Membrane Lipid Organization Is Critical for Human Neutrophil Polarization*

Lynda M. Pierini‡, Robert J. Eddy‡, Michele Fuortes§, Stéphanie Seveau‡, Carlo Casulo‡,¶ and Frederick R. Maxfield‡,¶

From the Departments of ¶Biochemistry and §Cell Biology, Weill Medical College of Cornell University, New York, New York 10021

Received for publication, December 5, 2002
Published, JBC Papers in Press, January 8, 2003, DOI 10.1074/jbc.M212386200

In response to chemoattractants neutrophils extend an actin-rich pseudopod, which imparts morphological polarity and is required for migration. Even when stimulated by an isotropic bath of chemoattractant, neutrophils exhibit persistent polarization and continued lamellipod formation at the front, suggesting that the cells establish an internal polarity. In this report, we show that perturbing lipid organization by depleting plasma membrane cholesterol levels reversibly inhibits cell polarization and migration. Among other receptor-mediated responses, β2 integrin up-regulation was unaffected, and initial calcium mobilization was only partially reduced by cholesterol depletion, indicating that this treatment did not abrogate initial receptor-mediated signal transduction. Interestingly, cholesterol depletion did not prevent initial activation of the GTPase Rac or an initial burst of actin polymerization, but rather it inhibited prolonged activation of Rac and sustained actin polymerization. Collectively, these findings support a model in which the plasma membrane is organized into domains that aid in amplifying the chemoattractant gradient and maintaining cell polarization.

Over the past decade, compelling evidence has emerged that challenges the notion of the plasma membrane lipid bilayer as a homogeneous passive entity that merely provides a scaffold for protein-mediated signaling (1, 2). This evidence suggests that certain lipids preferentially associate and form lateral heterogeneities in the membrane (3–6). One type of membrane domain has been described as rafts, glycolipid-enriched membrane domains (GEMs), or detergent-resistant membrane domains (DRMs) because they are enriched in glycosphingolipids, sphingomyelin, and cholesterol, which make them resistant to solubilization by cold non-ionic detergents (7). These lipid domains have been postulated to serve as centers for some signal transduction processes by virtue of their copurification with signaling molecules following cell lysis with cold nondenaturing detergents and flotation on sucrose density gradients (3). Because the direct visualization of lipid domains has been elusive, it is thought that they typically exist as dynamic submicron-sized regions within the plasma membrane (6, 8–10). However, most of the surface area of cells such as fibroblasts and neutrophils is resistant to extraction by cold Triton X-100 (11–13), suggesting that lipid organization may be more complex than depicted in a simple version of the raft model. In fact, several studies now indicate that multiple types of membrane microdomains co-exist within the plasma membranes of cells (14–16) and may coalesce into dense assemblies to form larger domain structures (1, 2, 11, 15).

Cholesterol is the most abundant lipid component of the plasma membrane, and it plays an important role in lipid organization. Extraction of cholesterol from the plasma membrane using the synthetic cholesterol chelator methyl-β-cyclo-dextrin (MβCD) causes reorganization of lipids with preferences for both ordered and disordered domains (13). While the effects of cholesterol depletion on ordered, raft-like domains have been emphasized, additional effects on the overall organization of lipids also occur (13). Cholesterol depletion has been used to examine the role of lipid domains in a multitude of cellular processes, including cell migration (15, 17) and phagocytosis (18–20). Disruption of lipid domain organization by MβCD treatment resulted in inhibition of motility of breast cancer-derived cells (17) and T cells (15), and abrogation of the phagocytosis of Escherichia coli by mast cells (18) and macrophages by macrophages (20) and neutrophils (19). Because cell motility and phagocytosis are mechanistically related, with both processes involving directional extension of actin-rich membranes, it seems likely that their inhibition following domain disruption may have the same underlying molecular basis.

Migration of neutrophils and other immune cells is contingent upon their ability to adopt a polarized morphology in response to chemotactic stimulation. In the presence of chemoattractants, neutrophils rapidly transform from roughly spherical resting cells to migratory ones with distinctive leading and trailing edges. Actin polymerization occurs most exclusively at the leading edge, resulting in a dramatic accumulation of F-actin at just one end of the cells (21). Notably, this remarkable asymmetry occurs even when the external chemoattractant signal is uniform, suggesting that at least one signaling step leads to an internal polarization of the cell. Given that chemoattractant receptors are typically distributed uniformly across the cell surface even after polarization (22), this internal signal occurs somewhere between receptor occupancy and actin

* This work was supported by National Institutes of Health Grants DK 27083 (to F. R. M.), GM34770 (to F. R. M.), and GM19078 (to L. M. P.) and The Arthritis and Musculoskeletal Research Award from Pfizer/Parker Davis (to L. M. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by the Minority Access to Research Careers Program.

‡ To whom correspondence should be addressed: 1300 York Ave., New York, NY 10021. Tel.: 212-746-6405; Fax: 212-746-8875; E-mail: FRmaxfie@med.cornell.edu.

The abbreviations used are: MβCD, methyl-β-cyclo-dextrin; fMLF, formyl Met-Leu-Phe; MES, 4-morpholineethanesulfonic acid; PIP2, phosphatidylinositol bisphosphate; PMN, polymorphonuclear neutrophil; mAb, monoclonal antibody; TRITC, tetramethylrhodamine isothiocyanate.

This paper is available on line at http://www.jbc.org
polymerization. The exact point at which polarization is induced is not known. However, the possibilities have been narrowed by an elegant study in neutrophil-differentiated HL-60 cells using the recruitment of a GFP-tagged pleckstrin homology domain to the plasma membrane as a readout for polarization (22). This study showed that amplification of polarization signals depends on one or more of the Rho GTPases, which anchor to the plasma membrane via lipid tails, and is downstream of the lipid products of phosphatidylinositol 3-kinase (22). In other words, amplification of polarization occurs at or near the plasma membrane. This, together with our finding that the plasma membrane of neutrophils segregates into distinct lipid domains that comprise either pole of migrating neutrophils (11), led us to hypothesize that signal amplification is dependent on plasma membrane domains. To test this, we used MjCD to disrupt membrane domain organization in human neutrophils and studied its effects on polarization and signaling.

MATERIALS AND METHODS

Neutrophil Preparation and Stimulation—Polymorphonuclear neutrophils (PMNs) were isolated from whole blood donated by healthy volunteers by centrifugation through Polymorphprep (Invitrogen). After lysis of contaminating erythrocytes by 30 s hypotonic treatment on at least three different days. Cells were then stimulated in the continued presence of ML-7 for 15 min at 37 °C.

Cholesterol Depletion and Repletion—Plasma membrane cholesterol was depleted by incubating PMNs (1.2 × 10^7/ml) in incubation buffer containing 10 μM MjCD (Sigma) for 15 min at 37 °C. At the end of this 15-min treatment, cells were diluted to 2.4 × 10^6/ml with incubation buffer (depleted). The extent of cholesterol depletion was determined by an enzymatic method using a commercially available kit (Free cholesterol C; Wako Biochemicals, Osaka). The cholesterol content of MjCD-treated cells was measured to be 20.9 ± 3.6% (mean ± s.d., n = 5) less than that of control cells. To replete membrane cholesterol, cholesterol-depleted cells were incubated with 5 mM Chol-MjCD for 2.5 min at 37 °C (repleted cells).

Immunofluorescence—After stimulation with FMLF, cells were fixed with 3.3% paraformaldehyde in the presence of 0.25 mg/ml saponin for 5 min at room temperature. When it was important to preserve the labile pools of F-actin, excess fluorescently conjugated phalloidin (Molecular Probes, Eugene, OR) was included during fixation and throughout the remaining labeling steps. Rac was visualized with a mouse monoclonal antibody against Rac1 and Rac2 (clone 23A8, Upstate Biotechnology, Placid, NY), which was directly conjugated to AlexaFluor 488 goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR). Anti-CD44-labeled cells were removed and diluted into ice-cold incubation buffer to stop exocytosis. Cells were then stimulated in the continued presence of ML-7 for 4 min using a Leica DMRB (Leica Mikroskopie und Systeme GmbH, Germany) set up for differential interference contrast microscopy. Time-lapse images were acquired with a cooled CCD camera driven by Metamorph Imaging System software. Migrating PMNs were defined as those that show tail and leading lamella moved at least 5 μm from their initial starting position within 240 s. Separate dishes were used for each treatment, and at least three dishes were monitored for each treatment on at least three different days. To monitor changes in intracellular free calcium levels ([Ca 2+]), PMNs were loaded with the ratiometric fluorescent indicator, fura-2 as described (24). Briefly, motility of plated and stimulated PMNs was monitored for 4 min using a Leica DMRB (Leica Mikroskopie und Systeme GmbH, Germany) set up for differential interference contrast microscopy. Image analysis software was acquired with a Zeiss LSM510 laser scanning confocal microscope (Jena, Germany) or a Leica DMRB widefield microscope, and then analyzed with MetaMorph image analysis software (Universal Imaging Corporation, Downingtown, PA).

In the absence of stimulation, the average fluorescence intensity per cell was measured for over 150 cells per condition from three experiments on different days. The average fluorescence intensity per cell for cholesterol-depleted cells was 46 ± 8% (S.D.) of the value for control cells.

Functional Assays—Motility assays were performed as described (23). Briefly, motility of plated and stimulated PMNs was monitored for 4 min using a Leica DMRB (Leica Mikroskopie und Systeme GmbH, Germany) set up for differential interference contrast microscopy. Time-lapse images were acquired with a cooled CCD camera driven by Metamorph Imaging System software. Migrating PMNs were defined as those that show tail and leading lamella moved at least 5 μm from their initial starting position within 240 s. Separate dishes were used for each treatment, and at least three dishes were monitored for each treatment on at least three different days.

To verify the ability of PMNs to upregulate their integrins in response to FMLF, PMNs were incubated at 37 °C with Fab fragments of an anti-β5 integrin antibody (IB4) that was directly conjugated to AlexaFluor 488 in the presence or absence of FMLF, as indicated. At various time points after the start of the incubation period, aliquots of cells were removed and diluted into ice-cold incubation buffer to stop exocytosis. Cells were then stimulated by an AB2, monitored using an LSC and analyzed by fluorescence-activated cell sorter (FACS) XL analytical flow cytometer. To account for any nonspecific binding of the IB4 Fab, fluorescence intensity of PMNs incubated with an irrelevant control AlexaFluor 488-conjugated Fab was set to an arbitrary value, and all other samples were measured relative to this value. Each data point represents measurements from 2,000 cells, and the data is a composite of at least three experiments performed in triplicate.

Inhibition of Actin-Myosin Contraction—During plating, neutrophils were incubated with 10 μM myosin light chain kinase inhibitor, ML-7 (26). Cells were then stimulated in the continued presence of ML-7 for the indicated times.
Asterisks interfere contrast (DIC, Triton X-100 to reveal membrane domain organization. Differential a, CD44, and then stimulated for 0 (jugated monoclonal antibody against the membrane domain marker, d) PMNs were labeled with a fluorescently con- d cholesterol-depleted (f), which localizes to raft-like domains in PMNs (11). Fig. 1 (a–c′) shows the progression of domain organization as control cells polarize in response to fMLF. As we reported previously, rafts are evenly distributed around the periphery of unstimulated cells (Fig. 1a′), form larger patches after 15–30 s of stimulation (Fig. 1b′), and finally coalesce into a cap toward the cell rear (Fig. 1c′) (11).

After the plasma membranes of cells have been cholesterol-depleted with MβCD (Fig. 1, d–f), the raft component CD44 is less well retained following detergent extraction; the average fluorescence intensity per cholesterol-depleted cell was decreased by 40.8% ± 3.2% (mean ± S.D.) compared with control cells. (The intensities of panels d′–f′ have been enhanced relative to panels a′–c′ to allow visualization of the CD44 distribution.) In contrast to control cells, fMLF stimulation does not induce capping of CD44 in cholesterol-depleted cells; rather, the CD44 that is retained remains relatively uniformly distributed around the cell periphery (Fig. 1, d′–f′). That is, cholesterol depletion inhibits the redistribution of detergent-resistant raft components into a cap at the cell rear, indicating that MβCD treatment inhibits large scale lipid organization in addition to disrupting microdomains.

Acute depletion of cholesterol by treatment with MβCD has dramatic effects on neutrophil polarization and migration (Fig. 2). When cellular cholesterol is depleted by just −21% (see “Materials and Methods”), PMN migration is inhibited by >90% (Fig. 2a), consistent with the report by Manes et al. (17) that depletion of membrane cholesterol inhibits migration of MCF-7 cells. However, in contrast to the interpretation reported in Manes et al., we find that cholesterol depletion abolishes lamellipod formation (Fig. 2b, compare center to left panel). Inhibition of migration and polarization can be attributed to effects of cholesterol modulation as opposed to nonspecific effects of MβCD treatment since the ability to polarize and migrate is restored to previously cholesterol-depleted cells upon replenishing membrane cholesterol with cholesterol-cholesterol-depleted MβCD (cholesterol, Fig. 2, a and b, repleted).

Because it has been reported, based on observation of cells by light microscopy, that cholesterol depletion does not affect membrane extension and ruffling (17, 20), we further investigated whether lamellipod extension in PMNs was affected by changes in cholesterol levels. Scanning electron microscopy reveals that cholesterol-depleted cells are unable to form membrane extensions or ruffles in response to fMLF (compare Fig. 3a, b to a). The lack of membrane ruffling in cholesterol-depleted cells is accompanied by an inhibition of stimulated actin polymerization. Following cholesterol depletion and stimulation with fMLF, cells were simultaneously fixed, permeabilized, and stained for F-actin with fluorescently conjugated phallolidin. Projections of confocal images show that control cells exhibited dramatic F-actin-rich ruffles within their lamellae (Fig. 3c), whereas cholesterol-depleted cells had no F-actin-rich projections (Fig. 3d). Using flow cytometry, we quantified the fluorescent phallolidin binding to F-actin in parallel samples of
suspended cholesterol-depleted and control cells, which had been stimulated with fMLF, or left unstimulated, for 5 min at 37 °C. Typically in response to fMLF PMNs increased their F-actin content by 2.5-3-fold (Fig. 3e, control). In contrast, fMLF stimulation of cholesterol-depleted cells led to only a 1.5-fold increase in F-actin content over unstimulated cholesterol-depleted cells (Fig. 3e, depleted). In these experiments, the final F-actin content of fMLF-stimulated cholesterol-depleted cells was 49% of the value for fMLF-stimulated control cells. We obtained similar results for adherent cells in which we measured the F-actin content of fMLF-stimulated cholesterol-depleted cells to be 46% that of stimulated control cells (data not shown). Aliquots of the flow cytometry samples of unstimulated PMNs were plated on a slide, and then imaged by confocal microscopy to examine the effects of MβCD treatment on F-actin structure. Fig. 3, f and g show the F-actin structure in unstimulated control and cholesterol-depleted cells, respectively. MβCD treatment causes a slight disruption in the cortical F-actin structure in unstimulated PMNs, with only an insignificant effect on the total F-actin content of unstimulated cells (Fig. 3e, compare gray bars).

Accumulation of F-actin at the leading edge of human neutrophils and subsequent lamellipodia formation and membrane ruffling, is at least partially due to activation of the small GTPases Rac1 and Rac2 (henceforth, collectively referred to as Rac, albeit Rac2 is the predominant isoform (>96%) in neutrophils) (36–40). In light of reports implicating an important role for Rac in stimulated actin polymerization in neutrophils (40, 41), and because the Rho GTPases associate with membranes via C-terminal lipid modifications, we tested whether Rac targeting was affected by changes in cholesterol levels. F-actin within newly formed lamellae is structurally and spatially distinct from cortical F-actin within the cell body (42) and is observed as short spikes or fingers when labeled with fluorophore-conjugated phalloidin (Fig. 4c’). It has been shown that establishment and maintenance of this asymmetrical distribution of F-actin spikes involves the Arp2/3 complex, and it was proposed that recruitment of Arp2/3 might be achieved through a Rho family member (43). Indeed, actin nucleation in neutrophils in response to fMLF has been shown to occur via both Cdc42- and Rac-dependent pathways (40), but spatial control of these signaling molecules was not shown. Using indirect im-

![Fig. 2. Disrupting lipid organization by depleting plasma membrane cholesterol reversibly inhibits cell polarization and migration.](http://www.jbc.org/)

![Fig. 3. Depletion of plasma membrane cholesterol inhibits chemoattractant-stimulated lamellipod extension and actin polymerization in PMNs.](http://www.jbc.org/)
munofluorescence we visualized endogenous Rac in unstimulated and fMLF-stimulated PMNs. In unstimulated PMNs (Fig. 4a), Rac (Fig. 4a") is found throughout the cytoplasm and bounded within the cortical F-actin ring (Fig. 4a'). This observation is consistent with biochemical analyses of membrane and cytosol fractions from human neutrophils that have shown that Rac in unstimulated cells is nearly entirely cytosolic, with no detectable Rac associated with membrane fractions (44). Within 15–30 s of stimulation, PMNs form multiple lamellipodia (Fig. 4b), which protrude in all directions and are rich in F-actin spikes (Fig. 4b'). Strikingly, each of the F-actin-containing membrane extensions stains brightly for Rac (Fig. 4b"'). The large increase in fluorescence intensity for the Rac staining in stimulated compared with resting cells is likely because activated membrane-bound Rac is better retained than cytosolic Rac after cell permeabilization. After 60 s of stimulation, when PMNs have become fully polarized (Fig. 4c), endogenous Rac is asymmetrically distributed to a band at the leading edge.

**Fig. 4.** Depletion of plasma membrane cholesterol inhibits the retention of Rac at the membrane of stimulated PMNs. Control (a–c) or cholesterol-depleted (d–f) PMNs were stimulated for 0 (a, d), 30 (b, e), or 60 (c, f) s with fMLF and then fixed and labeled with AlexaFluor 488-phalloidin (a'–f') and an anti-Rac mAb followed by an AlexaFluor 546-conjugated secondary antibody (a"–f"'). Fluorescence images shown in panels a'–f' and a"–f" are single confocal slices through the center of the cells. Corresponding differential interference contrast (DIC) images are shown (a–f). In the color overlays, F-actin is shown in green, and Rac is shown in red. Asterisks in panel c indicate cell uropods. Bars, 10 μm. g, quantification of digital wide-field fluorescence images shows that the fluorescence intensity due to Rac labeling increases dramatically by 30 s after fMLF stimulation in both control and depleted cells. By 120 s after stimulation, when labeling for Rac is still intense in control cells, Rac intensity is markedly diminished in depleted cells. The average fluorescence intensity per cell was quantified for four fields of cells (>100 cells) per condition. Bars represent S.E. The differences in intensity values for control and depleted cells are significantly different for the 120 s time point (p < 0.0001).
the surface expression of fMLF-stimulated secretion, we used flow cytometry to monitor this possibility, as well as to test the effects of MfMLF-stimulated secretion in these cells (Fig. 4, e). Localization of Rac in cholesterol-depleted cells suggests an explanation for the lack of membrane ruffling and actin polymerization in these cells (Fig. 4, d–f). As for control cells (Fig. 4, a–c), cholesterol-depleted cells undergo a burst of actin polymerization and Rac recruitment to the membrane within 15–30 s of stimulation (compare Fig. 4, d′ and e′ to d″ and e″). However, unlike control cells, distinct lamellipodia are not observed in these cells, and the F-actin appears circumferentially distributed (Fig. 4e″). Also, there is little correlation between Rac localization (Fig. 4e″) and F-actin (Fig. 4e′ and overlay), where there are brighter regions of F-actin staining in depleted cells, there are not necessarily bright regions of Rac staining, whereas there is a one-to-one correlation in control cells. After 60 s of stimulation (Fig. 4f), F-actin in cholesterol-depleted cells is still localized around the cell perimeter (Fig. 4f′) and is much less intense than that found in corresponding control cells (Fig. 4e″). Interestingly, in contrast to control cells, the Rac staining (Fig. 4f″) has diminished in intensity compared with cells that have been stimulated for just 15–30 s (Fig. 4e″), suggesting that Rac in these cells was only transiently activated.

Quantifying wide-field fluorescence images of cells labeled for Rac provides a means to assess the effect of cholesterol depletion on stimulated Rac recruitment to the membrane (Fig. 4g). Consistent with the images shown in Fig. 4a(a–c), stimulation of control cells with fMLF causes a marked and sustained increase in the intensity of Rac staining compared with unstimulated cells. The intensity of Rac labeling in control cells remained elevated until at least 120 s after stimulation. In contrast, fMLF stimulation of cholesterol-depleted cells caused only a transient increase in Rac labeling intensity, with Rac intensity diminishing to close to the starting level by 120 s after stimulation.

The dramatic inhibition of fMLF-stimulated actin polymerization in cholesterol-depleted PMNs could result from a general inhibition of receptor-mediated signaling. To investigate this, and to further investigate the observed effect of MβCD on cell morphology, we examined the effect of MβCD treatment on fMLF-stimulated β2 integrin up-regulation. β2 integrins are the most abundant integrins in PMNs, and their up-regulation at the surface and activation are required for cell spreading and migration (46). Consequently, another explanation for the absence of shape change of cholesterol-depleted PMNs could be inhibition of β2 integrin transport to the surface. To exclude this possibility, as well as to test the effects of MβCD on fMLF-stimulated secretion, we used flow cytometry to monitor the surface expression of β2 integrins in control and cholesterol-depleted cells over time. In these experiments, fMLF-stimulated integrin up-regulation was detected with fluorescently conjugated Fab fragments of a monoclonal antibody against β2 integrins. Fab fragments were used as opposed to whole antibody because cross-linking of β2 integrins can induce secretion in the absence of fMLF stimulation. We verified that the fluorescently conjugated anti-β2 Fab fragments did not induce secretion on their own (Fig. 5a, open symbols). Despite the profound effects on spreading and migration, cholesterol depletion had no effect on the extent or kinetics of fMLF-stimulated β2 integrin up-regulation (Fig. 5a, filled symbols), indicating that cholesterol-depleted cells are able to carry out regulated secretion in response to fMLF. To visually verify the flow cytometry data, aliquots of cells were washed, fixed, and imaged by fluorescence microscopy. Fig. 5b shows images of PMNs before and after 15 min of stimulation with fMLF. As demonstrated by flow cytometry (Fig. 5a), control and cholesterol-depleted PMNs show a comparable increase in expression of β2 integrins follow-
an fMLF stimulation. Activation of integrins is also apparently unaffected by cholesterol depletion as there was no obvious difference in adhesion between cholesterol-depleted and control cells (not shown). These results show that several consequences of stimulation by fMLF are not affected significantly by the cholesterol depletion protocol we used.

To further examine whether MβCD inhibits responses other than actin polymerization and cell polarization, we tested the effects of MβCD treatment on an early fMLF-mediated signaling event, elevation of intracellular free calcium levels ([Ca\(^{2+}\)]\(_i\)). Fig. 6 shows representative experiments in which we monitored fMLF-induced changes in [Ca\(^{2+}\)]\(_i\), over time for both control and cholesterol-depleted cells. For these studies, PMNs were loaded with the ratiometric indicator, fura-2, treated with MβCD (or not), and then monitored by spectrofluorometry. As shown in Fig. 6a, MβCD-treated cells are able to mobilize [Ca\(^{2+}\)]\(_i\), in response to 10 nM fMLF, but the response is diminished compared with control cells. The initial rise of cholesterol-depleted cells was 78.7 ± 7.8% (S.E., n = 9) of control cells. Interestingly, although the peak calcium response in cholesterol-depleted cells was only partially reduced compared with control cells, the sustained response was almost completely inhibited. These findings are generally similar to those reported by Barabe et al. (47) but they are not identical. In that study, cholesterol depletion with MβCD was likewise shown to inhibit the sustained calcium response induced by fMLF in PMNs; however, in contrast to our findings, it had no effect on the peak response. In those studies, PMNs were stimulated with 100 nM fMLF as opposed to 10 nM fMLF, as used in Fig. 6a. To determine if this difference in dose could account for the difference in our findings, we performed the same experiment using 100 nM rather than 10 nM fMLF. Fig. 6b shows the peak response of cholesterol-depleted cells is not diminished when the cells are stimulated with this higher dose of fMLF, even though the sustained phase of the response is inhibited as observed for stimulation with 10 nM fMLF.

The sustained phase of calcium elevation following fMLF stimulation includes contributions from both capacitative calcium entry across the plasma membrane and repeated transient releases from internal stores (49). To determine if the effect of cholesterol depletion on the sustained calcium response was due to an effect on calcium entry across the plasma membrane or an effect on the release from internal stores, cholesterol-depleted and control PMNs were stimulated in the absence of extracellular calcium (Fig. 6, a and b, dotted lines). Both the peak and sustained responses of control cells are diminished when extracellular calcium is removed (compare dotted to solid black lines). Similarly, the peak response of cholesterol-depleted cells in the absence of extracellular calcium was less than that of cholesterol-depleted cells in calcium-containing medium (compare dotted to solid gray lines), and less than control cells in calcium-free medium (compare dotted gray to dotted black line, Fig. 6a). Our results with 100 nM fMLF are similar to those reported by Barabe et al. (47) However, it should be noted that in all of our experiments, the differences between the calcium responses of control and cholesterol-depleted cells were always greater when the cells were stimulated with 10 nM fMLF than when cells were stimulated with 100 nM fMLF, and we found that the peak response of cholesterol-depleted cells was almost always smaller than that been stimulated with 10 or 100 nM fMLF for 60 s in the presence of extracellular calcium. Also shown in panel d is an image of PMNs that were pretreated with 100 nM thapsigargin for 3 min and then stimulated with 10 nM fMLF in the continued presence of thapsigargin, but in the absence of extracellular calcium.

**Fig. 6.** Cholesterol depletion diminishes the fMLF-stimulated calcium response of PMNs. The change in the intracellular calcium levels of control (black lines) or cholesterol-depleted (gray lines) PMNs was measured over time. Cells were stimulated with 10 nM fMLF (a), 100 nM fMLF (b), or 100 nM thapsigargin (c), at the indicated time points (arrows), in the presence (solid lines) or absence (dotted lines) of extracellular calcium. For experiments without extracellular calcium, PMNs were resuspended in calcium-free buffer containing 2 mM EGTA. The ratio values of the fluorescence intensities at 340 nm and 380 nm excitation (340/380 Ratio) are expressed in arbitrary units (a.u.) on the left axes. These 340/380 ratio values correspond to the intracellular calcium concentrations shown on the right axes. The intracellular calcium concentrations were calculated as described in Grynkiewicz et al. (25). The results in panels a–c are from a single experiment. For each condition, the same set of experiments was performed on at least three different days with cells from different donors. Panel d shows transmitted light images of control and cholesterol-depleted PMNs that have
of control cells when the cells were stimulated with 10 nM fMLF in the presence of EGTA.

One possible explanation for the diminution of the peak calcium response of cholesterol-depleted cells compared with control cells under both calcium-free and replete conditions is that cholesterol depletion affects the endoplasmic reticulum (ER) membrane as well as the plasma membrane, and thus affects internal calcium stores. To test this possibility, cholesterol-depleted and control PMNs were treated with 100 nM thapsigargin, which depletes ER calcium stores by preventing calcium reuptake. For the first several minutes, the increase in intracellular calcium induced by thapsigargin is identical in control and cholesterol-depleted cells, but then the responses diverge, with the calcium level in control cells continuing to rise, while that in depleted cells plateaus (compare solid black to gray traces in Fig. 6c). The deviation in responses likely results from differences in calcium entry across the plasma membrane, and, in fact, the responses of cholesterol-depleted and control cells to thapsigargin are indistinguishable when the cells are monitored in calcium-free medium (Fig. 6, dotted lines). These results are consistent with the findings by Barabe et al. (47) that Mn\(^{2+}\) influx is blocked by M\(\beta\)CD treatment, and they support the idea that M\(\beta\)CD treatment reduces capacitative calcium entry across the plasma membrane.

Since cholesterol depletion altered some calcium responses of PMNs, we explored whether the lack of shape changes in cholesterol-depleted cells is a consequence of weakened calcium responses in these cells. First, we tested the ability of cholesterol-depleted cells to undergo shape changes in response to stimulation with 100 nM fMLF. Fig. 6d shows that 100 nM fMLF does not induce shape changes in cholesterol-depleted PMNs, even though the magnitudes of the initial calcium response to this dose of fMLF are comparable in control and M\(\beta\)CD-treated cells (see Fig. 6b).

As a second test, we considered whether inhibition of calcium entry and depletion of intracellular calcium stores would produce effects similar to cholesterol depletion. We had observed previously that PMNs polarize and extend lamellae in the absence of external calcium (47). Pretreatment of PMNs with 100 nM thapsigargin for 3 min was used to deplete intracellular calcium stores (47), and extracellular EGTA was used to prevent influx of calcium across the plasma membrane. Fig. 6d shows that thapsigargin-treated cells in the absence of extracellular calcium readily spread and undergo shape changes within 60 s of stimulation with 10 nM fMLF. At later times after stimulation, these cells extended lamellae and became polarized in response to fMLF, although the process was somewhat slower than in control cells (not shown). This result, which is consistent with previous observations of [Ca\(^{2+}\)]\(_i\)-buffered PMNs (49), suggests that the effects of cholesterol depletion on lamellar extension are not due to depletion of intracellular calcium stores or to inhibition of calcium influx.

The results obtained from our cholesterol depletion studies suggest that membrane domain organization is important for cell polarization. To further test this hypothesis, we wanted a second distinct method of disrupting domain organization. We have shown previously that when myosin was inhibited by treatment with ML-7, CD44-containing membrane domains did not redistribute upon stimulation of PMNs (11). That is, CD44-containing domains did not form caps, but rather remained distributed in patches around the cells and intermingled with CD44-negative domains. Using this independent method to disrupt large-scale domain organization, we looked at the effects on F-actin and Rac localization (Fig. 7). In striking contrast to the effect of depleting plasma membrane cholesterol, inhibiting actin-myosin contraction by treatment of PMNs with ML-7 leads to the circumferential extension of a single large lamella following fMLF stimulation (26) (Fig. 7, a and b). Unlike fMLF-stimulated control cells (Fig. 4c), F-actin in stimulated ML-7-treated cells (Fig. 7, b’ and c’) is distributed evenly around the cells, without a vectorial bias. Fig. 7, b’ and c’ show that Rac, similar to the F-actin, is distributed around the circumference of the cells, demonstrating that Rac activation is not inhibited by ML-7 treatment, but its polarized localization is disrupted.

**DISCUSSION**

When neutrophils and other leukocytes polarize, the lipids at the front and rear of the cells take on different properties (11). This can be most clearly seen by the difference in sensitivity to solubilization by cold Triton X-100, with the rear of the cells being largely resistant to extraction (11). Although it has been recognized for some time that the plasma membrane can form localized submicron scale regions with different compositions and properties, the co-existence of many types of membrane domains and their ability to coalesce into larger structures is just beginning to be appreciated (1, 2, 15). Even detergent-resistant ordered domains (i.e. rafts) have been found to be heterogeneous in their composition and properties, including detergent sensitivity (14–16, 50, 51). For migratory cells, both the anterior (lamellipod) and posterior (uropod) of cells have been reported to be composed of subtypes of rafts that apparently coalesce to form micron-sized domains (see Fig. 8 and Refs 11, 15, 17, 27, 52). It is likely that there are numerous types of submicron scale domains within these larger regions at the front and rear of polarized neutrophils, but the exact nature (size, composition, dynamics, etc.) of these domains is unknown (2). Evidence to date supports a model in which cell uropods are composed of CD44-rich microdomains that are tightly anchored to the underlying cortical actin cytoskeleton, while lamellae are composed of different types of domains, which mediate dynamic actin reorganization, are on average
Membrane Domains and Neutrophil Polarization

We have used MβCD depletion of cholesterol to alter the lipid properties in neutrophils responding to fMLF. We have shown that depleting plasma membrane cholesterol prevents chemotactrant-stimulated actin polymerization, cell polarization, and formation of large-scale domains (Figs. 1–3). Restoring cellular cholesterol resulted in a significant restoration of these responses. While MβCD treatment is often used with the intention of disrupting cholesterol-rich rafts, it actually has profound effects on the distribution and order of lipid probes that partition into both ordered and disordered domains (13). For this reason, it is important to interpret results with MβCD in the context of a more complex model of membrane organization. For example, considering that raft-like domains are small but occupy a large fraction of the surface (11–13), it follows that a large fraction of the cell surface is in or near raft boundaries (2). This view of membrane organization has implications for interpreting the effects of cholesterol depletion; cholesterol depletion reduces the raft boundary area and/or changes the properties of the boundaries, as seen in model membrane studies and in studies of cells labeled with fluorescent lipid analogues (13, 56). If boundary regions are important for the recruitment/retention of certain signaling molecules to non-raft regions of the plasma membrane, then cholesterol depletion would affect these non-raft membrane proteins as well as raft membrane proteins.

MβCD treatment has been used to investigate the role of lipid organization in signaling through G-protein-coupled receptors, including the bradykinin receptor (54) and the high-affinity receptor for IgE, FceRI (28). We found that delivery of β3 integrins to the cell surface following stimulation of the G-protein-coupled fMLF receptors was not inhibited significantly by cholesterol depletion. We also found that cholesterol depletion had only a partial effect on the initial rise in \([Ca^{2+}]_c\), induced by 10 nM fMLF stimulation, and it had no significant effect on the initial \([Ca^{2+}]_c\) response when 100 nM fMLF was used. The sustained calcium response induced by either dose of fMLF was strongly inhibited by cholesterol depletion. Our findings using 100 nM fMLF are in complete agreement with those reported by Barabe et al. (47). Barabe et al. (47) showed that abrogation of the sustained response can be attributed to inhibition of capacitative calcium entry, while release of calcium from intracellular stores is largely unaffected by cholesterol depletion. We confirmed these findings (Fig. 6, a–c) and showed that the effects of MβCD on cell polarization cannot be attributed to the altered calcium response in these cells (Fig. 6d). Instead, we suggest that cholesterol-depleted neutrophils are unable to polarize because Rac is not retained at the membrane.

Rac was transiently recruited to the plasma membrane in MβCD treated cells, and there was a brief period of stimulated actin polymerization (Fig. 4, d–f). However, in the cholesterol-depleted cells, actin polymerization did not persist and Rac did not remain membrane associated. In agreement with this finding, it has been reported that cholesterol depletion inhibits phorbol ester-stimulated membrane recruitment of Rac, but not Rac activation, in A431 cells (57). It remains to be resolved if interruption of Rac retention caused inhibition of actin polymerization or vice versa. In support of the former scenario, the Rho GTPase-dependent asymmetric recruitment of the pleckstrin homology domain of AKT (protein kinase B) to the plasma membrane of neutrophil-like HL60 cells can occur in the absence of actin polymerization (22). In either case, the effects of cholesterol depletion on stimulated actin polymerization and Rac retention provide a mechanistic explanation for effects on both phagocytosis (18–20) and migration (this report and Ref. 17).

There is little information on how the organization of outer leaflet membrane components into microdomains might cause compartmentalization of lipids and proteins associated with the inner leaflet of the plasma membrane. However, the functional coupling of inner and outer leaflet membrane domain components has been well documented for the immune receptors, FceRI and the T cell receptor (58–61). Indirect evidence that microdomains extend over both leaflets of the plasma membrane consists of co-patching experiments in whole cells. Aggregation of outer leaflet-anchored (e.g. GPI-anchored proteins) or transmembrane (e.g. CD44, FceRI) raft components causes co-aggregation of inner membrane-associated signaling molecules (e.g. Lyn, Lck, Fyn, annexin II), and vice versa in the case of CD44 and annexin II (10, 28, 50, 51, 62, 63). Cholesterol depletion inhibits the coupling between transmembrane or GPI-anchored receptors and membrane domain components at the cytoplasmic face of the plasma membrane, apparently because it disrupts both inner and outer leaflet membrane domain integrity; neither inner leaflet (e.g. Lyn, Lck) nor transmembrane (e.g. CD44) domain components partition into the low density fraction of sucrose gradients after cholesterol depletion, and inner and outer leaflet domain components no longer co-cluster on intact cells (11, 28, 50, 51).

Our data show that during polarization and migration Rac is recruited stably to membrane regions that are excluded from CD44-containing raft-like domains. Stable incorporation of Rac to more disordered lipid domains is consistent with model membrane and whole cell studies, which showed that peptides with various types of isoprenyl-based lipid anchors preferentially partition into liquid-disordered domains as compared with co-existing liquid-ordered domains (64–66). MβCD treatment disrupts both raft and non-raft lipids (presumably on both sides of the membrane), and it has profound effects on the extent of boundary regions in the membrane (13). This change in lipid order appears to alter the ability of Rac to be incorporated stably into the membrane.

We also show here that Rac is recruited stably to the membrane in the ML-7-treated cells (Fig. 7, b″ and c″). In these cells Rac is found all around the cell, and, as noted previously, a circumferential lamellipod is formed (26). One interpretation of this is that coalescence of CD44-rich rafts at the rear of the cell
acts to exclude disordered domains (and thus minimize boundaries), and in this way stable recruitment of Rac and lamellipod formation is prevented. In the ML-7 treated cells, raft-like regions that contain CD44 are kept dispersed and interspersed with detergent-soluble regions all around the cell (11). It is interesting to compare the phenotypes of ML-7-treated and cholesterol-depleted cells: neither can polarize, but for very different reasons. In the cholesterol-depleted cells, stable Rac recruitment and actin assembly do not occur, while in ML-7-treated cells the cell rear is kept unstrained. In the plasma membranes of PMNs, actin-myosin contraction, polarized recruitment of Rac to the cell rear is prevented. In the ML-7 treated cells, raft-like domains to the cell rear is prevented by inhibition of actin-myosin contraction. Polarized recruitment of Rac to the membrane and polarized actin polymerization are also prevented. In our model, the plasma membranes of PMNs contain several types of microdomains, some of which are anchored to the actin cytoskeleton via transmembrane proteins such as CD44 (1, 11, 56). CD44-containing membrane domains would redistribute to the cell rear during polarization and act to exclude certain signaling molecules (e.g. Rac) or recruit others (1, 11). At the same time lipid structures, possibly PI(4,5)P2-enriched microdomains (55) and/or boundary regions, that are favorable for Rac recruitment are established at the front of the cell, and these mediate signals leading to actin polymerization.

Acknowledgments—We thank Dr. David Holowka for many helpful discussions, and we thank Drs. William Muller, David Holowka, Melanie Brazil, and Robert J. Vasquez for critical reading of the manuscript. We also thank Leon Cohen-Gould for technical expertise and assistance with scanning electron microscopy. Flow cytometry, scanning electron microscopy, and confocal microscopy were performed within core facilities provided by Weill Medical College of Cornell University.

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