Phospholipase A₂ inhibition results in sequestration of plasma membrane into electronlucent vesicles during IgG-mediated phagocytosis

Michelle R. Lennartz¹,*, Anson F. C. Yuen¹, Shannon McKenzie Masi¹, David G. Russell², Karolyn F. Buttle³ and Jennifer J. Smith¹

¹Department of Physiology and Cell Biology, Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208, USA
²Department of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Avenue, St Louis, MO 63110, USA
³New York State Department of Health, Wadsworth Center for Laboratories & Research, Albany, NY USA 12202-0509, USA

*Author for correspondence (e-mail: mlennartz@ccgateway.amc.edu)

SUMMARY

Arachidonic acid is essential for antibody-mediated phagocytosis but its role in this process has not been defined. The phospholipase A₂ inhibitor bromoenol lactone decreases arachidonic acid release and arrests phagocytosis; this effect is bypassed by the addition of arachidonic acid to brmoeanol lactone-treated cells. In this morphological study, monocytes treated with bromoanol lactone accumulate electronlucent vesicles in the cytoplasm underlying bound targets. The vesicles are not contiguous with the plasma membrane as they are not labeled with cationized ferritin and are not connected to the plasma membrane as determined by high voltage electron microscopy imaging. However, if the plasma membrane is decorated with wheat germ agglutinin-gold prior to vesicle formation, virtually all vesicles contain the gold marker, indicating that they are plasma membrane-derived. The number of vesicles decreases dramatically upon addition of arachidonic acid to phospholipase A₂-inhibited monocytes and phagocytosis is restored. Time course studies reveal electronlucent regions surrounding targets at early timepoints and a morphology consistent with fusion of electronlucent vesicles into the developing phagosome. These results are consistent with the following model: during the early stages of antibody-mediated phagocytosis, plasma membrane is sequestered in intracellular vesicles that provide membrane for the forming phagosome via fusion events that require arachidonic acid.

Key words: Arachidonic acid, IgG, Monocyte, Phagocytosis, Phospholipase A₂, Microscopy

INTRODUCTION

Maintenance of a disease-free state requires recognition and removal of invading pathogens. Recognition is signaled by the binding of antibodies (IgG) to the foreign organism. These IgG-opsonized particles are then taken up and destroyed by neutrophils, monocytes or macrophages in a highly coordinated sequence of events known as phagocytosis (reviewed by Swanson and Baer, 1995). IgG-mediated phagocytosis is initiated by the binding of a circumferentially-opsonized target to Fc receptors (FcR) on a phagocytic cell. Reorganization of the cytoskeleton in the vicinity of target binding results in extension of membranous pseudopods around the target. Electron microscopy (EM) revealed that the phagocyte extends pseudopods around the target; sequential binding of receptors to surface ligands directs pseudopod elongation around the target in a zipper-like fashion (Griffin et al., 1975; Kaplan, 1977). Once the target is surrounded by membrane, a fusion event isolates it in a phagosome. The phagosome then matures by fusion with endosomal and lysosomal compartments and the target is destroyed by lysosomal hydrolases and the reactive oxygen products of the respiratory burst (Greenberg and Silverstein, 1993).

Much of our understanding of phagocytosis derives from the identification of essential proteins and enzymes. Results from several laboratories have reported that IgG-mediated phagocytosis occurs in the apparent absence of calcium, imposing a considerable constraint on the enzymes of the signal transduction cascade (Di Virgilio et al., 1988; Lennartz et al., 1993; Lew et al., 1985). Previous work has demonstrated that tyrosine phosphorylation of FcR II (Greenberg, 1995), activation of protein kinase C (PKC; Zheleznyak and Brown, 1992) and arachidonic acid (AA) release (Lennartz et al., 1991, 1993) are required for phagocytosis. The characterization of a calcium-independent phospholipase that releases AA from phosphatidylethanolamine during phagocytosis (termed ‘phagocytic phospholipase’) provides a potential candidate enzyme for mediating AA release (Lennartz et al., 1993). This phospholipase activity is inhibited by bromoenol lactone (BEL), a selective inhibitor of calcium-independent phospholipase A₂ (PLA₂; Ackermann et al., 1995; Hazen et al., 1991). BEL decreases AA release and arrests phagocytosis in a coordinated fashion without altering the surface expression of FcR I or FcRII or the apparent affinity of IgG-opsonized targets for the monocyte (Lennartz et al., 1993). It was recently shown that PKC activation is required for AA release and results in
selective activation of the membrane-associated phagocytic phospholipase (Karimi and Lennartz, 1995), providing evidence for a PKC→PLA₂ signaling pathway. However, the role of AA in target ingestion has yet to be addressed.

Experiments with isolated endosomes have shown that endosomes form and dock in the presence of BEL but do not fuse; fusion is restored by the addition of AA (Mayorga et al., 1993). These findings suggest that a BEL-sensitive PLA₂, more specifically the PLA₂ product AA, is involved in membrane fusion. This evidence is strengthened by the characterization of an endosome-associated PLA₂ that is both calcium-independent and BEL inhabitable (Bette-Bobillo and Vidal, 1995) and several reports demonstrating that AA release/PLA₂ activation is associated with degranulation and secretion (Creutz, 1992; Karli et al., 1990). Application of these findings to phagocytosis suggests that the AA released during particle ingestion is utilized for membrane fusion, potentially for closure of the phagosome. We used a morphological approach to consider this hypothesis. The results obtained demonstrate that PLA₂ inhibition with BEL results in accumulation of electronlucent vesicles (ELV) in the cytoplasm of monocytes. The addition of AA to inhibited cells restores phagocytosis and results in a dramatic decrease in the number of these vesicles. Based on these findings we propose that ELV represent sequestered membrane that fuses into the plasma membrane in an AA-dependent manner to provide membrane for the developing phagosome. These results provide evidence that a novel intracellular structure, the ELV, may be involved in membrane movement during phagocytosis.

MATERIALS AND METHODS

Reagents and buffers
Bromoenol lactone was synthesized according to published protocols (Daniels et al., 1983; Durley et al., 1992). The authenticity of the reagent was verified by NMR analysis. Diphosphonic ester, tetraethyl (2-[2-(1-tridecyl)phenyl]ethenylidene) bisphosphonate (DPE), Monsanto US patent #5,504,237, was a generous gift from Dr Patrick Lennon (Monsanto Company, St Louis MO). Arachidonic acid was obtained from BioMol (Plymouth Meeting, PA) and complexed with BSA according to published protocols (Lennartz and Brown, 1991). Cationized ferritin and wheat germ agglutinin conjugated to 10 nm gold particles (WGA-Au) were purchased from Sigma Chemical Co (St Louis, MO).

HBSS*: Hanks’ balanced salt solution (Life Technologies, Grand Island, NY), pH 7.6, containing 4 mM sodium bicarbonate and 10 mM Hepes (both from Whittaker M.A. Bioproducts, Walkersville, MD), 1.5 mM each CaCl₂ and MgCl₂ (Sigma Chemical Co., St Louis MO) and 0.5% human serum albumin (Albany Medical College Pharmacy, Albany, NY). All experiments were performed in this buffer. Millonig’s buffer: 0.12 M NaPO₄, 0.1 M NaOH, 3.2 mM sucrose, 0.3 M glycine, pH 7.4.

Cells and targets
Human monocytes were purified from leukopheresis products by countercurrent elutriation as previously described (Karimi and Lennartz, 1995). Preparations were >90% monocytes as determined by forward and side angle light scattering and CD14 staining. Cells were used on the day of purification or were maintained on ice for use the following day. There were no differences between the results obtained on fresh or day-old cells. IgG-opsonized sheep erythrocytes (ElG) were prepared as described (Lennartz and Brown, 1991).

Phagocytosis assay
Phagocytosis assays were performed as previously described (Lennartz and Brown, 1991). Target ingestion was quantified as phagocytic index, the number of targets ingested per 100 monocytes. Unless otherwise noted, monocytes were incubated with targets for 30 minutes (37°C). For inhibitor assays, monocytes were treated with the indicated concentrations of BEL or diphosphonic ester (DPE) for 30 minutes, 37°C prior to the addition of targets (10:1, targets:monocytes). For AA recovery studies, monocytes were pretreated with BEL; AA and targets were then added and phagocytosis was allowed to proceed for 60 minutes (37°C). For electron microscopy (EM), phagocytosis assays were carried out in a total volume of 1 ml containing 2×10⁵ monocytes. Because BEL is easily hydrolyzed, the effective concentration of drug varied with each new preparation. Thus, for each experiment, we used the concentration of BEL that elicited 70-85% inhibition of phagocytosis and the same concentration of drug was used for the replicate assays in each experiment. The concentrations of BEL used for each experiment are noted.

Membrane labeling
For plasma membrane labeling with cationized ferritin or WGA-Au, monocytes were pretreated with BEL (30 minutes, 37°C). To determine whether or not ELV were contiguous with the plasma membrane, BEL-treated monocytes (2×10⁶ cells) were incubated with ElG in the standard phagocytosis assay. Samples were cooled in an ice bath for 10 minutes prior to the addition of cationized ferritin (1 mg) and glutaraldehyde (1.5%). Fixation in the presence of cationized ferritin was done for 1 hour on ice and the samples were processed for EM as described below. Colloidal gold conjugated to wheat germ agglutinin (WGA-Au) labels the plasma membrane with distinct particles which are more easily detectable than cationized ferritin. For this reason, we used WGA-Au as a membrane probe to establish that ELV were plasma membrane derived. BEL-treated samples were cooled on ice (10 minutes) and treated with WGA-Au (1:5 dilution, 60 minutes on ice). ElG were added and a standard phagocytosis assay was run. Following phagocytosis, the samples were fixed and processed for EM.

Electron microscopy
Samples were prepared for EM by a modification of our previously published protocol (Hirsch et al., 1994). Briefly, phagocytosis was terminated by the addition of glutaraldehyde to a final concentration of 1.5%. Fixation was done at room temperature for 1 hour after which time the fixative was replaced with Millonig’s buffer containing 1% glutaraldehyde and 3% paraformaldehyde. Samples were kept at 4°C until processed for EM. Processing included post-fixation in 1% each osmium tetroxide and potassium ferrocyanide (1 hour, room temperature) followed by en bloc staining with 2% uranyl acetate, dehydration through increasing concentrations of ethanol and embedding in SPURRS resin (Hirsch et al., 1994). Blocks were sectioned on a LKB ultratome (80 nm sections), stained with 2% uranyl acetate/3% lead citrate and viewed with a JEOL 100 CX TEM. For quantitation of vesicles, random fields were photographed at low magnification (3-5 fields/grid, at least 2 grids/condition/experiment, 3 experiments) and enlarged prints generated. Vesicles were quantitated by counting the vesicles present in each cell profile. To be included in the quantitation, the entire cell profile had to be visible in the photograph and contain the characteristic dense-staining monocytic nucleus (preparations contained less than 10% contaminating cells). All cells fulfilling these criteria were counted. Results are expressed as the average number of vesicles per cell profile. For high voltage electron microscopy (HVEM), 1 μm sections were cut, mounted on Formvar coated slot grids and stained with 4% uranyl acetate, 0.3% Tween-20 (1:1) (60°C, 1 hour) then Reynolds’ lead citrate (15 minutes, room temperature). Microscopy was carried out on an AEI high-voltage EM at 1.0 MeV. Stereopairs of negatives were taken with angular separations of 4-16 degrees depending on the thickness and magnification.
Role of arachidonic acid in phagocytosis (Hudson and Makin, 1970). The use of stereo pairs is the most common technique for 3-D visualization in HVEM. At least 10 cells from each of 4 separate experiments were photographed.

RESULTS

Bromoenol lactone caused a dose dependent arrest in monocyte phagocytosis of IgG-opsonized sheep erythrocytes (EiG) that was restored by the addition of exogenous AA to inhibited cells. This recovery was dose dependent and biphasic with 2-5 μM AA eliciting the maximal response (Fig. 1). The reason for the biphasic nature of the recovery is unclear but may reflect changes in the fluidity of the membrane due to incorporation of arachidonic acid into the bilayer. Recovery was detected at all concentrations of BEL tested and reflected not only an increase in the average number of targets ingested per monocyte but also an increase in the number of monocytes ingesting at least one target. For example, the percentage of cells ingesting at least 1 target is 25±3.4 for BEL alone but increased to 50±2.6 in the presence of BEL + 2 M AA; for uninhibited cells, the value was 45±9.4 (values are mean ± s.d., n=4 experiments, at least 100 cells counted/condition/experiment). Arachidonic acid had no effect on phagocytosis in the absence of BEL (Lennartz et al., 1993) nor did the arachidonic acid carrier heptane under any condition.

The effect of BEL on monocyte morphology was assessed by EM. Monocytes were incubated in the absence or presence of BEL prior to the addition of targets. Phagocytosis was

**Fig. 1.** Bromoenol lactone inhibits EiG phagocytosis. (A) Monocytes were preincubated with varying concentrations of BEL (30 minutes, 37°C) and phagocytosis was assessed as described in Materials and Methods. Results are presented as percentage control to allow comparisons between different monocyte preparations. (B) Monocytes were pretreated with 20 μM BEL (70% inhibition) prior to the addition of EiG in the presence of varying concentrations of arachidonic acid. Phagocytosis was carried out for 60 minutes (37°C) and quantified as described. Phagocytic indices (PI) for control samples = 100-200. Data are presented as mean ± s.e.m.; n=7 (A) and 4 (B). *P<0.05 (compared to no added arachidonic acid) as determined by Student’s t-test.

**Fig. 2.** Bromoenol lactone alters the morphology of human monocytes. Monocytes were preincubated in the absence (A) or presence (B) of 20 μM BEL (70% inhibition) and subjected to the standard phagocytosis assay. BEL-treated cells exhibit electronlucent structures in the cytosol which were polarized to the target; such structures are not apparent in controls. Bar, 2 μm.
Fig. 3. Bromoenol lactone elicits electronlucent structures only in the presence of phagocytic targets. Monocytes were preincubated in the absence (A,C,E) or presence (B,D,F) of 25 μM BEL (75% inhibition) as detailed in Materials and Methods. A second incubation with no targets (A,B), unopsonized erythrocytes (C,D) or ElgG (E,F) was carried out for 30 minutes (37°C). Samples were then fixed and processed for EM. BEL had no apparent effect on monocyte morphology in the absence of ingestible targets; electronlucent structures are seen only in samples containing both BEL and ElgG (F). Bar, 5 μm.
allowed to proceed for 30 minutes after which the process was stopped by fixation with glutaraldehyde and the samples were processed for EM. BEL-treated cells exhibit cytoplasmic electronlucent vesicles that were often seen underlying bound targets (Fig. 2B); few such structures were evident in untreated controls (Fig. 2A). Occasionally a few vesicles were seen at sites of contact with other monocytes (Figs 2B, 7A). While the explanation for this is unclear, similar structures were observed in untreated monocytes and thus are probably not due to the presence of BEL (data not shown).

To determine whether or not ELV were artifacts of drug treatment, we studied the effects of targets on the ultrastructure of control or BEL-treated monocytes (Fig. 3). BEL-treated monocytes did not appear significantly different from control cells in the absence of targets (Fig. 3A vs B). The surface contours of both preparations were similar and a few large electronlucent regions were apparent in the periphery of some cells. Due to their location and large size, these structures may result from pseudopodia folding back onto the cell surface, an activity that has been previously described for macrophages (Swanson and Baer, 1995). Additionally, control and BEL-treated monocytes exhibit similarly ruffled membranes when examined by scanning EM (data not shown). The addition of unopsonized sheep erythrocytes did not substantially alter the morphology of either inhibited or control cells (Fig. 3C vs D). In contrast, incubation of monocytes with ElgG led to the appearance of electronlucent structures in many (60-75%) of the BEL-treated monocytes (Fig. 3E,F). Often these structures were localized near targets. However, vesicle clusters lacking surface-associated targets were observed and may represent instances where targets were bound but subsequently lost during processing for EM.

A similar morphology was observed using a diphosphonic ester (DPE) PLA2 inhibitor (Fig. 4B). DPE inhibits phagocytosis in intact cells, the phagocytic phospholipase in in vitro studies (data not shown), and endosome-endosome fusion (Mayorga et al., 1993). In contrast to BEL, which is an irreversible suicide inhibitor of calcium-independent PLA2s, DPE exhibits potent but reversible calcium independent PLA2 inhibition. The observation that two PLA2 inhibitors, with different mechanisms of action, generate the same morphology, suggests that ELV are formed as a result of PLA2 inhibition and are not unique to BEL.

Note that the majority of vesicles in BEL-treated cells are localized to that part of the cell bearing bound targets (Figs 2B, 3F). This observation raises the possibility that the vesicles were generated (or recruited) in response to a signal. We suggest that the signal is the ElgG binding and the vesicles may be formed from the membrane perturbations that accompany target binding.

Because target binding elicits membrane ruffling in phagocytes, we addressed the possibility that ELV were actually membrane invaginations that appeared as vesicles due to the plane of sectioning. This was done using cationized ferritin, which binds to the plasma membrane and decorates it with electron dense granules. Monocytes were pretreated with BEL and incubated with ElgG to generate ELV (Figs 2B, 3F). Following ELV formation, the cells were cooled on ice to prevent further membrane internalization. Cationized ferritin was added and the cells were fixed on ice. Under these conditions, ferritin selectively labels the plasma membrane (Grönblad et al., 1982; Nilsson et al., 1983). As ferritin is a soluble protein, it would be expected to label any membrane accessible to the medium. Because the ELV were formed before the addition of ferritin, if they were discrete they should not have access to the ferritin. Conversely, if they were membrane invaginations, they would contain the ferritin label. Fig. 5 illustrates the results obtained by this protocol. Although ferritin can be seen decorating the plasma membrane of the monocytes (arrows), none is visible in the vesicles, even at high magnification (Fig. 5B). These results suggest that ELV are not contiguous with the plasma membrane. However, it could be argued that the vesicles are invaginations of the plasma membrane with narrow necks inaccessible to ferritin or that the density of ferritin on the surface is not sufficient to label membrane invaginations. Thus, we used high voltage electron microscopy (HVEM) to verify the ferritin results. This technique permits imaging of thick sections (1 μm), capturing a greater percentage of the cell in a single section than is...
possible by conventional EM (80 nm thick sections). Monocytes were treated with BEL and ElG and processed for EM as for the ferritin experiments. Thick sections were then made and analyzed by HVEM. Stereo images were generated and examined for the presence of connections between ELV and the plasma membrane. A minimum of 10 cells from each of 4 different cell preparations were analyzed. Although some vesicles were in close proximity to the plasma membrane (stars, Fig. 6A,B), in no case was a connection apparent (i.e. the membranes remained discrete). Taken together, these results provide evidence that ELV are not contiguous with the plasma membrane.

The localization of ELV in the periphery of the cytosol is consistent with a plasma membrane origin for the vesicles. We modified the cationized ferritin technique to ask whether or not ELV originate in the plasma membrane. Monocytes were pretreated with BEL alone, a condition that should not result in the accumulation ELV (Fig. 3B). The cells were then cooled on ice to prevent membrane internalization and incubated with WGA-Au to label the plasma membrane. The temperature was then raised to 37°C in the presence of ElG and the incubation continued for 30 minutes in the standard phagocytosis assay. This second 37°C incubation should result in the appearance of ELV (Fig. 3F). Because the WGA-Au was added before ELV formation, if the ELV contain the gold, they must have originated at the plasma membrane. Conversely, a lack of label would suggest that they were derived from intracellular pools. Under these conditions, the majority of the ELV contained gold, supporting the hypothesis that they are formed from the plasma membrane (Fig. 7). The fact that the specificity of WGA binding is for the complex carbohydrates on glycoproteins allows us to conclude from these data that ELV contain plasma membrane-derived proteins.

We have shown that the inhibition of phagocytosis by BEL can be reversed by exogenous AA (Lennartz et al., 1993 and Fig. 1B). The addition of AA to BEL-treated cells results in recovery of phagocytosis and a significant decrease in the number of ELV/cell profile (25 µM BEL: 28.9±3.9, 25 µM BEL + 2 µM AA: 10±1.1, P<0.01 compared to BEL alone, control lacking BEL and AA: 3.7±0.5, all values mean ± s.e.m., data were accumulated from 3 experiments). The phagosomes of recovered monocytes have electronlucent regions between the target and phagosomal membrane in contrast to the tightly apposed membrane seen in control cells (Fig. 8E,F vs A,B). Additionally, ELV-like structures are seen in close proximity to the phagosome, a morphology consistent with docked endosome (Mayorga et al., 1993).

If ELV are involved in the normal process of IgG-mediated phagocytosis, they should be apparent in uninhibited cells during the early stages of particle ingestion. Indeed, time course studies revealed ELV-like structures localized to targets at early time points. Although many of the ELV were present in the cell body (Fig. 9, arrows), vesicles were also observed...
Role of arachidonic acid in phagocytosis

in pseudopods (Fig. 9, arrowheads), raising the possibility that ELV may provide membrane for pseudopod extension. Also, the size and location of these vesicles is similar to that seen in PLA₂-inhibited cells (compare Fig. 9A-D with Figs 2 and 4), suggesting that the ELV accumulated by PLA₂ inhibition represent a blockade in the normal phagocytic process. In addition, we observed electronlucent regions around targets in both fully and partially formed phagosomes (Fig. 9, asterisks) that are reminiscent of the ‘loose’ phagosome morphology of recovered cells (Fig. 8E-H). By 4 minutes, the classic phagosome morphology (i.e. particles with tightly apposed membranes) is evident in many cells. These findings provide strong evidence that ELV are generated during the normal phagocytic process.

Fig. 6. Stereoimaging of BEL-treated monocytes. Monocytes were pretreated with 50 μM BEL (70% inhibition), incubated with ElgG and processed for HVEM. 0.5-1 μm sections were taken and cells were photographed as stereopairs. A total of 45 vesicle-containing cells from 4 independent experiments were analyzed. Representative cells from two different preparations are presented. Note the presence of vesicles in the cytosol near targets (T) in (A). Although vesicles are seen very near the plasma membrane (stars), no connections between the two membranes were detected. N, nucleus. Bars, 1 μm.
DISCUSSION

The zipper model for IgG-mediated phagocytosis has driven research in this field for the past 20 years. The receptors and ligands have been characterized and cloned and more recent advances have addressed the intracellular pathways involved in transducing the signal. However, little is known about the mechanism(s) of pseudopod extension during this process. We have previously shown that AA is required for IgG-mediated phagocytosis in monocytes and that AA release is mediated by a calcium independent phospholipase (Lennartz and Brown, 1991; Lennartz et al., 1993). More recent studies link AA release with upstream activation of protein kinase C (Karimi and Lennartz, 1995). Our current data addresses the question: what is the role of AA in phagocytosis? Results demonstrating that AA is involved in endosome-endosome fusion provided a working hypothesis that the released AA mediates membrane fusion (Mayorga et al., 1993). Our results are consistent with this finding.

Inhibition of phagocytosis with BEL or DPE results in the accumulation of intracellular electronlucent vesicles (Figs 2 and 4). Many of these vesicles are localized to the cytoplasm underlying surface-associated targets. The fact that such vesicles are not seen when monocytes are incubated with BEL alone or in the presence of unopsonized targets (Fig. 3) is consistent with the interpretation that vesicles are formed as the cells try (unsuccessfully) to ingest targets and are not an artifact of drug treatment.

The combined cationized ferritin labeling and HVEM data (Figs 5 and 6, respectively) demonstrate that ELV are discrete structures and not invaginations of the plasma membrane. The plasma membrane origin of the vesicles was determined by WGA-Au labeling of the plasma membrane prior to vesicle formation. Additionally, we assessed the presence of extracel-

Fig. 7. ELV are derived from the plasma membrane. Monocytes were preincubated with 75 μM BEL (85% inhibition), the cells were cooled to prevent membrane internalization and treated with WGA conjugated to 10 nm gold particles to mark the plasma membrane. ElgG were then added and cells incubated to permit ELV formation. The majority of ELV contain the gold label under these conditions, indicating that they arise from the plasma membrane. (A) Low magnification image showing polarized vesicles. Bracketed region is enlarged in B. (B-D) Higher magnifications of ELV regions from three cells. Bars: 1 μm (A); 0.5 μm (B-D).
**Fig. 8.** Loss of electronlucent vesicles in the presence of BEL and arachidonic acid. Monocytes were incubated in the presence (C-F) or absence (A,B) of 75 μM BEL (85% inhibition). ElgG ± 2 μM AA were then added and phagocytosis allowed to proceed for an additional 60 minutes; samples were then processed for EM. Two representative cells are shown for each condition. Uninhibited monocytes exhibit classical phagosomes with tightly opposed membranes (A,B), BEL-treated cells contain ELV (C-F). ELV are apparent in samples treated with BEL alone and no internalized targets are present (C,D), the number of vesicles decreases upon the addition of AA and targets are detected in phagosomes containing electronlucent regions (E,F). Thus, AA restores phagocytosis to BEL-inhibited monocytes, possibly by allowing ELV to fuse into the forming phagosome (arrows). Bar, 1 μm.
Fig. 9. ELV are present during uninhibited phagocytosis. Uninhibited monocytes were mixed with ElgG, spun gently to increase monocyte/target interaction (150 g, 5 minutes) and incubated with ElgG for 0 (A), 1 (B), 2 (C) or 4 (D) minutes (37°C) prior to fixation and processing for EM. Two representative fields from each time point are presented. ELV-like structures (arrows) in the cell body and in pseudopod extensions (arrowheads) can be seen immediately; electronlucent regions around targets (asterisks) are apparent by 2 minutes. Also note that the location and appearance of ELV in these uninhibited cells is similar to that seen in Pla2-inhibited cells (Figs 2 and 4). Results from 1 experiment, representative of 3, are shown. These findings suggest that ELV are normal constituents of phagocytosis and are present in pseudopods. Bar, 2 µm.

Although we have yet to establish the final disposition of the ELV membranes, we suggest that they reinsert into the developing phagosome in an AA-dependent manner. In this scenario, AA would promote fusion of ELV by a process that involves contact and fusion of the cytoplasmic face of the ELV with the plasma membrane. This cytoplasmic fusion may be similar to the endosome/endosome fusion that we have shown to be AA dependent and BEL-inhibitable (Mayorga et al., 1993). Thus, we propose that the AA requirement for phagocytosis derives from its involvement in membrane fusion, specifically its role in mediating fusion of ELV with the monocyte membrane.

Based on these data and our earlier results (Lennartz et al., 1993; Mayorga et al., 1993), we propose the following models for membrane movement during phagocytosis of IgG-opsonized targets. Ligation and aggregation of Fc receptors elicits localized membrane ruffling, as evidenced by the selective presence of membrane extensions near targets (Fig. 9A,B) and as previously reported (Silverstein et al., 1989). Ruffling generates plasma membrane-derived electronlucent vesicles in the cytosol underlying bound targets. Alternatively, vesicles may be formed from distal segments of the plasma membrane and be recruited to forming phagosomes. Vesicles then insert into the plasma membrane in an AA-dependent fashion to provide membrane for the growing pseudopods. Insertion may occur at regions of the plasma membrane close to the boundary of contact, into the phagocytic cup or into the pseudopod proper. The presence of ELV in pseudopods suggests that they may insert into the pseudod (Fig. 9, arrowheads). By limiting AA availability, BEL interferes with this fusion event, resulting in ELV accumulation within the cytosol. These models assume that the ELV fuse back into the plasma membrane and serve as a sequestered pool of membrane for regulated insertion into the forming phagosome. Our results are consistent with this model but direct evidence for reinsertion of ELV into the membrane and localization of ELV insertion requires selective labeling of ELV and determining their final disposition upon recovery of phagocytosis with AA. We are currently developing the techniques to address this issue.

These proposed models raise several intriguing questions with regards to the ELV, such as composition, contents and regulation of formation and final disposition in the phagosome. The answers to these questions will contribute to our overall understanding of membrane movement during phagocytosis and the phagocytic process in general.

The authors are indebted to Dr Peter Weber for synthesis of BEL, Dr Patrick Lennon of the Monsanto Company for supplying the diphosphonic ester, Ms Corrine Guinta for her expertise in the preparation of samples and electron microscopy, and Ms Mary Beth Frewin and Mr Demian Singleton for monocyte elutriation. The electron microscopy facility at the Stratton Veterans Administration Medical Center, under the direction of Dr Jaswant Singh, was used for these studies. Many thanks to Drs Philip Stahl and Carmen Alvarez-Dominguez for insightful discussion and the BSA-gold. This work was supported by grants from the National Institute of General Medical Sciences (45983) and The American Heart Association, New York affiliate (93-310G8) (MRL) and by grant RR01219 awarded by the Biotechnology Area, National Center for Research Resources (DHH/PHS), to support the Wadsworth Center’s Biological
REFERENCES


(Received 20 December 1996 - Accepted 17 June 1997)