Cholesterol-modulating Agents Selectively Inhibit Calcium Influx Induced by Chemoattractants in Human Neutrophils*

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The effects of cholesterol-perturbing agents on the mobilization of calcium induced upon the stimulation of human neutrophils by chemoattractive factors were tested. Methyl-β-cyclodextrin and filipin did not alter the initial peak of calcium mobilization but shortened the duration of the calcium spike that followed the addition of fMet-Leu-Phe. These agents also inhibited the influx of Mn2+ induced by fMet-Leu-Phe or thapsigargin. Methyl-β-cyclodextrin and filipin completely abrogated the mobilization of calcium induced by 10−7 M platelet-activating factor, which at this concentration depends to a major extent on an influx of calcium as well as the influx of calcium induced by 10−7 M platelet-activating factor. On the other hand, methyl-β-cyclodextrin and filipin enhanced the mobilization of calcium induced by ligandation of FcγRIIA, an agonist that did not induce a detectable influx of calcium. Finally, methyl-β-cyclodextrin and filipin enhanced the stimulation of the profile of tyrosine phosphorylation, the activity of phospholipase D (PLD), and the production of superoxide anions induced by fMet-Leu-Phe. These results suggest that the calcium channels utilized by chemoattractive factors in human neutrophils are either located in cholesterol-rich regions of the plasma membrane, or that the mechanisms that lead to their opening depend on the integrity of these microdomains.

The migration of human polymorphonuclear leukocytes (neutrophils) to sites of infection or tissue injury is an essential element of their role in the first line of immune defense. It is orchestrated by the local accumulation of chemoattractive factors that may be of endogenous or exogenous origin. Examples of the former include complement-derived factors (e.g. C5a), lipid-derived mediators such as leukotriene B4 and platelet-activating factor (PAF),1 and tissue-derived chemokines (interleukin-8 (IL-8) in particular). Small formylated peptides of which fMet-Leu-Phe is the archetypical member represent examples of bacterially derived products known to attract neutrophils (1).

Most neutrophil chemoattractive factors including those named above interact with plasma membrane receptors that belong to the superfamily of G protein-coupled receptors (2–4). Their occupancy results in the G protein-dependent activation of several signaling pathways (PLCβ, adenylate cyclase, tyrosine phosphorylation) that lead to the generation of multiple second messengers including transient increases in the level of cytoplasmic free calcium (5, 6). These are known to be involved in the initiation and modulation of several critical neutrophil responses including phagocytosis (7–9) and the activation of the NADPH oxidase (10–12).

The increases in the level of free cytoplasmic calcium induced by chemoattractive factors result from the intracellular release of calcium mediated by PLCβ-derived inositol 3,4,5-trisphosphate coupled in a poorly understood manner to an increased influx of calcium from the extracellular medium (13, 14). Although the identity of the calcium channels responsible for the influx of calcium in nonexcitable cells is still under investigation (15, 16), recent data has implicated one or more homologs of Drosophila Trp (transient receptor potential) in this process (16–19), some of which may partition into lipid raft domains (20). A role for cyclic ADP-ribose in the regulation of the influx of calcium has also been postulated (21).

The present study was initiated to investigate the potential involvement of cholesterol-rich membrane microdomains in the modulation of the mobilization of calcium induced by chemoattractive factors in human neutrophils. The results to be described provide evidence that agents that interfere with plasma membrane cholesterol selectively inhibit the chemoattractant-stimulated influx of calcium without altering the release of calcium from intracellular stores. These results suggest that the calcium channels activated by these agonists are either located in cholesterol-rich membrane microdomains or that the mechanisms that couple intracellular store depletion to their activation are dependent on the integrity of the microdomains.

MATERIALS AND METHODS

Reagents and Antibodies—Methyl-β-cyclodextrin (MβCD), Filipin III, thapsigargin, and FMLP were purchased from Sigma. Pura-2/AM and Fluo-3/AM were obtained from Molecular Probes. PAF was kindly provided by Dr. Pierre Borgeat (Laval University, Québec, Canada).

The anti-FcγRIIA monoclonal antibody IV.3 was purified from the ascites of mice inoculated with hybridoma HB 217 obtained from the American Type Culture Collection. The anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology. The goat F(ab′)2 fragments directed against mouse F(ab′)2 used for cross-linking were purchased from Jackson ImmunoResearch Laboratories. F(ab′)2 fragments of antibody IV.3 were prepared essentially as described in the Pierce catalog.

Neutrophil Purification—Venous blood was collected in isocitrate

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androantagonist from healthy adult volunteers, and neutrophils were steriley purified as previously described (22). Neutrophils at 1 \times 10^7 cells/ml were resuspended in Hanks’ balanced salt solution (HBSS) containing 1.6 mM CaCl_2 but no magnesium.

**Measurement of Cytoplasmic Free Calcium Concentration**—Neutrophils (1 \times 10^7 Cells/ml) were incubated at 37°C for 30 min with 10 mM MgCD, 0.5 \mu g/ml filipin or their solvent (Me_2SO and ethanol, respectively), and with 1 \mu M Fura-2/AM or 1 \mu M Fluo-3/AM. The cells were washed and resuspended at 5 \times 10^6 cells/ml in HBSS with 1.6 mM CaCl_2. Neutrophils were then kept at 37°C and transferred to the thermostatted cuvette compartment of a spectrofluorimeter (SLM 8000C). Neutrophils were stimulated with the indicated agonists at the times indicated by the arrows in the individual figures. In the case of stimulation of FcγRIIA, the cell suspensions were incubated for 2 min with antibody IV.3 (2.5 \mu g/ml of F(ab’)_2 fragments), and FcγRIIA was then cross-linked upon the addition of 25 \mu g/ml of goat F(ab’)_2 fragments against mouse F(ab’)_2. The fluorescence of the cells was monitored at an excitation wavelength of 340 nm and an emission wavelength of 510 nm for the Fura-2 probe. In the experiments where Fluo-3 was used, the wavelengths used for excitation and emission were 506 and 526 nm, respectively. The internal calcium concentrations were calculated as described by Grynkiewicz et al. (23).

**Manganese Influx Measurement**—Neutrophils were preincubated as described above with MgCD, filipin, or their diluents and with 1 \mu M Fura-2/AM. The cells were washed and resuspended in HBSS without calcium and magnesium. The wavelengths used for these experiments were 360 and 505 nm for excitation and emission, respectively. After 10 s, 200 \mu M MnCl_2 was added to the cell suspensions that were then stimulated with the indicated agonists at 75 s. Adenosine deaminase (0.1 units/ml) was added 5 min before MnCl_2 in those experiments in which the effects of thapsigargin were monitored. Manganese influx is reported in arbitrary fluorescence units.

**Tyrosine Phosphorylation**—Neutrophils, at 1 \times 10^7 cells/ml, were incubated with MgCD, filipin, or their solvents as described above. The cells were washed and stimulated with FMLP (10^-7 M) at 37°C for the indicated times. The reactions were stopped by transferring 100 \mu l of the cell suspensions to an equal volume of boiling 2× Laemmli sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 5% 2-mercaptoethanol, 8.5% glycerol, 2.5 mM orthovandanate, 10 mM paraarachidonophosphate, 10 \mu g/ml leupeptin, 10 \mu g/ml aprotinin, 0.025% bromphenol blue) and boiled for 7 min. The samples were then subjected to 7.5–20% SDS-PAGE gradients and transferred to immobil polyvinylidene fluoride membranes (Millipore). Immunoblotting was performed using the 4G10 antiphosphotyrosine antibody (final dilution 1:4000) and revealed by the ECL detection system as previously described (24).

**Phospholipase D Measurements**—Neutrophils were prelabelled with 1-O-[3H]alkyl-2-lyso-phosphatidylcholine (2 \mu Ci/10^7 cells) for 90 min as described previously (25–27). For the last 30 min, 10 mM MgCD were added to the cells. Neutrophils were then washed and resuspended at 8 \times 10^6 cells/ml. Ethanol (final concentration, 1.0% v/v) was added immediately preceding the addition of FMLP (10^-7 mol/liter, 10 min). The incubations were stopped by adding 1.8 ml of cold chloroform/methanol/HCl (50:100:1, v/v/v) and unlabeled phosphatidylethanolamine (PEt) as a standard. The lipids were extracted, dried under nitrogen, and spotted on prewashed silica gel 60 thin-layer chromatographic (TLC) plates. PEt was separated from the other lipids with the solvent mixture chloroform/methanol/acetic acid (85:15:2, v/v/v). Lipids were visualized by Coomassie Brilliant Blue staining, and the different lipid classes were scraped off the plates. Radioactivity in PEt was monitored by liquid scintillation counting, and the results were corrected for background radioactivity and quenching.

**Superoxide Measurements**—Superoxide production was measured using the reduction of cytochrome c as previously described (28). Briefly, the cells were resuspended at 10^6 cells/ml with 130 mM cytochrome c and stimulated for 5 min at 37°C with fMet-Leu-Phe. The tubes were then transferred to an ice bucket for 15 min and centrifuged for 10 min at 1,500 \times g. The optical density of the supernatants was read at 540 and 550 nm, and the amounts of superoxide produced were calculated from the differences between the optical readings at 550 nm and those at 540 nm using an extinction coefficient of 21.1.

**RESULTS**

**Effects of Methyl-β-cyclodextrin on the Mobilization of Calcium Induced by fMet-Leu-Phe**—As previously reported (6) the addition of 10^{-7} M fMet-Leu-Phe to a suspension of human neutrophils led to increases in the concentration of cytoplasmic free calcium as monitored using the fluorescent calcium probe Fura-2 (Fig. 1). This increase was rapid and transient with levels of cytosolic free calcium returning to near basal values by 2–3 min poststimulation. Pretreating the cells with 10 mM MgCD, a cholesterol-binding agent, had little effect on the peak of calcium mobilization induced by fMet-Leu-Phe. In contrast, it significantly reduced the duration of the calcium spike affecting mostly the maintenance of the elevated calcium levels (Fig. 1A). The concentrations of MgCD used were similar to those that had previously been characterized in other cell types as effective in reducing plasma membrane cholesterol levels with minimal overall cell damage (29–32). MgCD did not affect cell viability under the experimental conditions used as evidenced by trypan blue exclusion (data not shown), the maintenance of normal levels of resting cytoplasmic calcium, and the ability to respond to fMet-Leu-Phe (initial phase of calcium mobilization, superoxide production, activation of PLD, tyrosine phosphorylation (see Fig. 7)). A second agent affecting cholesterol-rich membranes, the cholesterol-sequestering antibiotic (33, 34) filipin, was tested to corroborate the results obtained with MgCD using Fluo-3 as a probe to circumvent optical interferences between Fura-2 and filipin. The results shown in Fig. 1B indicate that filipin had no effect on the early peak of cytoplasmic free calcium induced by fMet-Leu-Phe, but it reduced the duration of the maintenance of the elevation of cytoplasmic free calcium much as MgCD did (Fig. 1A).

The modifications to the shape of the calcium spike brought about by pretreatment with MgCD and filipin suggested that these compounds were acting principally on the influx of calcium that follows the depletion of intracellular calcium stores induced upon activation of chemotactic factor receptors. Two sets of experiments were carried out to test this hypothesis as directly as possible.

First, calcium influx was monitored by following the quenching of intracellular Fura-2 that results from the entry of Mn^{2+}.
through the calcium channels (35, 36). The results of these studies are illustrated in Fig. 2. As expected, the addition of fMet-Leu-Phe to a suspension of neutrophils loaded with Fura-2 increased within about 30 s, the rate of quenching of the calcium probe. This effect is a reflection of the increased plasma membrane permeability to divalent cations (increased calcium influx). Of particular relevance to the present study is the practically complete inhibition of the fMet-Leu-Phe-induced increased rate of quenching of Fura-2 that is observed following pretreatment with MβCD (panel A) and filipin (panel B), effects that are indicative of an inhibition of the opening of the calcium channels.

Second, the mobilization of calcium induced by fMet-Leu-Phe was monitored immediately following chelation of extracellular calcium by EGTA. Under those conditions of low extracellular calcium most if not all of the increases in the levels of cytosolic free calcium can be assumed to be derived from the emptying of the intracellular stores of calcium. As shown in Fig. 3 the addition of 2 mM EGTA to the cells prior to stimulation with fMet-Leu-Phe significantly reduced the maintenance of the elevated calcium levels without significantly affecting the initial peak of mobilization of calcium. These modifications in the pattern of cytoplasmic free calcium mobilization induced by the addition of EGTA are similar to those induced by MβCD. It is also important to point out that adding EGTA to MβCD-treated cells was without any significant additional effect on the mobilization of calcium induced by fMet-Leu-Phe (compare the MβCD tracing to that of MβCD + EGTA).

Effects of Methyl-β-cyclodextrin on the Mobilization of Calcium Induced by PAF—Having observed that the influx of calcium induced by fMet-Leu-Phe was inhibited in MβCD-treated cells, we next examined the effects of this agent on neutrophil agonists that either were very dependent on an influx of calcium or, alternatively, did not rely to a significant extent on this response.

Previous studies provide evidence that the mobilization of calcium induced by low concentrations of the lipid mediator PAF differs from that stimulated by fMet-Leu-Phe in that it was nearly completely dependent on an influx of calcium from the extracellular medium (37, 38). Neutrophils were therefore pretreated with MβCD, loaded with Fura-2, and treated with 10−10 M PAF. As shown in Fig. 4A, PAF at this concentration was still able to significantly increase the level of cytoplasmic free calcium. MβCD completely inhibited the mobilization of calcium induced by 10−10 M PAF. The data in panel B show that the influx of calcium induced by 10−10 M PAF was completely inhibited by MβCD.

At higher concentrations the mobilization of calcium induced by PAF closely resembles that of other chemoattractants, i.e. an immediate spike due to a release of intracellular calcium followed by a wave of calcium derived from the extracellular medium. MβCD treatment inhibited the second phase of calcium mobilization induced by 10−5 M PAF (Fig. 4C) much as it had that induced by fMet-Leu-Phe (Fig. 1). The influx of calcium induced by 10−3 M PAF was inhibited by MβCD (panel D) as was observed at low concentrations of PAF (panel B).

Effects of Methyl-β-cyclodextrin on the Mobilization of Calcium Induced by Ligation of FcγRIIA—The mobilization of calcium induced by the FcγRIIA in contrast to that induced by chemotactic factors (Fig. 1) is not coupled to an influx of calcium and relies to a much larger extent on the recruitment of calcium from intracellular stores. Cross-linking of FcγRIIA induced a rapid and transient mobilization of calcium (Fig. 5, panel A) that was not inhibited by MβCD. The extent of the mobilization of calcium induced by ligation of FcγRIIA was relatively unaffected by the addition of EGTA (data not shown), and no evidence for increased Mn2+-dependent quenching of Fura-2 was obtained (Fig. 6B) indicating that most if not all of the calcium was derived from intracellular stores. It should be noted that these experiments did not address the question of the nature of the calcium stores called upon by these receptors and their relationship to those mobilized by chemotactic factors.

Effects of Cholesterol-modulating Agents on the Influx of Calcium Induced by Thapsigargin—One of the strongest pieces of evidence for the presence of store-operated calcium (SOC) channels in cells is provided by the ability of the Ca2+-ATPase inhibitor thapsigargin to stimulate an influx of Mn2+ subsequent to the depletion of intracellular calcium stores (39, 40).
As shown in Fig. 6 this effect is readily detectable in neutrophils as evidenced by the increased rate of Mn$^{2+}$-dependent quenching of Fura-2 following the addition of thapsigargin. Pretreatment with MβCD or filipin nearly abolished the stimulation of the influx of Mn$^{2+}$ induced by thapsigargin.

Effects of Methyl-β-cyclodextrin on the Tyrosine Phosphorylation, Phospholipase D Activation, and Oxidative Responses to fMet-Leu-Phe—The effects of MβCD on other neutrophil responses to fMet-Leu-Phe were tested next. As shown in Fig. 7A, MβCD enhanced the stimulation of the pattern of tyrosine phosphorylation elicited by the chemotactic factor at all time points tested, and this effect was also observed with the cholesterol-sequestering agent filipin (data not shown). The enhancing effects of MβCD and filipin on the levels of tyrosine phosphorylation were not reproduced by inhibiting the influx of calcium with EGTA (data not shown) indicating that the former did not result from the inhibition of the entry of calcium per se. Increases in the pattern of tyrosine phosphorylation were also observed in MβCD-treated cells stimulated with 10$^{-7}$ M PAF (data not shown). MβCD had a similar stimulatory effect on the enhancement of the activity of phospholipase D (Fig. 7B) and the activation of the oxidative burst (Fig. 7C) induced by fMet-Leu-Phe.

FIG. 4. Effect of MβCD on the mobilization of calcium and the influx of Mn$^{2+}$ induced by PAF. The incubation conditions with MβCD were as in Fig. 1. The cells were stimulated with 10$^{-10}$ M (panels A and B) or 10$^{-7}$ M (panels C and D) PAF. The results are from separate experiments, each carried out in duplicate on cells from three or more donors.

FIG. 5. Effect of MβCD on the mobilization of calcium and the influx of Mn$^{2+}$ induced by ligation of FcγRIIA. The incubation conditions with MβCD were as described in the legend to Fig. 1. Details of the FcγRIIA ligation procedure are described under "Materials and Methods." The results in the two panels are from separate experiments, each carried out in duplicate on cells from three or more donors.

FIG. 6. Effect of MβCD and filipin on the influx of Mn$^{2+}$ induced by thapsigargin. The incubation conditions with MβCD and filipin were as in Fig. 1. Thapsigargin was added at the time indicated by the dotted arrow. The results are from a single experiment, representative of duplicate determinations on cells from three or more donors.

**DISCUSSION**

The results of the present investigation provide evidence for a role for cholesterol-rich membrane microdomains in the link between the emptying of intracellular calcium stores and the opening of the calcium channels as stimulated by G protein-coupled receptors in human neutrophils.

The cholesterol-modifying depleting agents MβCD and filipin inhibited the stimulation of the influx of calcium induced by fMet-Leu-Phe and PAF. The receptors of these chemottractants belong to the superfamily of G protein-coupled receptors (4). They are thought to share many of the steps leading to the
mobilization of calcium including reliance on the same intracellular stores of calcium and, although this had not been directly established, the same mechanisms of coupling to the plasma membrane calcium channels or SOCs (capacitative calcium entry). The common sensitivity of the calcium influx stimulated by these agonists to inhibition by MβCD and filipin is consistent with this view.

Several lines of evidence indicate that the effects of MβCD and filipin are not due to a general deleterious effect on cell viability. First, no differences in trypan blue exclusion were noted between treated and untreated cells under the conditions reported here (data not shown). Second, neither agent signifi-

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**Fig. 7.** Effect of MβCD on various responses of human neutrophils to fMet-Leu-Phe. The ability of fMet-Leu-Phe to stimulate the tyrosine phosphorylation (panel A) activity of PLD (panel B) and the production of superoxide anions (panel C) in cells preincubated, or not, with MβCD was monitored. The procedures are detailed under “Materials and Methods.” Asterisk, statistically different from the values of the fMet-Leu-Phe samples (p < 0.05, Wilcoxon matched pairs test).

Cconsistently altered the resting levels of cytoplasmic calcium, a sensitive indicator of cell integrity. Third, the initial mobilization of calcium induced by fMet-Leu-Phe was not affected by these agents indicating that the steps leading to the Ins(3,4,5)P₃-mediated release of calcium, including the integrity of the calcium stores, were intact. Fourth, the effects of MβCD and of filipin were limited to interference with the mobilization of calcium as several neutrophil responses (tyrosine phosphorylation, O₂⁻ generation, activation of PLD) were not inhibited but in fact were enhanced. Finally, the mobilization of calcium induced by the cross-linking of FcyRIIA was also enhanced. Taken together, these data provide strong evidence for a selective and nontoxic effect of cholesterol-modulating agents on the responses of human neutrophils to the activation of chemotactic factor receptors under the experimental conditions tested here.

The effects of MβCD and filipin resided primarily at the level of the activation of the SOCs. Evidence for this conclusion comes most specifically from the following: (i) the lack of effect on the magnitude of the initial spike of calcium mobilization, an indicator of calcium release from intracellular stores, (ii) the lack of effect of MβCD on the mobilization of calcium observed in the absence of extracellular calcium, and (iii) the inhibition of the uptake of Mn²⁺, a classic index of inward-directed calcium permeability, and in particular following the addition of the Ca²⁺-ATPase inhibitor thapsigargin. It is consistent with the sensitivity of the mobilization of calcium induced by low concentrations of PAF to MβCD, concentrations at which the lipid mediator relies to a major extent on increases in plasma membrane permeability to calcium and only marginally on release from intracellular stores (37, 38). Finally, the relative resistance of the calcium mobilization induced by ligation of FcyRIIA to the cholesterol-perturbing agents also supports this conclusion as the activation of the latter receptor does not appear to be tightly coupled to increases in calcium influx. This may be related to the possible utilization of different pools of intracellular calcium by chemotactic factor and opsonin receptors (42). Finally, the present results differ from the previously described interference with calcium signaling through the Fc receptor in RBL cells by cholesterol-depleting agents (43) indicating that the DRM dependence of the various Fc receptors vary as a function of cell or receptor type. In addition, they are unlikely to be explained by direct interference with the partitioning of G protein subunits in DRMs such as has been described for Gα₁ (44), because this would have resulted in the inhibition of all responses to fMet-Leu-Phe and PAF. On the other hand, they are consistent with the results of Petrie et al. (31) who observed that although cholesterol sequestration disrupted lipid rafts in B cells and prevented B cell receptor (BCR) redistribution, it did not inhibit tyrosine kinase activation or phosphorylation of extracellular regulated kinase. The effects of MβCD and filipin on the calcium response to cross-linking of FcyRIIA also resemble those of Petrie et al. (31) who observed that raft disruption enhanced the release of calcium from intracellular stores following BCR stimulation.

The results presented are consistent with the interpretation that the link between the emptying of the Ins(3,4,5)P₃-sensitive stores and subsequent opening of plasma membrane calcium (capacitative calcium entry) is dependent on the integrity of the plasma membrane microdomains rich in cholesterol. These are known by various names including detergent insoluble glycogolden enriched membrane fractions (DIGs), DRMs, and lipid rafts, a multiplicity that may reflect subpopulations of these microdomains (45) that are thought to act as hubs of concentration of signaling molecules. Their involvement in the regulation of calcium channels in human neutrophils is to the best
of our knowledge undocumented. However, these results are consistent with the recent identification of hTrp1, a putative component of SOCs, in the low density fraction of Triton X-100-extracted human submandibular gland cell membranes (20), which is an empirical definition of DRMs. The very recent observations that the calcium influx induced by fMet-Leu-Phe was inhibited in CD38– neutrophils (46) and that CD38 was associated with lipid rafts in T lymphocytes (47) raise the intriguing possibility of a relationship between CD38 and our results.

Finally, although this study focused on the effects of cholesterol-perturbing agents on the mobilization of calcium, it also uncovered an unexpected up-regulation of several neutrophil responses upon the addition of either MβCD or filipin. The three responses examined, tyrosine phosphorylation, activation of PLD, and production of superoxide anions are experimentally independent but may be causally related. Indeed, the activation of PLD is thought to be controlled by one or more tyrosine phosphorylation-dependent steps (48), and several lines of investigation indicate that the production of superoxide anions lies downstream from the activation of PLD (49). It is therefore conceivable that the enhancements of the stimulation of the activity of PLD (several isoforms of which have been localized to DRMs, Refs. 41, 50, 51) and of the production of superoxide anions are secondary to that of the profile of tyrosine phosphorylation. A corollary of this interpretation is that components of cholesterol-rich microdomains (tyrosine phosphatases?) may act to limit the extent of the activation of human neutrophils by chemotactic factors.

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