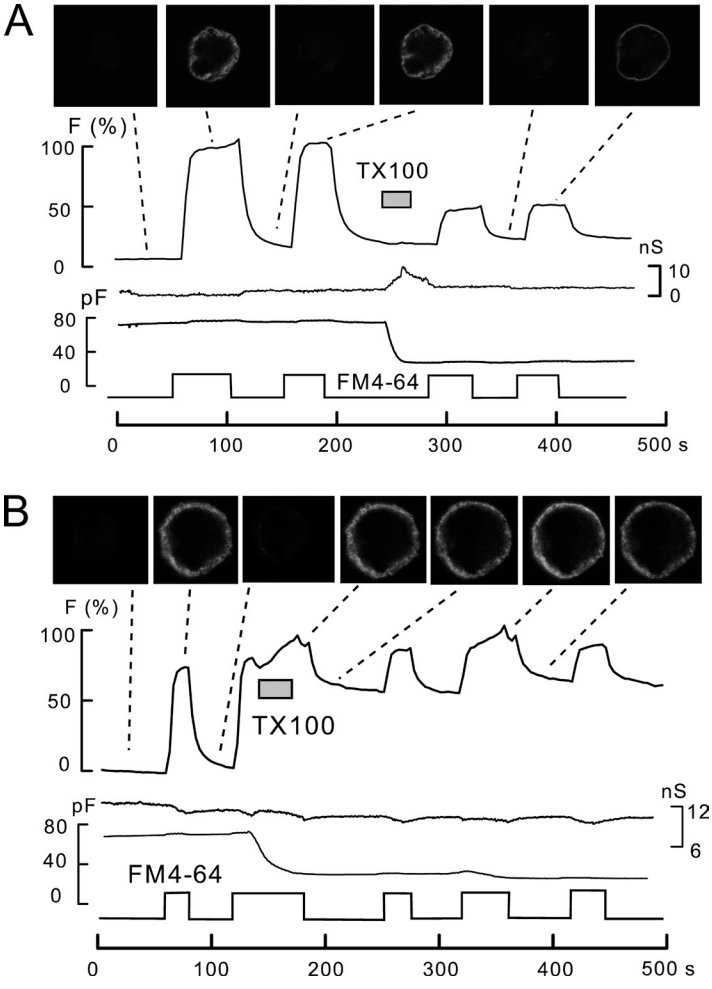

Figure 3. Characterization of TX100-induced MEND using simultaneous optical and electrical recordings in BHK cells. The pipette solutions contained 2 mM ATP and 0.2 mM GTP. (A) 5 µM FM 4–64 was applied and removed twice, and then 120 µM TX100 was applied for 30 s, inducing a 66% MEND response. FM dye was applied and removed twice more. Membrane binding of FM dye decreases 73% after MEND. (B) 5 µM FM 4–64 was applied once and washed. During the second application, 120 µM TX100 was applied for 30 s, inducing a 65% MEND response. After MEND, FM dye was applied and removed three more times. FM fluorescence remaining in the cell does not wash off and amounts to 70% of pre-MEND fluorescence, corresponding roughly to the amount of membrane internalized as per capacitive measurements.

membrane lost from the cell surface during the application of TX100 can be accounted for by internalized membrane. This result indicates further that FM 4–64 binds roughly equally well to the membrane that internalizes and that which remains at the cell surface. As described in our companion paper (Hilgemann and Fine, 2011), this is the exception rather than the rule for fluorescent membrane probes.

As described in relation to Fig. 2, we verified repeatedly that nonionic detergents cause MEND in cells without cytoplasmic ATP, with AMP-PNP (e.g., Fig. 2 D), and with high cytoplasmic Ca concentrations that can cause membrane blebbing. Therefore, we next tested more rigorously whether MEND occurs without requirement for nucleotides or an intact membrane cytoskeleton. Fig. 4 A shows an experiment in which the cytoplasmic solution contained 2 mM AMP-PNP, no ATP, no GTP, a high concentration of latrunculin (5 µM), and a high concentration of Ca (2 µM of free Ca using 10 mM EGTA plus 8.5 mM Ca) to promote disruption of cytoskeleton. As shown by the bright field images, the cell membrane became nearly smooth with this cytoplasmic solution and the cell appears swollen. As shown in the continuous fluorescence record, 6 µM FM dye was applied and removed three times with good reversal of fluorescence each time. Then, the dye was applied together with TX100, and the C_m response amounted to a loss of 75% of initial C_m. Thereafter, 85% of the FM fluorescence signal could not be washed off and formed a bright rim at the cell periphery, similar to results described previously. Thus, disruption of membrane cytoskeleton, removal of nucleotides, and blockade of ATP-hydrolyzing mechanisms do not block, and may actually promote, MEND responses to nonionic detergents.

Further characteristics of MEND induced by 120 µM TX100 are shown in Fig. 4 B using bar graphs to present composite results. The average response to this concentration of TX100 was a 60% decrease of C_m. The MEND response to TX100 was not significantly affected by including 0.2 mM guanosine 5′-[γ-thio]triphosphate (GTPγS) in the cytoplasmic solution to hinder G protein cycling. It was not affected by including 5 µM latrunculin and 2 mM AMP-PNP to disrupt actin cytoskeleton, block ATPase activities, and deplete phosphatidylinositiol-bis 4,5-phosphate. And MEND responses were not affected by inhibiting dynamin activity with an unmyristolated dynamin inhibitory peptide (50 µM; DynPep; Tocris Bioscience) or the organic dynamin inhibitor, dynasore (0.2 mM). Finally, we point out that high extracellular Ca (2–6 mM) effectively blocks detergent-activated MEND, independent of Ca influx and cytoplasmic Ca changes, whereby the block can be overcome with higher detergent concentrations. The block probably reflects Ca binding by the outer membrane monolayer,

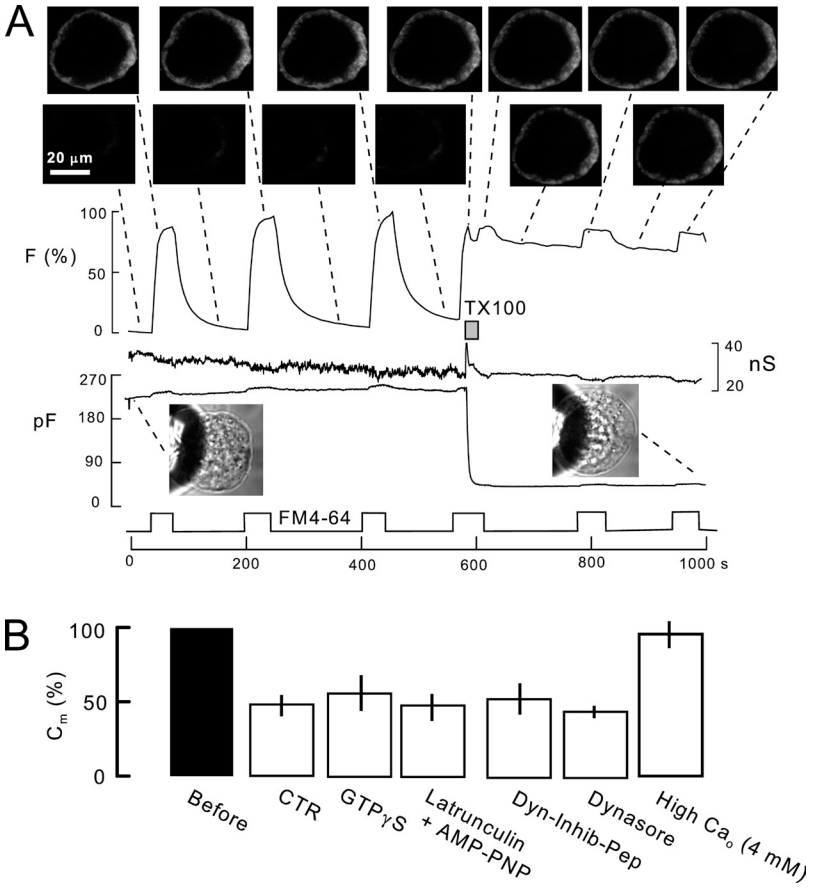

Figure 4. TX100-induced MEND does not depend on canonical endocytic proteins. (A) TX100-induced MEND after extensive disruption of cytoskeleton, phosphoinositides, and ATP-dependent processes (5 µM latrunculin with 2 µM Ca, no ATP or GTP, and 2 mM AMP-PNP). FM 4–64 was applied three times before MEND. During the fourth application, 120 µM TX100 was applied, causing a 77% MEND response. Thereafter, 85% of FM dye fluorescence was retained, and FM dye was reapplied twice. The reversible FM fluorescence amounts to 15% of pre-MEND fluorescence. (B) Further characteristics of MEND induced by 120 µM. As indicated by the open bar graphs, TX100-induced MEND was not inhibited when the cytoplasmic solution contained 0.2 mM guanosine 5′-[γ-thio]triphosphate (GTPγS), 5 µM latrunculin with 2 mM AMP-PNP, 50 µM of an unmyristolated dynamin inhibitory peptide (DynPep; Tocris Bioscience), or 0.2 mM of the organic dynamin inhibitor, dynasore.

which decreases the apparent affinity of the membrane for amphipaths. An example is given in Fig. S1 of our companion paper (Hilgemann and Fine, 2011). In addition, Figs. S1 and S2 illustrate that TX100-induced MEND is partially blocked by β-cyclodextrin treatments, but it is not fully blocked by even extensive treatments that extract a large fraction of the total membrane. Fig. S3 illustrates that agents commonly used as "membrane fluidizers," which can partially disrupt domain formation in some model membranes (Maula et al., 2009), do not disrupt TX100-induced MEND at nondisruptive concentrations and even promote TX100-induced MEND.

Structural basis of amphipath-induced MEND

To visualize the morphology of cells and vesicles formed during detergent-induced MEND, we performed a scanning (SEM) and a transmission electron microscopy analysis of HEK293 cells. Cells were removed from dishes, as in electrophysiology experiments, incubated for 3 min in standard extracellular solution, incubated further with or without 150 µM NP-40 for 1 min, and then fixed as described in Materials and methods. To verify that membrane internalization was occurring in this same protocol, cells were incubated with the fixable dye, FM 1-43FX (5 µM), for 3 min, incubated further with or without 150 µM NP-40 for 1 min with dye, and then fixed in dye-free solution. As shown in Fig. 5 A, treatment with detergent generates a bright rim of fluorescence at the cell surface, corresponding to internalized surface membrane. Fig. 5 B shows SEM micrographs of cells treated in this same way. As evident in Fig. 5 B (left), representative of >20 observations, extensive membrane ruffling is a hallmark of these cells when removed from dishes. As evident in Fig. 5 B (right), ruffles are lost and cells become nearly smooth upon treatment with detergent. To examine the ultrastructure of vesicles formed during MEND, we incubated cells with 10 mg/ml HRP during a 1-min detergent treatment, as described in Materials and methods and our companion paper (Lariccia et al., 2011). As shown in Fig. 5 C, numerous stained vesicles with diverse morphologies were found in detergent-treated cells just under the outer surface of cells, whereby some showed a clearly multi-laminar structure. As shown in Fig. 5 D for 26 control and 28 treated cells, NP-40 treatment increased the number of clearly stained vesicles by 2.5-fold.

Amphipath-activated MEND occurs in cells with "physiological" morphology and connects to physiological membrane-trafficking processes

Results presented thus far demonstrate that amphipath-activated MEND is extremely powerful in cells with extensive ruffle structures that have been removed from dishes by proteolysis. Thus, both proteolysis of the cell surface and ruffling might importantly promote the MEND process. Therefore, we performed both electrophysiological and optical experiments to test whether TX100 exposure induces MEND in BHK cells growing on coverslips. As described in Fig. S4, TX100 indeed induces MEND with similar characteristics to those reported above in cells growing on dishes without previous treatment with proteases. As documented in Fig. S4, MEND induced by 200 µM TX100 amounted to 41% of cell capacitance on average, could be repeated multiple times in the same cell, and resulted in equivalent losses of FM dye binding and FM dye uptake.

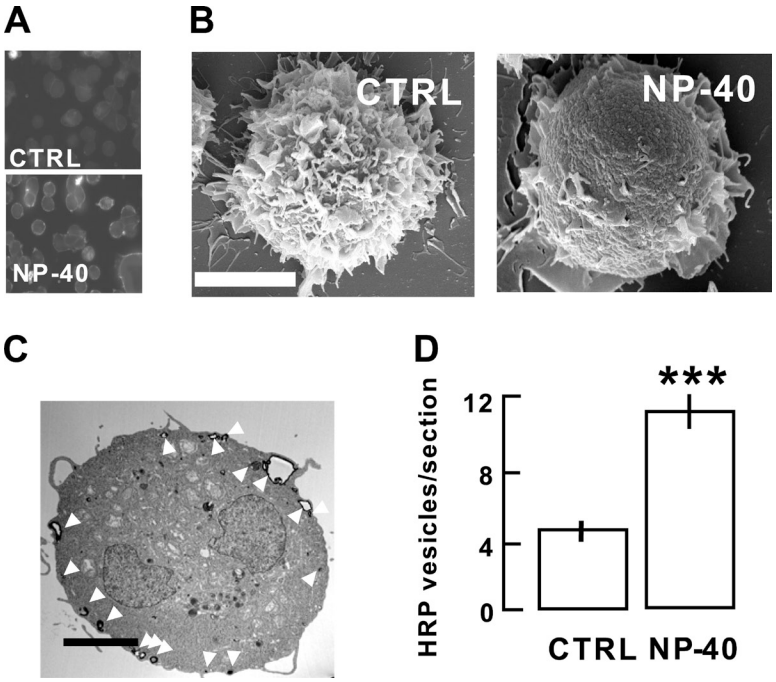

Figure 5. Analysis of NP-40 responses of HEK293 cells by scanning and transmission elecronmicroscopy (SEM and TEM). (A) Demonstration of NP-40–induced MEND in a cell population. Cells were incubated with FM 1-43FX during NP-40 application for 30 s, and then washed and fixed as described in Materials and methods without FM dye. A bright rim of fluorescence was generated in cells treated with detergent, thereby verifying the operation of MEND in this protocol. (B) Scanning electron micrographs of HEK293 cells before (left) and after (right) treatment with NP-40. Control cells show an extensive mesh of membrane ruffles, whereas NP-40 cells appear nearly smooth in comparison. Bar, 10 µm. (C) TEM image of an NP-40–treated HEK293 cell. Detergent-treated cells show large DAB-stained vacuoles (>20 observations). Bar, 5 µm. Stained vesicles are indicated with arrows. (D) DAB-stained vacuoles are significantly increased in HEK293 cells after treatment with NP-40 for 60 s ($n \geq 26$; $P < 0.001$).

That detergent-activated MEND can be repeated with recovery of capacitance occurring over several minutes is similar to both Ca-activated and SMase-activated MEND (Lariccia et al., 2011). Using detached cells, as described in Fig. 6 A, we examined the reversal of MEND using several different cytoplasmic solution compositions. We found that the modified standard cytoplasmic solution, used in myocyte experiments (Lariccia et al., 2011), was very effective to promote the reversal of MEND when used with 8 mM ATP and 0.2 mM GTP. This solution contains 60 mM Na and 60 mM K, with no Li, and a higher EGTA concentration (1 mM), with lower free Ca (0.2 mM of total Ca; 90 nM of free Ca) than standard solution. As illustrated by Fig. 6 A, it was routine to obtain complete plasmalemma recovery from MEND responses >70%, and multiple cycles of MEND and recovery could be performed in single experiments. Maximal recovery rates were in the range of 15% of internalized membrane per minute. As shown in Fig. 6 B, recovery was abolished when a nonhydrolyzable ATP analogue, AMP-PNP (2 mM), was used instead of ATP (>10 observations). Fig. 6 C illustrates that recovery was equally abolished by 0.5 mM *N*-ethylmaleimide (NEM; five observations). We note that NEM was included in both extracellular and intracellular solutions, as it crosses membranes rapidly. Fig. 6 D illustrates that recovery was slowed severalfold (<4 vs. 15% per minute) and was incomplete or negligible when a low concentration of the NO donor, nitroprusside (16 µM; four similar observations), was included in cytoplasmic solutions with standard nucleotides. Because 0.3 mM cGMP (dotted record, scaled to the nitroprusside record) had no effect (five

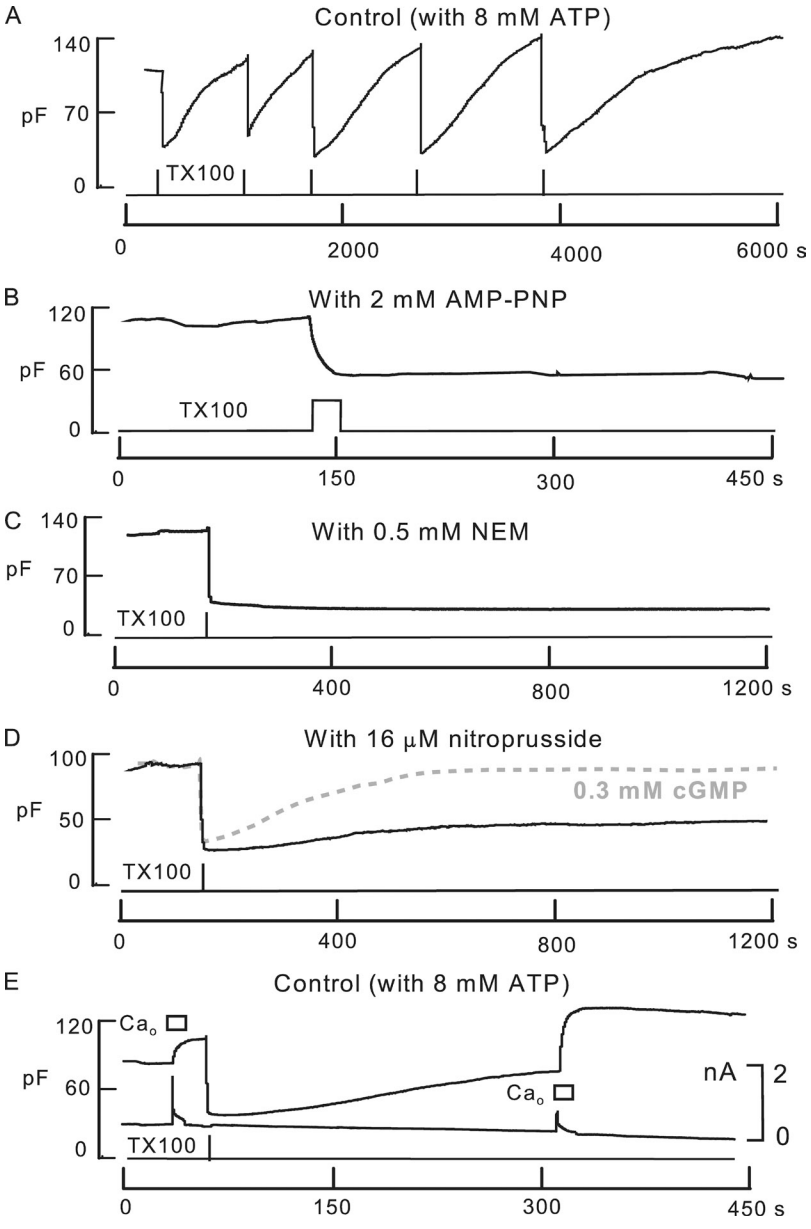

Figure 6. Reversal of TX100-induced MEND in BHK cells. (A) TX100-induced MEND reverses completely when the cytoplasmic solution contains 8 mM ATP, 0.2 mM GTP, no lithium, and a low free cytoplasmic Ca concentration (1 mM EGTA with 0.2 mM total Ca; free Ca calculated to be 0.15 µM; >20 observations). In this recording, MEND is induced with 200 µM TX100, and it reverses with a progressively longer halftime of 5–16 min over five repetitions. (B) MEND does not recover in the absence of ATP and the presence of a nonhydrolyzable ATP analogue (2 mM; AMP-PNP). (C) MEND recovery is completely blocked by 0.5 mM NEM added to both extracellular and cytoplasmic solutions (five similar observations). (D) MEND recovery is strongly inhibited by cytoplasmic application of 16 µM of the NO donor, nitroprusside (four similar observations), whereas it is unaffected by 0.3 mM cGMP (five similar observations). (E) When MEND recovery has progressed partially for 3 min, a Ca transient can induce complete recovery of membrane area in seconds. In this experiment, a Ca transient was evoked before MEND to determine the immediately available membrane pool for exocytosis. Thereafter, MEND amounts to a loss of 66% of the cell surface, and after 3 min, a Ca transient causes exocytosis of a three times greater amount of membrane than was initially available.

observations), NO is probably acting via oxidative stress. All of these results are consistent with a role for the oxidation-sensitive NEM-sensitive factor (Lowenstein and Tsuda, 2006) in cycling membrane back into the plasmalemma in these experiments.

Fig. 6 E illustrates that vesicles that recycle in these experiments may become available for Ca-induced membrane fusion before they are reinserted into the cell surface during the recovery process illustrated in panel A. In this experiment, exocytosis was activated by a Ca transient before inducing MEND with 200 µM TX100. The exocytic response amounts to 25% of C_m, and the MEND response amounts to 66% of C_m. Membrane area recovers partially within 4 min, as usual with 8 mM ATP. A Ca transient then results in complete recover of C_m. There is no further increase of C_m afterward, suggesting that the membrane that was being inserted slowly over many minutes may have been inserted within seconds during the Ca transient.

Fig. 7 A illustrates that C_m changes and NCX1 activity changes occur in parallel during repeated MEND responses. In these experiments, BHK cells were employed using standard cytoplasmic solution with a high cytoplasmic EGTA concentration (20 mM with 5 mM Ca; 0.3 µM of free Ca) to effectively buffer NCX1-mediated cytoplasmic Ca transients. NCX1 current was activated multiple times for 1 s, as indicated before and after the application of 150 µM NP-40. In response to three applications of NP-40, C_m decreased by 44, 38, and 25%, whereas peak NCX1 currents decreased by 36, 48, and 36%, respectively. C_m recovered by 65% after the first two NP-40 exposures, whereas exchange currents recovered by ~50%, suggesting that membrane recycling can bring internalized vesicles back to the cell surface. Exchange current magnitudes follow qualitatively the changes of membrane area, as if exchangers internalize and recycle with the plasmalemma. However, we note that exchange currents did not recover as completely as C_m after a MEND response.

As described in Fig. 7 B, we used HEK293 cells expressing NCX1–pHluorin fusion protein to quantify NCX1 internalization. Extracellular NCX1 fluorescence was determined by acidifying the medium to pH 6.0 and then switching to pH 8. With the application of 150 µM NP-40 for 10 s, the fluorescence that is rapidly induced by alkalinizing from pH 6.0 to 8 decreased by 37%, whereas C_m decreased by 48%. Similar to results for Ca-induced and SMase-induced MEND (Lariccia et al., 2011), there is an increase of fluorescence at pH 6.0. Thus, vesicles formed do not immediately acidify, as this would have resulted in a large decrease of fluorescence at all three pH values. In addition, it is important that the maximal fluorescence at pH 8.0 decreases by only 10%. This indicates that exchangers were not lost from the cell to the medium as a result of membrane shedding. Rather, they must have been internalized to nearly the same extent as the plasmalemma.

Differential Na/K pump sensitivity to MEND in myocytes, BHK cells, and HEK293 cells

In our companion paper (Hilgemann and Fine, 2011), we present evidence that detergent-activated MEND internalizes preferentially liquid-ordered membrane domains, with disordered membrane staying at the cell surface.

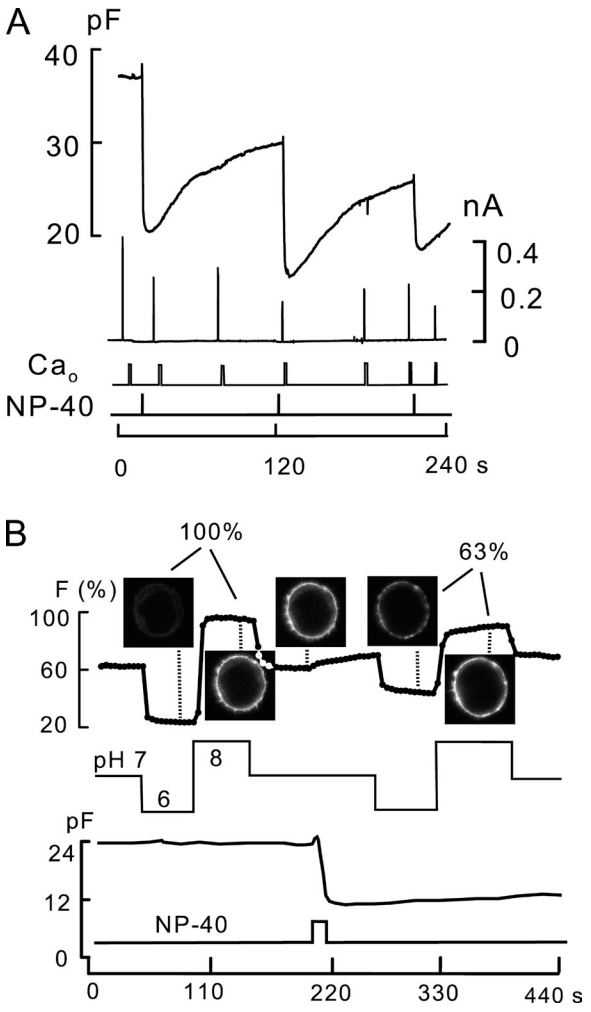

Figure 7. Functional and optical analysis of the influence of MEND on NCX1. (A) MEND responses and NCX1 currents in a BHK cell perfused with a high (20 mM) EGTA concentration to negate Ca transients and high ATP (8 mM) to promote MEND reversal. For the most part, MEND reverses within 2 min. We note that this experiment was performed with standard solutions, not modified standard solutions that promote recovery. NCX1 current is decreased after each MEND response and recovers partially during recovery of C_m. (B) Time-lapse confocal imaging of an HEK293 cell stably expressing NCX1–pHluorin fusion protein before and after exposing the cell to 150 µM NP-40 for 10 s. Fluorescence arising from NCX1 at the cell surface is determined as the fluorescence activated by jumping extracellular pH from 6.0 to 8.0. MEND induced by NP-40 causes a C_m drop of 48%, whereas the fluorescence intensity of cell surface NCX1 (i.e., fluorescence jump on changing solutions from pH 6.0 to 8.0) decreases by 37%. The presence of fluorescence at pH 6.0 after MEND indicates that NCX1 has entered an internal compartment that does not immediately acidify.

Accordingly, protocols similar to those just described should allow for studies of membrane protein trafficking with respect to membrane domains. Overall, it should be possible in live cells to determine whether individual channels or transporters prefer to reside in ordered domains, and it should be possible to determine factors that influence their residence in ordered versus disordered membrane. As an initial example, we present in Fig. 8 functional results for Na/K pump activities in mouse myocytes, BHK cells, and HEK293 cells.

Fig. 8 A illustrates first that TX100 induces rapid MEND in mouse myocytes of similar magnitude to results for BHK and HEK293 cells. Using mouse and rat myocytes, the application of 200 µM TX100 for 10 s causes a >40% drop of C_m (>20 observations). Second, the record illustrates, in stark contrast to results for BHK and HE293 cells, that C_m does not readily recover in myocytes. No exception to this pattern was obtained in >20 observations, indicating that membrane trafficking occurs very differently in myocytes and the other cells used. Third, the record illustrates changes of Na/K pump activities in myocytes in response to TX100-induced MEND. Pump activity is activated by the application of 6 mM KCl in exchange for 6 mM NaCl in these experiments. Transport activity is reflected in the outward current activated upon applying potassium, and a negative capacitive signal reflects charge movements related to ion binding at the extracellular surface of Na/K pumps (Lu et al., 1995; Holmgren et al., 2000). With the solutions used, this ouabain-sensitive signal is

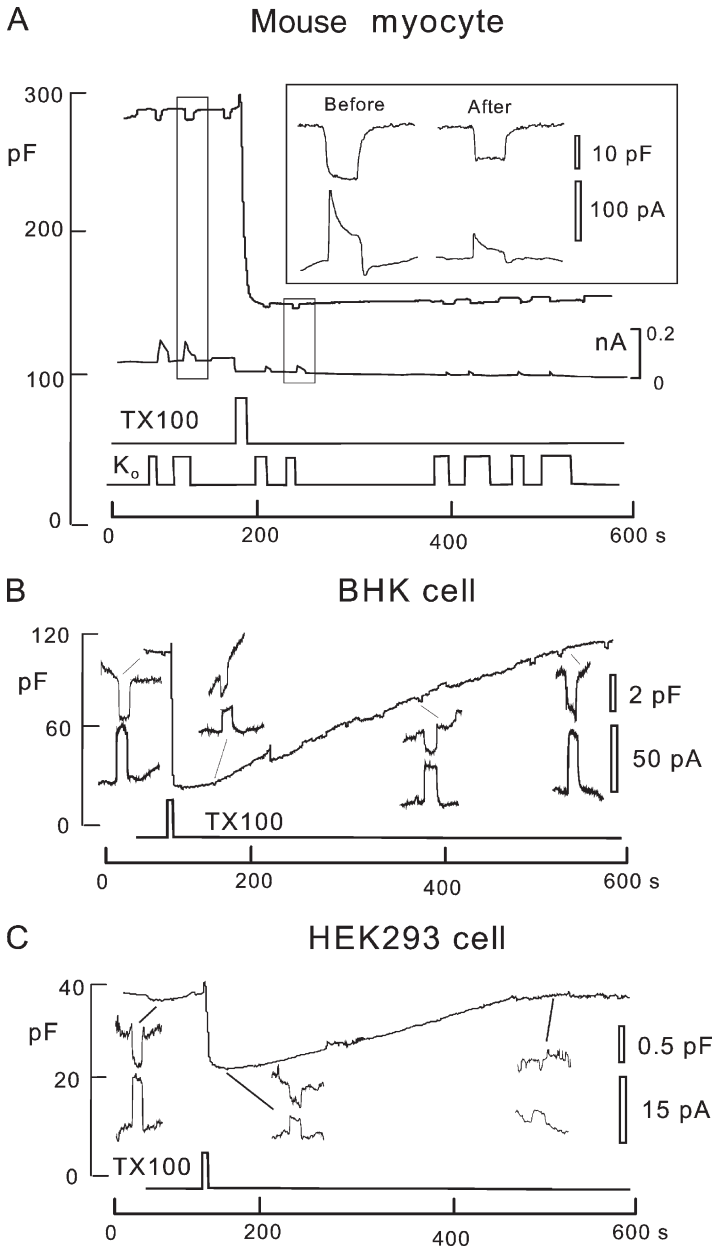

Figure 8. Comparison of TX100-induced MEND and its influence on Na/K pump activity in a mouse myocyte, a BHK cell, and an HEK293 cell. All results use modified cytoplasmic solution (Lariccia et al., 2011) with 8 mM ATP and 0.2 mM GTP, and MEND is induced with 200 µM TX100. Pump currents are activated by applying and removing 6 mM KCl in exchange for 6 mM NaCl. Pump activation is evaluated both as pump current and as a capacitive signal that reflects a decrease of extracellular cation binding during pump activity. (A) Mouse myocyte. TX100 induces a 42% MEND response (>10 observations), whereas pump signals are decreased by 62%. Neither membrane area nor pump activity recovers over a 7-min observation period. (B) BHK cell. MEND amounts to 70% of membrane area, pump activity decreases by the same percentage, and both membrane area and pump activity recover over 8 min (four observations). (C) HEK293 cell. Na/K pump activity decreases over-proportionally to the loss of membrane area associated with MEND, and pump activity does not recover during the recovery of membrane area over 8 min (10 observations).

primarily caused by lithium binding to E2 pump configurations. The signal decreases when pumps are activated by potassium, and the average configuration of pumps shifts toward one with binding sites open to the cytoplasmic side (i.e., the E1 configuration).

As indicated in Fig. 8 A, potassium was applied and removed eight times during the myocyte record, twice before and six times after the application of TX100. Current and capacitance records before and after TX100, demarcated by rectangles, are shown in the inset of Fig. 8 A. The peak pump current is decreased by 62%, whereas the capacitive pump signal is decreased by 55%. Thus, Na/K pumps are potentially internalized, with some preference in this protocol (five similar observations).

Fig. 8 (B and C) illustrates the equivalent experiments using HEK293 and BHK cells, whereby apparent differences were verified in >10 recordings from each cell type. As illustrated in Fig. 8 B, pump currents in BHK cells decrease roughly in proportion to C_m, and currents recover as C_m recovers after MEND (six similar observations). In the HEK293 cell, capacitance decreases by 45%, whereas pump currents and capacitive pump signals decrease by 74 and 71%, respectively. In a series of 11 experiments, pump currents in HEK293 cells decreased by 76 ± 3% when C_m was reduced by 49 ± 5%. Thus, Na/K pumps seem to be preferentially internalized during TX100-activated MEND in HEK293 cells, as well as in mouse myocytes. Each cell type shows characteristic patterns of C_m and Na/K pump activity changes during these protocols, presumably indicative of different membrane-trafficking patterns.

Characteristics of MEND induced by other detergents and amphipathic agents

It does not seem to be established in the literature if detergents besides nonionic detergents cause phase separations and domain growth in liposomes. Thus, it seemed important to explore whether other detergents, and other amphipathic compounds, induce MEND and with what characteristics. The examples, presented in Figs. 9–11 and in Figs. S5–S7, show that MEND can be activated by many different amphipathic compounds in two fundamentally different patterns.

Fig. 9 A shows the response of a BHK cell to 50 µM SDS (>50 observations), and it is illustrated in Fig. 10 C that dodecylglucoside (DDG) acts similarly. C_m was never observed to decrease during SDS application. Rather, the detergent causes a slow increase of C_m during its application, and C_m then falls precipitously within a few seconds after detergent is removed. Here, MEND amounts to 65% of C_m. As shown in Fig. 9 B, SDS is without effect when applied by pipette perfusion to the cytoplasmic side, in this example at a concentration five times greater than needed to induce MEND from outside (i.e., 150 vs. 50 µM). Because SDS is effectively membrane impermeable, whereas nonionic detergents can translocate across membranes in seconds to minutes (Heerklotz, 2008), these experiments establish firmly that SDS is promoting MEND from the extracellular side. Presumably, it blocks the final fission of event until it is removed. Clearly, cytoplasmic SDS does not block membrane fusion or subsequent MEND responses induced by 50 µM SDS from the extracellular side. Thus, detergent gradients across the bilayer play no evident role in MEND. In Fig. S8, it is documented that SDS not only blocks endocytosis during its application but also appears to promote recycling of membrane back into the cell surface, subsequent to a MEND.

Figs. S5 and S6 describe the MEND responses induced by DDM and hexyltriphenylphosphonium (C6TPP). C6TPP acts like SDS, whereas the DDM pattern is intermediate between that of nonionic detergents and SDS. We describe further in Fig. S9 that many detergents did not induce MEND when applied at concentrations just less than those causing disruption of experiments. These detergents included octylglucoside, octylsulfonate, deoxycholate, CHAPS, tauro-deoxycholate, glycodeoxycholate, β-escin, saponin, pluronic, and Lipofectamine 2000. Overall, it can be summarized that detergents of

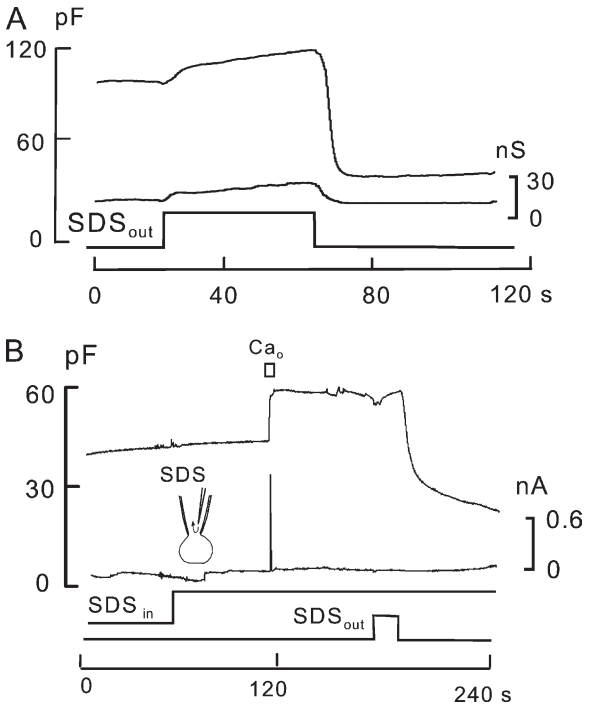

Figure 9. MEND responses induced by the application and removal of SDS. Cytosolic solutions contained 2 mM ATP and 0.2 mM GTP. (A) Within a few seconds, 50 µM SDS caused a small increase of C_m, and then C_m continued to rise nearly linearly. MEND occurred within seconds upon SDS removal. (B) Rapid pipette perfusion of 150 µM SDS into a BHK cell with 2 mM ATP was without effect on C_m. Thereafter, membrane fusion occurred normally upon the activation of Ca influx by NCX1. Finally, the application and removal of 50 µM of extracellular SDS caused a 50% MEND response over 20 s after SDS was removed.

low molecular weight, with alkyl chains <10 carbons long and/or with large head groups, usually disrupted experiments before inducing MEND. The commonalities of detergents that cause MEND upon their removal appear to be relatively small hydrophilic (or ionic) head groups and/or short alkyl side chains. At least, in the triphenylphosphonium series, a longer (C12) side chain versus a shorter (C6) side chain promotes immediate MEND. There is no evident correlation between MEND pattern and the relative abilities of detergents to cross the membrane.

Capacitive binding signals of detergents before and after MEND

As noted in the Introduction, TX100 segregates to fluid membrane domains in complex artificial membranes containing cholesterol, sphingomyelin, and phosphatidylcholine (Heerklotz et al., 2003). Assuming that MEND depends on growth of domains, followed by internalization of ordered membrane domains (Hilgemann and Fine, 2011), TX100 should segregate to domains that remain at the cell surface. How other detergents segregate is not known, except that different detergents can generate different detergent-resistant membranes with substantially different protein and lipid compositions (Lingwood and Simons, 2007). In our experiments, detergents that interact with both ordered and disordered membrane domains might dissociate faster from ordered domains than disordered domains. Upon washout, then, detergent remaining bound to disordered domains would transiently promote MEND, similar to TX100. Alternatively, such detergents might promote domain growth but hinder the final fission of ordered domains. Upon washout of detergent, expanded ordered domains would outlive the presence of detergent in the membrane, and fission would then proceed after detergents dissociate from both ordered and disordered domains.

To better understand how different detergents induce MEND, we have analyzed the capacitive signals that detergents generate upon binding to the cell surface. These signals, subsequently called capacitive binding signals, may reflect both an expansion and a thinning of the membrane as amphipaths intercalate into the outer cell monolayer. Fig. 10 presents results for four detergents: TX100, two detergents that cause MEND on wash-off, namely SDS and DDG, and deoxycholate, which does not cause MEND. In the experiment described in Fig. 10 A, TX100 was applied and removed many times at a concentration (70 µM) that did not induce MEND. The capacitive response to applying and removing TX100 amounts to ~5 pF. After inducing a 60% MEND response with a higher TX100 concentration (200 µM), the lower concentration of TX100 was applied and removed again multiple times. The magnitude of the capacitive signal

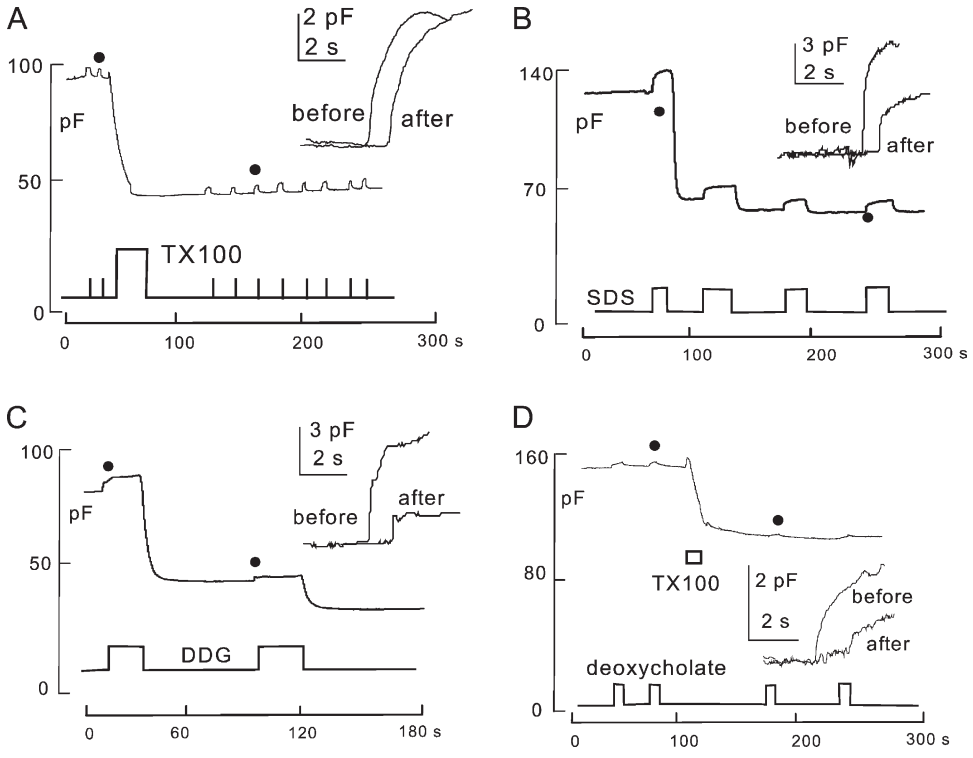

Figure 10. Capacitive binding signals of detergents before and after MEND. Standard cytoplasmic solutions with 2 mM ATP and 0.2 mM GTP in the cytoplasmic solution. In each panel, the rising phases of capacitive binding signals, indicated by filled circles in the complete C_m records, are shown in the inset at higher resolution. (A) TX100 capacitive binding signals are unchanged by MEND, causing a 60% loss of plasma membrane. As indicated below the Cm record, a low concentration of TX100 (70 µM) was applied and removed multiple times, with each application giving rise to an ~5-pF increase of C_m that reverses with a time constant of 2–3 s. MEND was induced by applying 200 µM TX100 for 25 s, and thereafter the capacitive binding signals for the low TX100 concentration were unchanged (five similar observations). (B) Capacitive binding signals for 30 µM SDS are markedly decreased by SDS-induced MEND. Because SDS does not cause MEND during its application, the capacitive binding signal can be evaluated with the same detergent application that induces MEND. The capacitive binding signals decrease by a fractional amount that is similar to the fractional loss of membrane. (C) Capacitive binding signals for 30 µM DDG are markedly decreased by DDG-induced MEND. (D) Capacitive binding signals for 500 µM deoxycholate are markedly decreased by TX100-induced MEND.

is unchanged (seven similar experiments and routine observations, as highlighted by Fig. 2 D). To illustrate the insensitivity of signals to MEND, the rising phases of capacitive signals before and after MEND are shown in the inset of Fig. 10 A. As shown in Fig. 10 B, the equivalent capacitive binding signals for SDS (30 µM) behave very differently. After a 60% MEND response, the initial binding signal upon applying SDS is decreased by a nearly equivalent amount (five similar observations). As shown in Fig. 10 C, the equivalent experiment for 60 µM DDG gives a very similar result. A 40% decrease of C_m during MEND is accompanied by a decrease of the DDG capacitive binding signal by >50% (three similar observations). As shown in Fig. 10 D, the equivalent experiment for 0.5 mM deoxycholate, which does not induce MEND, also shows a large decrease of the capacitive binding signal after TX100-induced MEND (six similar observations). Thus, on the basis of capacitive binding signals, SDS, DDG, and deoxycholate all bind effectively to the membrane that internalizes, whereas TX100 does not. In our companion paper (Hilgemann and Fine, 2011), we use this same approach to analyze the membrane binding of dyes and show that capacitive binding data agree well with optical measurements of dye binding before and after MEND.

Common pharmacologically active hydrophobic agents are good MEND inducers

During these studies, we used several amphipathic reagents commonly used in cell biological experiments at rather high concentrations. Several were found to be powerful inducers of MEND. Fig. 11 describes results for four such compounds, each result being representative of at least five observations. The nonspecific phospholipase C inhibitor, U73122 (5 µM) (Horowitz et al., 2005) was the most effective (Fig. 11 A), causing on average a 35% C_m decline in <2 min ($n = 6$), whereas the "control compound" without phospholipase inhibitory action, U73343, was without effect ($n = 5$). We note in this connection that the active compound probably accumulates in the outer monolayer because it is cysteine reactive, whereas the control compound is not. We stress further that U73122, like other amphipaths, had no MEND-promoting effect when used in cytoplasmic solutions, as in Fig. 5 B of our companion paper (Lariccia et al., 2011). Two antiproliferative amphipaths, edelfosine and tamoxifen, were effective (Fig. 11, B and C). Edelfosine, which is an alkyl-lyso-phospholipid (van Blitterswijk and Verheij, 2008), was more effective. The physiological amphipath, LPC (Fig. 11 D), was effective at 15–40 µM, concentrations that are within twofold of those that disrupted recordings. As illustrated for LPC, the MEND-promoting action of all of these amphipaths stopped rather abruptly upon wash-off of amphipath-containing solutions. In Fig. S7, we describe similar responses to U73122 in cardiac myocytes.

DISCUSSION

Lipidic forces can drive endocytosis in intact cells

In our companion paper (Lariccia et al., 2011), it is demonstrated that bacterial SMases can cause rapid endocytic responses of very large magnitude when applied to the extracellular side of cells. The responses are likely related to the generation of ceramide-rich domains that develop negative curvature and vesiculate spontaneously into the cytoplasm. Nonionic detergents now provide a second means to induce similar, or even larger, MEND responses, but without biochemical modification

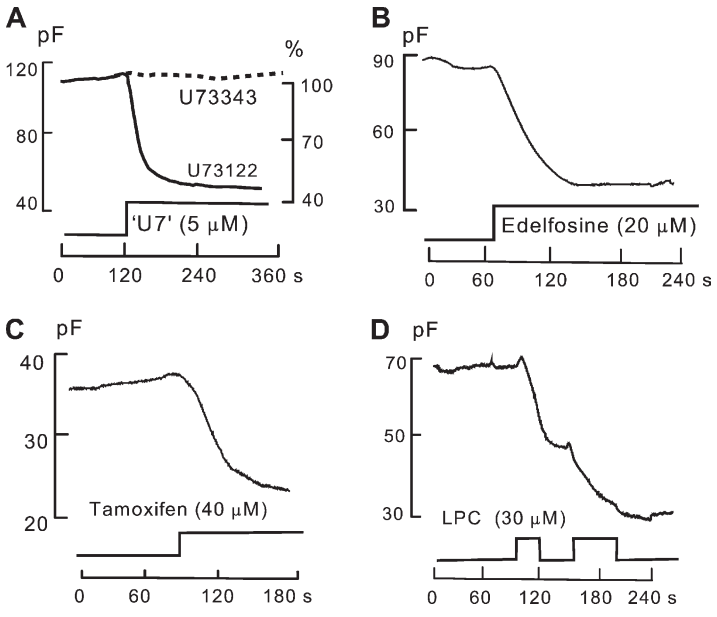

Figure 11. Activation of MEND by diverse amphipaths in BHK cells. Cytoplasmic solutions contain 2 mM ATP and 0.2 mM GTP. (A) 5 µM U73122, but not the inactive "control" analogue, U73343, induces MEND within 30 s. (B) 20 µM edelfosine induces MEND. (C) 40 µM tamoxifen induces MEND. (D) 20 µM LPC induces MEND. Similar to detergent-induced MEND, LPC-induced MEND stops abruptly upon the removal of LPC.

of the membrane. The growth of ordered membrane domains in response to low concentrations of TX100 was previously characterized in complex artificial membranes (Heerklotz et al., 2003), and the potential of domain growth, induced by detergents, to cause membrane vesiculation was also established in giant liposomes (Staneva et al., 2005; Hamada et al., 2007).

In spite of these precedents, our analysis of detergent actions in intact cells has generated multiple surprises. The magnitudes and speeds of detergent-induced vesiculation were unexpected, the clear directionality of detergent-induced vesiculation was unexpected, and it was unexpected that the vesicles formed would quickly couple to cellular processes that recycle them back into the cell surface. All of these results suggest that detergent-activated MEND can be related to endocytic processes occurring physiologically, and that amphipath-induced MEND can be extensively exploited to study physiological membrane-recycling mechanisms.

That the asymmetry of the plasmalemma plays a critical role in detergent-activated MEND is evident from the fact that detergents are inert from the cytoplasmic side of the plasmalemma (Figs. 2 E and 9 B). A primary role for the outer monolayer is also suggested by the rapidity with which MEND activates and deactivates upon applying and removing detergents (Fig. 2 D). The fact that membrane internalization occurs with no disruption of membrane integrity (Figs. 1, 3, and 4) fulfills an important prerequisite for detergent-activated MEND to be related to a physiological endocytic process. Perhaps most importantly, we find that capacitive signals related to TX100 and NP-40 binding are unaffected by the MEND responses caused by their presence in the membrane (Figs. 2 D and 10 A). This result establishes unambiguously that lipid domains are critically involved in detergent-induced MEND. Further, this result explains why vesicles formed during detergent-activated MEND can be recycled by physiological trafficking mechanisms. The vesicles formed are unlikely to contain large amounts of detergent and therefore may be similar to physiologically formed endocytic vesicles in these cells.

Given the fundamental roles that detergents have played in biological studies, it is surprising that large endocytic responses were not described previously. In fact, TX100 is presently used to reversibly open cells for delivery of impermeable reagents such as contrast agents (van de Ven et al., 2009). It is assumed in those studies that TX100 allows reagents free access to the cytoplasm. Given that the lowest possible detergent concentrations are used to allow reagent entry, it seems likely from the present study that MEND plays at least a significant role. Clearly, TX100 allows HRP to label vesicles and vacuoles without HRP reaching the cytoplasm (Fig. 5), and our electrical measurements indicate clearly that this happens without development of membrane leaks. We suspect that detergent-activated MEND went unnoticed up to now because nonionic detergents indeed cross membranes rapidly and exert diverse nonspecific intracellular effects, unless they are removed very quickly, as occurs in our experiments. We note that standard protease treatments, as used here to remove cells from dishes, are known to promote more efficacious transfections of cells by cationic detergents (Matheny et al., 2004). In contrast, TX100 induces MEND very effectively in cells that are not protease treated (Fig. S4), and standard transfection reagents do not induce MEND (Fig. S9).

Although the membrane itself clearly plays a central role in MEND, we cannot rule out roles for membrane proteins that may segregate with some specificity into membrane domains. With certainty, Ca transients play no immediate role in detergent-activated MEND because high cytoplasmic concentrations of EGTA (20 mM in Fig. 7 A) have no influence on MEND responses. Nor do ATP-, GTP-, actin-, or dynamin-dependent processes play a role (Fig. 4 B). From a biophysical viewpoint, the major question raised is how, in detail, formation (or growth) of membrane domains causes vectorial budding of membrane to the cytoplasmic side in cells. From a biological viewpoint, the major question raised is how, and if, these MEND responses are related to endocytic mechanisms taking place in cells. From a biochemical viewpoint, a major question raised is how amphipath-activated MEND is related to the generation of detergent-insoluble membrane fractions using 20–100 times higher concentrations of nonionic detergents.

The mechanism of amphipath-induced MEND

A tentative mechanistic model of amphipath-activated MEND is presented in Fig. 12. Previous studies show that TX100 at concentrations used in this study (100–200 µM) incorporate into phospholipid membranes at mole fractions in the range of 0.1 to as high as 0.5 (Kragh-Hansen et al., 1998). SDS at the concentrations used in Results (30–60 µM) can reach 0.2 mol per mole in cell membranes (Kragh-Hansen et al., 1998). Therefore, we speculated for some time that detergent incorporation might extract individual lipids from the outer monolayer without causing permeability changes. In this way, outer monolayer area would be decreased and generate negative curvature and fission to the cytoplasmic side. However, we have found no support for this idea in the literature. Furthermore, this hypothesis cannot explain why detergents are inert from the cytoplasmic side (i.e., fail to cause shedding), why detergent responses can be repeated many times in the same cell (Figs. 6 A and 7 A), or why some detergents are effective only after they are washed off (Figs. 9, 10 C, S5, and S8). Given these results, collectively, we conclude that detergents are acting to expand and reorganize the outer plasmalemma monolayer in a way that promotes its internalization.

In our companion paper (Hilgemann and Fine, 2011), we provide multiple lines of evidence that, with respect to the outer monolayer, it is primarily *Ld* membrane domains that expand and *Lo* membrane domains that are internalized.

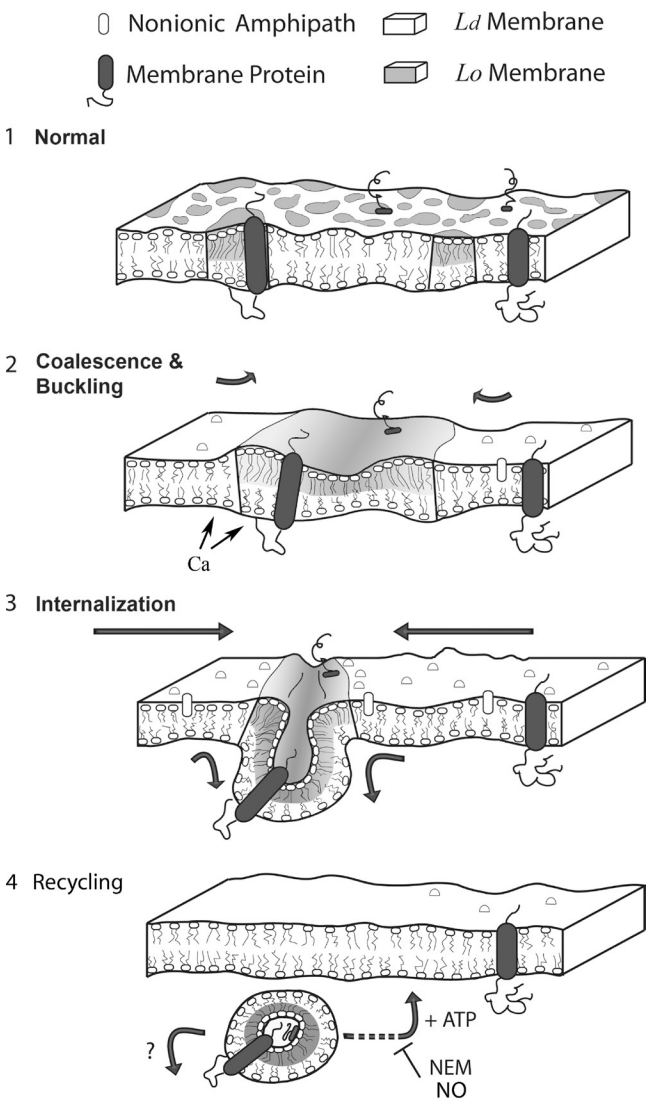

Figure 12. Endocytosis driven by lipidic forces: a hypothesis. (1) The outer monolayer consists of *Lo* and *Ld* domains of small size and equal prevalence. Lipids diffuse rapidly between domains, with affinity differences for *Lo* versus *Ld* domains being less than one log unit. "Lipid shells" around membrane proteins (Anderson and Jacobson, 2002) need not be synonymous with *Lo* and *Ld* domains. (2) Nonionic amphipaths expand *Ld* domains and promote cap formation with buckling of the plasmalemma, which is associated with domain coalescence and protein sorting. Ca transients may trigger the generation of endocytosis-promoting lipids and their movement into the outer monolayer (Hilgemann and Fine, 2011; Lariccia et al., 2011). (3) Membrane internalization. Coalescence of *Lo* domains within expanded *Ld* domains promotes negative curvature, vesiculation, and fission without adapters or dynamins, similar to the generation of ceramide domains via SMases (Lariccia et al., 2011). Nonionic amphipaths remain in *Ld* domains at the cell surface. (4) MEND generates vesicles that follow normal trafficking pathways to endosomes with recycling back to the plasmalemma via ATP-dependent processes that are inhibited by NEM and oxidative stress.

As discussed in more detail in our companion paper (Hilgemann and Fine, 2011), it is likely that *Lo* domains of intact cells are normally small, probably <5 nm on average (Lingwood and Simons, 2010). Accordingly, amphipaths must in some way cause ordered domains to coalesce before budding can proceed. As described for artificial membranes, one important factor is that detergents, certainly nonionic detergents, are cholesterol phobic (Heerklotz, 2008) and promote a segregation of cholesterol into *Lo* domains. Some detergents may mimic the ordering function of cholesterol, thereby generating unnatural ordered domains of large diameter, which would explain why β-cyclodextrins do not fully block detergent-activated MEND (see Figs. S1 and S2). One possible progression (see Fig. 12) is that the binding of detergents in more fluid membrane regions causes those regions to expand, coalesce, and form "caps" that in turn cause *Lo* domains to coalesce into valleys or troughs (Minami and Yamada, 2007). Presumably, the asymmetry of plasma membrane monolayers plays a key role in determining that vesiculation takes place to the cytoplasmic side. In the simplest case, ordered domains of the outer monolayer will develop negative curvature when they reach an appropriate size as a result of physical–chemical interactions that promote their collective tilt (Sarasij et al., 2007).

As noted in Results, it is not clearly established whether detergents besides nonionic ones cause phase transitions in complex liposomes at low concentrations. DDG and SDS are active at lower concentrations than TX100, and they cause MEND only after they are removed from the outside of cells (Figs. 9 and 10 C). In clear contrast to TX100, DDG and SDS binding is decreased after MEND (Fig. 10, B and C). Thus, they appear to interact with the membrane that internalizes in MEND. However, because MEND occurs only after their washout, the vesicles formed may be largely free of detergent, as expected for vesicles induced by TX100 exposure. As noted further in Results, it is unclear at this time whether membrane domains might coalesce during detergent application or in association with washout, or both. Independent of the details, these observations suggest that it may be possible to develop endocytosis inhibitors that act at the outside monolayer by inhibiting the final steps of fission.

To explain that MEND is activated by detergent in a few seconds, and that ongoing MEND stops in a few seconds (Fig. 2 D), it must be assumed that the domains generated by the presence of detergents have lifetimes of one to a few seconds. This is consistent with domain lifetimes of 13 s, determined for 0.2–2-μm *Lo* domains identified in arterial smooth muscle using 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine with single-molecule tracking (Schutz et al., 2000). From the perspective of the present work, such large domains would occur only

rarely in the surface membrane and represent only a small fraction of total "*Lo*" membrane. We point out further that this lifetime is nearly three log units longer than lifetimes predicted for nanometer-scale lipid domains (Subczynski and Kusumi, 2003).

Potential utility of amphipath-activated MEND for membrane-trafficking studies

The fact that detergent-activated MEND is reversible opens many new possibilities to study basic mechanisms of membrane recycling. Because the reversal of MEND requires ATP and is sensitively inhibited by NEM and the oxidative influence of nitric oxide donors (Fig. 6), it is likely that "NEM-sensitive factor" plays a key role (Lowenstein and Tsuda, 2006). It also appears likely that the vesicles that recycle can be reinserted by the Ca-dependent fusion mechanisms present in BHK cells (Fig. 6 E), and it will be of great interest to determine whether vesicles pass through endosomes in the time course of our experiments. A few possibilities to exploit detergent-activated MEND in membrane protein–trafficking studies, especially when combined with patch clamp, are hinted at by results obtained for Na/Ca exchange and Na/K pump function in relation to MEND (Figs. 7 and 8). Na/Ca exchangers are internalized roughly in parallel with the fraction of plasmalemma that internalizes. It is therewith a question of whether exchangers have no preference for residence in ordered or disordered membrane, or whether this apparent disregard for order can be altered by regulatory influences. In the case of Na/K pumps, there appears to be a preference for residence in and/or internalization within ordered membrane domains (Fig. 8). Although domains are rapidly recycled in the proliferative BHK and HEK293 cells, we see effectively no recycling of membrane internalized in detergent-activated MEND in myocytes. Interestingly, this observation contrasts to Ca-activated MEND in myocytes; membrane can be rapidly reinserted into the sarcolemma after Ca-activated MEND (Lariccia et al., 2011). All of these observations suggest that detergent-activated MEND provides new experimental handles on multiple membrane-trafficking processes in experiments using intact cells. Caveats encountered in the biochemical fractionation of membranes to analyze lipid raft compositions are well known (Lingwood and Simons, 2007). As a method to probe membrane organization, amphipath-activated MEND may have advantages because the concentrations of detergents used are less than critical micelle concentrations and because cells remain intact.

Finally, we have described here that multiple amphipathic compounds, often used at rather high concentrations in cell biological experiments, effectively induce MEND with similar characteristics to detergent-induced MEND (Fig. 11). Edelfosine is a proapoptotic lipid that has been used in cancer therapy (van der Luit et al., 2007), and we stress that nonionic detergents such as TX100 also initiate apoptosis (Sawai and Domae, 2009). The concentrations of these agents that effectively induce apoptosis (van der Luit et al., 2007; Sawai and Domae, 2009) are similar to those used here to cause MEND.

In conclusion, perturbation of the outer monolayer of cells by common detergents can cause very large and rapid endocytic responses that appear to be related to "lipid raft–dependent" endocytic mechanisms occurring physiologically. These responses do not require nucleotide hydrolysis, dynamins, other G proteins, or a functional membrane cytoskeleton. Only the outer monolayer responds to detergents by initiating MEND. Thus, it appears that outer monolayer phospholipids and cholesterol can develop lateral inhomogeneities (i.e., lipid domains) that preferentially and effectively vesiculate plasma membrane to the cytoplasmic side. Detergents induce MEND without being internalized, so that the composition of vesicles may be nearly physiological. In our companion paper (Hilgemann and Fine, 2011), multiple lines of evidence are presented showing that detergents are causing the internalization of ordered membrane domains and that Ca-activated MEND promotes internalization of the same membrane subset.

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Note added in proof. Because the LPC preparation used in Fig. 11 D contains multiple molecular species, we subsequently analyzed the characteristics of MEND induced by a series of synthetic LPCs from Avanti Polar Lipids, Inc. 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (C18:0) and 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine (C18:1) at concentrations of 1–4 µM induced large capacitive binding signals that reversed only very slowly over many minutes. 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (C16:0; C16-LPC) at concentrations of 3–6 µM induced MEND, with characteristics very similar to results in Fig. 11 D ($n = 6$), suggesting that C16-LPC is the major active species in the preparation used. 1-lauroyl-2-hydroxy-sn-glycero-3-phosphocholine (C12:0; C12-LPC) at concentrations of 20–60 µM induced MEND, with characteristics very similar to SDS in Fig. 9 ($n = 6$), with MEND occurring <3 s upon wash-out. 1-decanoyl-2-hydroxy-sn-glycero-3-phosphocholine (C10:0; C10-LPC) at concentrations up to 3 mM induced only capacitive binding signals that reversed within 2 s and that were decreased by >60% after inducing MEND with C16-LPC or TX100 ($n = 6$). Thus, C10-LPC appears to bind well to membrane that internalizes. These outcomes for LPCs are qualitatively similar to results for a series of alkyl triphenylphosphoniums presented in our companion paper (Hilgemann and Fine. 2011. *J. Gen. Physiol.* doi:10.1084/jgp.201010470). Within both series, the propensity of amphipaths to bind to membrane domains that internalize appears to increase as acyl chain length is decreased. At intermediate chain lengths, amphipaths appear to organize the membrane for MEND but hinder fission until they are removed. The optimal carbon chain length to induce MEND during amphipath application appears to vary from 12 to 16.

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