Imaging signal transduction during phagocytosis: phospholipids, surface charge, and electrostatic interactions

Sergio Grinstein
Cell Biology Program, The Hospital for Sick Children, Toronto, Canada
Submitted 23 August 2010; accepted in final form 23 August 2010

Grinstein S. Imaging signal transduction during phagocytosis: phospholipids, surface charge, and electrostatic interactions. Am J Physiol Cell Physiol 299: C876–C881, 2010. First published August 25, 2010; doi:10.1152/ajpcell.00342.2010.—Together with the development of genetically encoded fluorescent probes, digital imaging has provided great impetus to the study of cell signaling by providing enhanced sensitivity and much-improved spatial and temporal resolution. We have used phagocytosis as a paradigm of signal transduction, taking advantage of the generous size of phagosomes and of their comparatively leisurely rate of formation. Aided by the design of specific probes, we demonstrated a highly localized and elegantly choreographed sequence of changes in the level of several phosphoinositides and were able to also monitor the fate of phosphatidylserine. The net changes in the content of these anionic phospholipids are accompanied by marked alterations in the surface charge of the membrane of nascent phagosomes. These, in turn, cause the relocation of proteins that associate with the membrane by electrostatic interactions. Our studies suggest that anionic lipids control protein targeting not only through stereospecific recognition by specialized domains but also by electrostatic association mediated by polycationic motifs. The “electrostatic switch” can be turned on or off by altering the charge of the protein ligand (e.g., by phosphorylation) or, alternatively, by modifying the lipid composition of the target membrane.

phosphoinositide; phosphatidylserine; phagosome; macrophage
chemical and immunochemical methods to study signaling. New techniques were clearly necessary to assess the formation of fleeting, low-affinity complexes. Improved temporal resolution was also a priority and, ideally, much greater sensitivity would additionally provide the ability to monitor single cells, obviating the response averaging inherent to population-based measurements. Given enough sensitivity and spatial resolution, improved methods could define the precise subcellular location where the responses occur.

Most of these requirements are fulfilled by spectroscopic techniques: they combine high sensitivity with rapid responsiveness and, if used wisely, are minimally invasive. Fluorescence microscopy, in particular, provides remarkable sensitivity and requires the use of affordable hardware already in existence in many laboratories. The renaissance of fluorescence microscopy in modern cell physiology has been aided greatly by the advent of genetically encoded fluorescent proteins and by the development of novel synthetic fluorophores to take advantage of all regions of the visible spectrum (5).

PHAGOCYTOSIS AS A MODEL OF SIGNAL TRANSDUCTION

To take advantage of this newly developed wealth of technical resources, we chose phagocytosis as a paradigm of signal transduction. Phagocytosis, the receptor-mediated internalization of particulate material (≈0.5 μm in diameter), offers unique features and opportunities for the study of signaling by optical methods. Key events occur in comparably large structures well above the diffraction limit of the optical microscope. Indeed, the size of the active zone where signals are generated and conveyed, termed the phagocytic cup, can be manipulated experimentally by supplying cells with phagocytic targets of the desired diameter. In addition, unlike many of the phenomena studied by physiologists (such as electrophysiological responses or muscle contractility), phagocytosis develops relatively slowly over the course of many seconds to minutes. This property affords ample time for collection of a sufficient number of photons from even dim samples. When brighter samples are available, the comparatively slow course of phagocytosis enables serial optical sectioning by confocal microscopy, making it possible to obtain three-dimensional reconstructions at different times, a rare luxury.

Much of our effort has been directed to the study of lipids in signaling phagocytosis, a problem that, for the reasons discussed above, is almost intractable by conventional biochemical means. Progress was fostered by the explosive development of lipid-specific, genetically encoded probes, mainly by the laboratories of Drs. Tamas Balla and Tobias Meyer (8, 10, 12, 13), both of whom very generously shared their reagents and expertise. The principle underlying the design of virtually all such probes is similar: lipid head groups are identified in intact cells using domains derived from cellular proteins that evolved to interact with defined lipids in the context of their physiological function. Thus PH (pleckstrin homology), FYVE (named after Fab 1, YOTB, Vac 1, and EEA1), and PX (phox or phagocyte oxidase) domains have been identified to bind to specific phosphoinositides and exploited to monitor their distribution and metabolism. Similarly, C1 domains are used to detect diacylglycerol and discoidin C2 domains sense phosphatidylserine (PS). Access to the nucleotide sequences encoding such domains, together with the sequences of humanized fluorescent proteins, facilitated the generation of cDNA constructs for the expression of chimeric, lipid-specific fluorescent probes. Finally, the availability of different fluorescent proteins with varying spectral properties (5) enabled us and others to monitor two, and potentially more, different probes simultaneously. The table was set to make rapid progress in studying the signals that trigger the innate immune response.

ROLE OF PHOSPHOINOSITIDES IN PHAGOCYTOSIS

We performed most of our studies in macrophages, the phagocytic cell par excellence. We routinely use the murine macrophage line RAW264.7. These cells not only have an insatiable appetite but are moderately transfectable. For most of our experiments we coated particles with IgG, thereby targeting them for phagocytosis via Fcγ receptors (FcγR).

As does virtually every cell type, resting macrophages have abundant phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] in the inner leaflet of their plasma membrane. This was readily apparent in cells transfected with the PH domain of phospholipase C (PLC), a PI(4,5)P2-specific probe (2). What was rather unexpected was the biphasic change in PI(4,5)P2 that occurred upon engagement of IgG-coated particles: Botelho et al. (2) noted a modest, transient enrichment at the early stages of phagocytosis, followed by a marked depletion. Indeed, PI(4,5)P2 is undetectable in the membrane of the sealed phagosome, the vacuole that forms as a result of invagination of, and scission from, the surface membrane (see Fig. 1). When large particles are studied, disappearance of PI(4,5)P2 from the base of the (unsealed) phagocytic cup is clearly apparent, suggesting the presence of a diffusional barrier that prevents mixing of phagosomal constituents with lipids from the bulk (unengaged) plasma membrane during the seconds/minutes it takes for phagocytosis to be completed.

The mechanism accounting for the transient elevation of PI(4,5)P2 is not well understood, but local stimulation of plasmalemmal phosphatidylinositol 4-phosphate 5 kinases is most likely. By contrast, we know more about the processes that mediate the subsequent disappearance of PI(4,5)P2. Depletion is caused by a combination of at least three factors: conversion to phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3], hydrolysis by phospholipase Cγ, and reduced synthesis. Evidence in support of these conclusions was obtained by imaging phagocytosis in live cells. Marshall et al. and Botelho et al. (2, 6) found that the disappearance of PI(4,5)P2 was accompanied by the recruitment of the PH domains of Akt or Gab2, which report the formation of PI(3,4,5)P3, and also of the C1 domains of PKCζ or PKD, which indicate generation of diacylglycerol. It is rather likely that, as happens during endocytosis (3), some of the phagosomal PI(4,5)P2 is also degraded by phosphatases such as inositol polyphosphate-5-phosphatase (Inpp5) and/or oculocerebrorenal syndrome of Lowe (OCRL), but direct evidence is missing. At the same time, green fluorescent protein (GFP)-tagged constructs of the enzymes that generate PI(4,5)P2, the phosphatidylinositol 4-phosphate 5 kinases, which are constitutively associated with the membrane of resting cells, detached from the base of forming phagosomes. Thus the abrupt disappearance of PI(4,5)P2 from the forming phagosome results from a combination of its conver-
sion to other chemical species and the termination of its synthesis (Fig. 1).

These early changes in lipid metabolism are not inconsequential. On the contrary, they appear to be critical for the progression of the particle engulfment process: pharmacological inhibition of the enzymes that catalyze the phosphorylation of PI(4,5)P$_2$ (e.g., using wortmannin) or its hydrolysis (e.g., using the PLC inhibitor U73122) arrests phagocytosis (1, 9), as does the overexpression or enforced retention of phosphatidylinositol 4-phosphate 5 kinase at the phagosomal cup, as Gregory Fairn found (4). The products of PI(4,5)P$_2$ metabolism, PI(3,4,5)P$_3$, inositol 3,4,5-trisphosphate, and diacylglycerol (DAG), act as second messengers, recruiting and activating a variety of downstream effectors. In addition, the disappearance of PI(4,5)P$_2$ itself is likely required for actin remodelling (9) and possibly also for membrane scission.

PI(3,4,5)P$_3$ and DAG persist in the phagosomal membrane for about 1–2 min after sealing is completed, disappearing rapidly thereafter (Fig. 1). The phosphatases SH2 domain-containing inositol phosphatase (SHIP) and phosphatase and tensin homolog (PTEN) are thought to account for the hydrolysis of PI(3,4,5)P$_3$, while DAG-kinases and/or lipases metabolize DAG. Otilia Vieira (14) found that disappearance of PI(3,4,5)P$_3$ and DAG is followed, as if on cue, by the formation of phosphatidylinositol 3-phosphate [PI(3)P], the product of the class III phosphatidylinositol 3 kinase, also known as mVps34 (14). PI(3)P generation coincides with and seemingly depends on the recruitment of Rab5. Together, the inositide and GTPase recruit tethering proteins, exchange factors, and other effectors that initiate the conversion of the early phagosome to a late phagosome, and ultimately to a fully microbicidal phagolysosome. Interestingly, PI(3)P is also required to
terminate the action of Rab5 in the phagosome; wortmannin, which inhibits not only class I but also class III phosphatidylinositol 3 kinase, greatly extends the time of residence of active Rab5 on phagosomes and arrests their maturation (15).

**SURFACE CHARGE AND ROLE OF ELECTROSTATIC ASSOCIATIONS IN PROTEIN RECRUITMENT**

At physiological pH the phosphoinositides are negatively charged. Indeed, the number of charges increases with the degree of inositol phosphorylation; PI(4,5)P2 has a net charge of $\approx 3.5$ (i.e., it is a nearly equimolar mixture of tri- and tetravalent anionic species). Because of their polyvalency, together with their relative abundance, phosphatidylinositol is thought to constitute nearly 10% of the inner leaflet of the plasma membrane, while phosphatidylinositol 4-phosphate and PI(4,5)P2 contribute 2–5% each, the phosphoinositides are expected to contribute significantly to the surface charge (7). As discussed in great detail by McLaughlin et al. (7), the electrostatic attraction generated by this surface charge can be an important contributor to the recruitment of cationic proteins and alters the local concentration of inorganic cations in the immediate vicinity of the membrane. We found intriguing the possibility that, as the phosphoinositide composition of the membrane becomes locally remodeled during phagocytosis, the surface charge and hence the recruitment of cationic molecules could be significantly altered.

To investigate this possibility, we scoured the literature in search for methods to quantify the surface charge of biological membranes. Remarkably, while the surface charge of the external aspect of the plasma membrane had been measured by electrophoretic or spectroscopic means, we could find no reports of similar measurements in endomembranes. More depressing was the realization that the methods used for the external monolayer were not applicable to the inner leaflet or to other organelles in situ. We therefore set out to design means to measure endomembrane surface charge. In a nutshell, the method that Tony Yeung and John Silvius (18) developed uses genetically encoded fluorescent probes that act as coincidence detectors: the probes measure not only the presence of negative charge (i.e., not just the presence of a membrane) but require both of these determinants to interact with a target. The probes consist of three elements: 1) a hydrophobic determinant, either an acyl or polypreyl chain, that favors association of the probe with membranes; 2) a polycationic motif, either polysine or, to avoid complications introduced by ubiquitylation, polyarginine that attracts the probe to negative entities; and 3) a fluorescent moiety, either GFP or one of the assortment of red fluorescent proteins (18).

When such probes were constructed using varying numbers of cationic residues in tandem, we found that seven or eight charges directed the probes almost exclusively to the inner aspect of the plasma membrane. We interpret these results to mean that this monolayer represents the most negatively charged surface of the entire cell, presumably attributable to its elevated (poly)phosphoinositide content. In support of this notion we found that the probes detached rapidly from the membrane when PI(4,5)P2 was hydrolyzed by recruitment of phosphoinositide phosphatases using the rapalog system (4) or by activation of endogenous lipases and phosphatases upon elevation of cytosolic calcium (18).

Using these fluorescent coincidence detectors, we assessed whether the surface charge of the macrophage membrane undergoes significant changes during particle engulfment. Strikingly, we found that probes with an 8+ cationic motif that are heavily partitioned to the inner surface of the plasma membrane of resting macrophages detach almost entirely during phagosome formation. This observation, in all likelihood attributable to the concomitant phospholipid remodelling, has far-reaching implications. Proteins that are retained by the membrane at least partly by electrostatic means are likely to detach, with potentially serious functional consequences. Two examples vividly illustrate this point: all three isoforms of the phosphatidylinositol 4-phosphate 5 kinase associate electrostatically with the surface membrane of quiescent cells, where they maintain the resting level of PI(4,5)P2. As described above, Greg Fairn (4) found that these enzymes detach from the forming phagosome, accentuating the rate of PI(4,5)P2 depletion from the sealing vacuole. A second example is provided by Rac1, the GTPase that is primarily responsible for the polymerization of actin that drives pseudopod extension. As are other Rho family GTPases, Rac1 is prenylated near its COOH terminus, where it also bears a polycationic motif. This region of the protein serves to target and/or stabilize the active (GTP bound) form to the plasma membrane. Thus a constitutively active mutant of Rac1 that is continuously bound to GTP is found almost entirely at the plasmalemma. Of note, this active form of Rac1 detaches from the nascent phagosome, not as the surface charge drops (18). This implies that the function of Rac1 can be terminated when phagocytosis is completed, not by inactivation of the respective GEF nor by activation of a GAP, but merely by the detachment of the GTPase from its target membrane, which is an inevitable consequence of the change in surface charge.

**MONITORING PHOSPHATIDYLSERINE**

Whereas phosphoinositides are plentiful in the surface membrane, they are by no means the most abundant anionic phospholipid. That distinction belongs to phosphatidylserine (PS), which generally constitutes $\approx 20\%$ (and in some species upwards of 30%) of the lipid of the inner leaflet. Yet, for years, we and others neglected to study PS. This was at least in part a consequence of technical limitations; the probes in use to monitor PS in cells were not very satisfactory. Annexin V, which had been used to detect the appearance of PS on the cell surface, binds PS only when the concentration of calcium is near millimolar. This feature negates its application to the study of intracellular PS, since the cytosolic calcium concentration is orders on magnitude lower (submicromolar), and thus, insufficient to support PS recognition by annexin V. As an alternative, others used nitrobenz-2-oxa-1,3-diazole (NBD)-labeled PS. Introduction of the NBD moiety to one of the acyl chains of PS, however, grossly distorts the structure and hence the behavior of the phospholipid, making the determinations unreliable. Indeed, after a few minutes of uptake by mammalian cells, NBD-PS is found largely in the endoplasmic reticulum and is barely detectable in the surface membrane. This conflicts with the known distribution of endogenous PS, which was determined to be severalfold more abundant in the membrane than in the reticulum.
Once again, we were compelled to search for alternative means of analysis. In this instance, Tony Yeung (16) resorted to the approach that had proven so successful in the case of the phosphoinositides: we looked for a protein domain that evolution selected for association with PS but that, unlike annexin V, did not require calcium to do so. The discoidin-type C2 domains fit this description. Such domains are found in a number of secreted proteins, certain of which, e.g., factors V and VIII, participate in the initiation of blood coagulation. Gary Gilbert and Tony Yeung (16) performed in vitro experiments using recombinant C2 domains that verified their ability to bind PS and more importantly, demonstrated a high degree of specificity. No other lipids, including several anionic ones, bound the probe to a significant degree. This selectivity was confirmed by comparing the behavior of the probe in wild-type and PS-deficient yeast: the former showed a distinct association of C2-GFP with the plasma membrane, whereas the probe was largely soluble in the mutants lacking PS.

Having validated the usefulness of the probe to our satisfaction, we proceeded to study the distribution of PS in macrophages. In good agreement with the biochemical data, we found the plasma membrane to be a major repository of PS. In addition, a tubulovesicular endomembrane compartment was also observed to be rich in PS. Dual labeling experiments with organelle-specific markers revealed the PS-rich endomembranes to correspond to compartments of the endocytic pathway. Early and late endosomes, as well as lysosomes, were all decorated by the C2-GFP probes (16).

How about the phagosomes? Cells allowed to ingest IgG-coated targets clearly displayed PS on the nascent phagosomes. Unlike P(4,5)P₂, however, PS persisted on the phagosomal membrane throughout the engulffment and subsequent maturation process (Fig. 1). Indeed, bona fide phagolysosomes were endowed with an amount of PS that was indistinguishable from that in the membrane by the C2-GFP imaging method (17).

If the very abundant PS remains on the phagosomal membrane, is it not expected to maintain the negativity of the surface charge? This assertion is only qualitatively true. The large electrostatic potential of the inside aspect of the plasma membrane reflects the contribution of two main components: the phosphoinositides and PS. Thus depletion of polyphosphoinositides (as occurs during phagocytosis) is predicted to reduce, but not eliminate, the negativity entirely. We could validate this conjecture experimentally. Using a series of coincidence detector probes with hydrophobic tails plus polycationic motifs of varying charge, we were able to visualize not only the most negative membrane (the plasma membrane) but also to identify a sequence of progressively less electronegative surfaces. It is noteworthy that, after the plasma membrane, the tubulovesicular endocytic membranes present the next most negative surfaces to the cytosol. Phagosomes, which are in dynamic equilibrium with the endocytic pathway, behaved similarly (18). This can be readily accounted by their elevated PS content, despite their lower content of phosphoinositides.

An important inference from the preceding observations is that the complement of proteins that associate electrostatically with the plasma membrane will differ from those associated with endosomes or maturing phagosomes, which will in turn be different from those bound to mitochondria or to subcompartments of the secretory pathway, which have the least negative limiting membranes. In this manner, the varying surface charge can serve as an address code for the delivery and retention of cationic ligands, specific for each compartment.

PERSPECTIVES

Despite the progress made by a number of groups throughout the world, our current appreciation of lipid metabolism and redistribution in signal transduction in general, and during phagocytosis in particular, is surely fragmentary and woefully incomplete. Indeed, we know precious little about the formation and fate of phosphatidic acid, arachidonate, phosphatidylinositol 3,5-bisphosphate, and lysobisphosphatic acid, all suspected to play important roles in phagosome formation or maturation. Their study has been hampered by the paucity of adequate probes. Much will depend on the development of suitable reagents. This task will be a particular challenge for low-abundance species, since probes with extremely high affinity and specificity will be required. The pursuit for high affinity probes, however, will have to be tempered by the need for rapid reversibility, if kinetic studies are envisaged, and by the potential risk of interference with the endogenous physiological ligands. Indeed, excessive expression of high affinity probes can result in scavenging of the lipid target, with untoward functional consequences. Nevertheless, based on the progress made to date for other lipids, the future is promising!

ACKNOWLEDGMENTS

I am deeply indebted to Paul Paroutis for drawing the figure.

GRANTS

Original work in the author’s laboratory is supported by the Canadian Cystic Fibrosis Foundation and by the Canadian Institutes of Health Research Grant MOP-7075. S. Grinstein is the current holder of the Pitblado Chair in Cell Biology and is cross-appointed to the Department of Biochemistry of the University of Toronto.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


