methods

A targeted mass spectrometric analysis of phosphatidylinositol phosphate species

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Abstract The development of a new mass spectrometric lipid profiling methodology permits the identification of cellular phosphatidylinositol monophosphate/phosphatidylinositol bisphosphate/phosphatidylinositol trisphosphate (PIP/PIP_2/PIP_3) species that includes the fatty acyl composition. Using electrospray ionization mass spectrometry, we were able to resolve and identify 28 PIP and PIP_2 compounds as well as 8 PIP_3 compounds from RAW 264.7 or primary murine macrophage cell extracts. Analysis of PIP profiles after agonist stimulation of cells revealed the generation of differential PIP_3 species and permitted us to propose a novel means for regulation and specificity in signaling through PIP_3. This is the first reported identification of intact, cellular PIP_3 by mass spectral analysis. The ability to analyze the fatty acyl chain composition of signaling lipids initiates new venues for investigation of the processes by which specific polyphosphoinositide species mediate.—Milne, S. B., P. T. Ivanova, D. DeCamp, R. C. Hsueh, and H. A. Brown. **A targeted mass spectrometric analysis of phosphatidylinositol phosphate species. *J. Lipid Res.* 2005.** 46: **1796–1802.**

Supplementary key words lipidomics • phosphoinositides • electrospray ionization mass spectrometry

Phosphatidylinositol phosphates are versatile signaling lipids involved in multiple cellular functions. They and their metabolites are important elements of cellular processes, including cell motility, polarity, apoptosis, oncogenesis, vesicle transport, membrane fusion, and calcium mobilization (1–5). The production of phosphatidylinositol trisphosphate (PIP_3) is particularly notable as it has been shown to be a regulator of many signaling processes and downstream molecules such as serine/threonine kinase and mammalian target of rapamycin. The broad role of PIP_3 and related metabolites in signaling processes demands a worthy method for measuring its cellular activity. However, PIP_3 is present in such low concentrations within the cell that direct measurement has been difficult. Traditional methods for phosphoinositide detection in cell extracts have included receptor displacement assays, metabolic labeling, and chromatographic separation of the radiolabeled products after deacylation (6–8). To identify phosphatidylinositol monophosphate (PIP), phosphatidylinositol bisphosphate (PIP_2), and PIP_3 compounds in total cell extracts, we strategically combined a selective extraction and mass spectrometric protocol. Lipidomic analysis permits the detection and identification of important membrane signaling molecules that were formerly difficult to fully characterize by other methods (9–12). This includes the assessment of changes in cellular levels of polyphosphoinositides. Traditionally, these species have been analyzed as a deacylated "pool" of ^{32}P-labeled inositides by HPLC (7) or as intact compounds by TLC (13). These procedures can result in qualitative estimates of total PIP and PIP_2, but chemical identification of the species within the PIP/PIP_2 pools (i.e., species distinguishable from one another by their fatty acyl chains) in the context of other changes in lipid species has been challenging. Electrospray ionization mass spectrometry has been applied to the analysis of polyphosphoinositides (i.e., PIP and PIP_2) (14), but until now, the detection of PIP_3 and the identification of its chemical composition have been elusive.

Since its discovery in the late 1980s, PIP_3 has been intensively studied because of its important role as a mediator in signal transduction and in many physiologically and pathologically important processes. Practically undetectable in unstimulated cells, PIP_3 species formation increases rapidly in response to a variety of stimuli, including growth factors and oxidative stress. Reactive oxygen species function as mediators in cellular responses and are clinically

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Abbreviations: AfCS, Alliance for Cellular Signaling; LPA, lysophosphatidic acid; MCF, macrophage colony-stimulating factor; PIP, phosphatidylinositol monophosphate; PIP_2, phosphatidylinositol bisphosphate; PIP_3, phosphatidylinositol trisphosphate.

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important as factors for respiratory distress and other disorders. Although PIP_3 generated as a result of growth factor stimulation is synthesized by the action of phosphatidylinositol-3-kinase on phosphatidylinositol 4,5-bisphosphate, exposure of the cells to oxidative stress activates a novel synthetic pathway via type I PIP kinase acting on phosphatidylinositol 3,4-bisphosphate (**Fig. 1**) (15, 16). After the production of PIP_3, a host of signaling proteins with lipid recognition sites are localized to the plasma membrane and their signaling activity is enhanced or set in motion. Feedback loops that promote or control additional PIP_3 production have been described for processes such as cell polarity and chemotaxis (17). Clearly, PIP_3 is an important signaling molecule whose activity is central to many cellular processes, including those involved in disease (18). The implication of PIP_3-dependent signaling has received attention in cancer research, because deregulation of this signaling system [i.e., phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and SH2 (Src homology 2)-containing inositol phosphatase (SHIP)] facilitates tumor progression (19, 20). The lack of adequate methods has not previously permitted investigators to determine whether distinct species of PIP_3 might contribute to its broad signaling role or alter the formation of specialized proteolipidomic complexes that might be exploited pharmacologically. The importance of acyl chain specificity has been shown for diacylglycerol activation of protein kinase C (21, 22). Recent studies of the selectivity for phosphatidylinositol and phosphatidylcholine by phosphatidylinositol transfer protein suggests that head group chemistry is not the only determining factor in lipid binding affinity. Analysis of acyl chain length and saturation indicates that these are also critical factors in lipid binding and transfer (23, 24). For example, the increased levels of lysophosphatidic acid (LPA) observed in ovarian cancer patients have been implicated as important markers of the disease. An increased quantity of unsaturated fatty acid LPA species is found in patients with late-stage ovarian cancer, thus making it a potential target for cancer therapy (25). Interestingly, a recent report suggests that the acyl chain length of PIP_3 is an important determinant in the binding to the (CT-)SH_2 domain of the p85α subunit of phosphatidylinositol 3-kinase (26). The catalytic properties of type II phosphoinositide 5-phosphatases, and also their biological functions, are not only factors of the localization (or their regulatory domain) but are highly dependent on the fatty acid and head group composition of the lipid substrate (27).

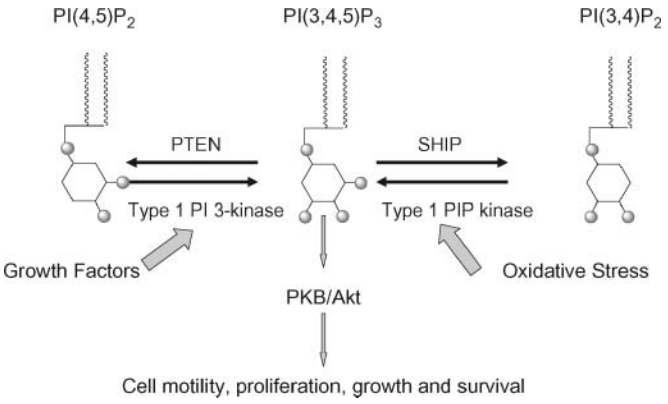

Fig. 1. Pathways of biosynthesis and metabolism of phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P_3]. PI, phosphatidylinositol; PIP, phosphatidylinositol monophosphate; PKB/Akt, serine/threonine kinase (also called protein kinase B); PTEN, phosphatase and tensin homologue deleted on chromosome 10; SHIP, SH2 (Src homology 2)-containing inositol phosphatase.

MATERIALS AND METHODS

RAW 264.7 cell and primary murine peritoneal macrophage protocols

RAW 264.7 cells are a macrophage-like, Abelson leukemia virus-transformed cell line derived from BALB/c mice. RAW 264.7 cells used by the Alliance for Cellular Signaling (AfCS) were obtained from the American Type Culture Collection (ATCC catalog number TIB-71, lot number 2263775), expanded, and stored in aliquots for use by AfCS laboratories. Stock vials of frozen AfCS cells were thawed and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES, and 2 mM L-glutamine at 37°C in a humidified atmosphere with 5% CO_2. For routine maintenance in culture (passage), cells were seeded at a confluence of ~10% (1×10^6 and 3×10^6 cells on 100 and 150 mm plates, respectively) and grown to a density of ~80%. For ligand stimulation, cells were seeded at 4×10^6 cells on 60 mm plates. Before assay, the medium was changed to DMEM supplemented with 20 mM HEPES, 2 mM L-glutamine, and 0.1 mg/ml BSA. After the addition of C5a, LPA, zymosan, macrophage colony-stimulating factor (MCF), or control medium (growth medium), the plates were placed on ice and the media were aspirated at the appropriate time points. The cells were washed with 1.5 ml of ice-cold PBS solution and pelleted, and PBS was aspirated. For comparison, reactions were also terminated by the addition of cold 10% trichloroacetic acid, which gave similar results (data not shown).

RAW 264.7 cells used by the LIPID MAPS consortium were obtained from the American Type Culture Collection (ATCC catalog number TIB-71, lot number 3002360). Likewise, a strain of RAW 264.7 cells commonly used at Vanderbilt Medical Center were originally obtained from the American Type Culture Collection (ATCC catalog number TIB-71). These additional sources for RAW 264.7 cell strains were all stored, grown and assayed according to the AfCS protocol described above.

Stimulation with LPA, C5a, and zymosan was carried out in all cultured and primary murine macrophage cells. MCF experiments were performed on RAW 264.7 cells used by the LIPID MAPS consortium. Peritoneal macrophages were harvested from CD-1/ICR mice (standard outbred strain). The lavage fluid from the mice was centrifuged to a pellet and resuspended in α-MEM containing 10% fetal calf serum and penicillin at 2 ml/mouse. The cells were plated onto 60 mm plates at 6 ml (three mice)/plate, incubated for 2 h, and then washed with warm PBS to remove the lymphocytes. The macrophages were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES, and 2 mM L-glutamine at 37°C in a humidified atmosphere with 5% CO_2 overnight.

Phosphatidylinositol cell pellet extraction procedure

Ice-cold 1:1 $CHCl_3/CH_3OH$ (400 μl) was added to each pellet and vortexed for 1 min, or until thoroughly mixed. Samples were

centrifuged at 9,000 rpm for 5 min at 4°C, supernatant decanted, and discarded. To the remainder of the cell pellet, 200 μl of 2:1 $CHCl_3/CH_3OH$ containing 0.25% 12 N HCl was added. Samples were vortexed for 5 min and then pulse spun. To the supernatant, 40 μl of 1 N HCl was added and vortexed for 15 s. Samples were centrifuged to separate the phases. The solvent from the collected lower layer was evaporated in a vacuum centrifuge (Labconco CentriVap Concentrator, Kansas City, MO), and lipid film was rapidly redissolved in 55 μl of 1:1:0.3 $CHCl_3/CH_3OH/H_2O$. Before analysis, 5 μl of 300 mM piperidine (28) was added, and the sample was vortexed and pulse spun.

Mass spectrometry analysis of phospholipid cell extracts

Mass spectral analysis was performed on either an MDS SCIEX 4000 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA) or a Finnigan TSQ Quantum triple quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA). Both instruments were equipped with a Harvard Apparatus syringe pump and an electrospray source. Samples were analyzed at an infusion rate of 10 μl/min in negative ionization mode over the range of *m/z* 400–1,200. Because of their very low abundance and detection only after stimulation, PIP_3 species cannot be quantitated or identified by traditional full scan or precursor ion scan (*m/z* 481) techniques. If much larger sample sizes were available, quantitation might be possible by precursor ion scan with the use of an internal standard such as 16:0 PIP_3 (phospholipids are presented with the class abbreviation preceded by xx:y, where xx is the total carbon atoms in the fatty acid chains and y is the number of double bonds). Instead, qualitative identification of the individual phosphatidylinositol phosphates present in the total lipid extracts was accomplished by electrospray ionization tandem mass spectrometry with a collision energy of 50 eV. Peaks corresponding to known PIP_3s were fragmented and inspected manually for the presence of the identification peaks (**Table 1**). A confirmed identification was achieved when key fragmentation peaks were greater than three times the signal-to-noise ratio. The lower limit of detection using this method was found to be <9 pmol/ml for 38:4 PIP_3 (Avanti Polar Lipids, Alabaster, AL). Data were collected with either the Analyst software package (Applied Biosystems) or the Xcalibur software package (ThermoFinnigan).

RESULTS AND DISCUSSION

PIP_3s constitute only ~0.25% of the inositol lipids in eukaryotic cell membranes. To minimize the interference of other phospholipids during analysis, a selective two-step extraction was used. First, the cell material was extracted with neutral solvents [somewhat similar to steps described previously (29)], and the resulting pellet was extracted with acidified solvents for quantitative recovery of polyphosphoinositides. The majority of noninositol phospholipids were extracted with neutral solvents, and no PIP_2 or PIP_3 species were detected in this extract.

Using electrospray ionization tandem mass spectrometry, we have surmounted many obstacles and can now identify a plethora of intact polyphosphoinositides, including PIP_3. Collision-induced dissociation of the peaks of interest yielded fragmentation patterns that were used to unambiguously identify the lipids present at a particular *m/z* value (**Fig. 2**) (30). The molecular weights of two PIP_3 compounds detected coincided with PIP_2 compounds (32:0 PIP_3/38:2 PIP_2 at *m/z* 1,049, and 32:1 PIP_3/38:3 PIP_2 at *m/z* 1,047). However, this in no way interfered with the identification process, because key fragments attributable only to PIP_3 species were used to classify these compounds. A list of the major fragments for the eight PIP_3 species observed to date is summarized in Table 1. A typical fragmentation pattern from a PIP_3 compound includes the two fatty acids (fatty acids 1 and 2), LPAs (LPA 1 and LPA 2) as well as their dehydration products (LPA-H_2O), and inositol-1,3,4,5-tetrakisphosphate fragments.

TABLE 1. Key fragments from the eight PIP_3 species identified by collision-induced dissociation tandem mass spectrometry

PIP_3	*m/z*	Fatty Acid 1 (*m/z*)	Fatty Acid 2 (*m/z*)	LPA 1 (*m/z*)	LPA 2 (*m/z*)	LPA-H_2O (*m/z*)	LPA-H_2O (*m/z*)
32:1	1,047	16:0 (255)	16:1 (253)	16:0 (409)		16:0 (391)	
32:0	1,049	16:0 (255)	16:0 (255)	16:0 (409)	16:0 (409)	16:0 (391)	16:0 (391)
34:1	1,075	16:0 (255)	18:1 (281)	18:0 (437)		16:0 (391)	18:1 (417)
		18:0 (283)	16:1 (253)			18:0 (419)	16:1 (389)
36:2	1,101	18:1 (281)	18:1 (281)	18:1 (435)	18:1 (435)	18:1 (417)	18:1 (417)
36:1	1,103	18:0 (283)	18:1 (281)			18:0 (419)	18:1 (417)
38:4	1,125	18:1 (281)	20:3 (305)	18:1 (435)			20:3 (441)
38:3	1,127	18:2 (279)		18:2 (433)			

PIP_3	303	321	339	383	401	419	463	481	499
32:1	+	+	+	+	+	+	+	+	+
32:0	+	+	+	+	+	+	+	+	+
34:1	+	+	+	+	+	+	+	+	
36:2	+	+	+	+	+	+	+	+	
36:1			+		+		+	+	
38:4	+		+		+		+	+	
38:3	+		+			+	+	+	

LPA, lysophosphatidic acid; PIP_3, phosphatidylinositol trisphosphate. The *m/z* 303, 321, and 339 peaks are inositol bisphosphate fragments [produced from phosphatidylinositol monophosphate (PIP), phosphatidylinositol bisphosphate (PIP_2), or PIP_3 species]; the *m/z* 383, 401, and 419 peaks are inositol trisphosphate fragments (produced from PIP_2 or PIP_3 species); and the *m/z* 463, 481, and 499 peaks are inositol-1,3,4,5-tetrakisphosphate fragments (only produced from PIP_3 species). + indicates that this fragment was observed during collision-induced dissociation tandem mass spectrometry analysis.

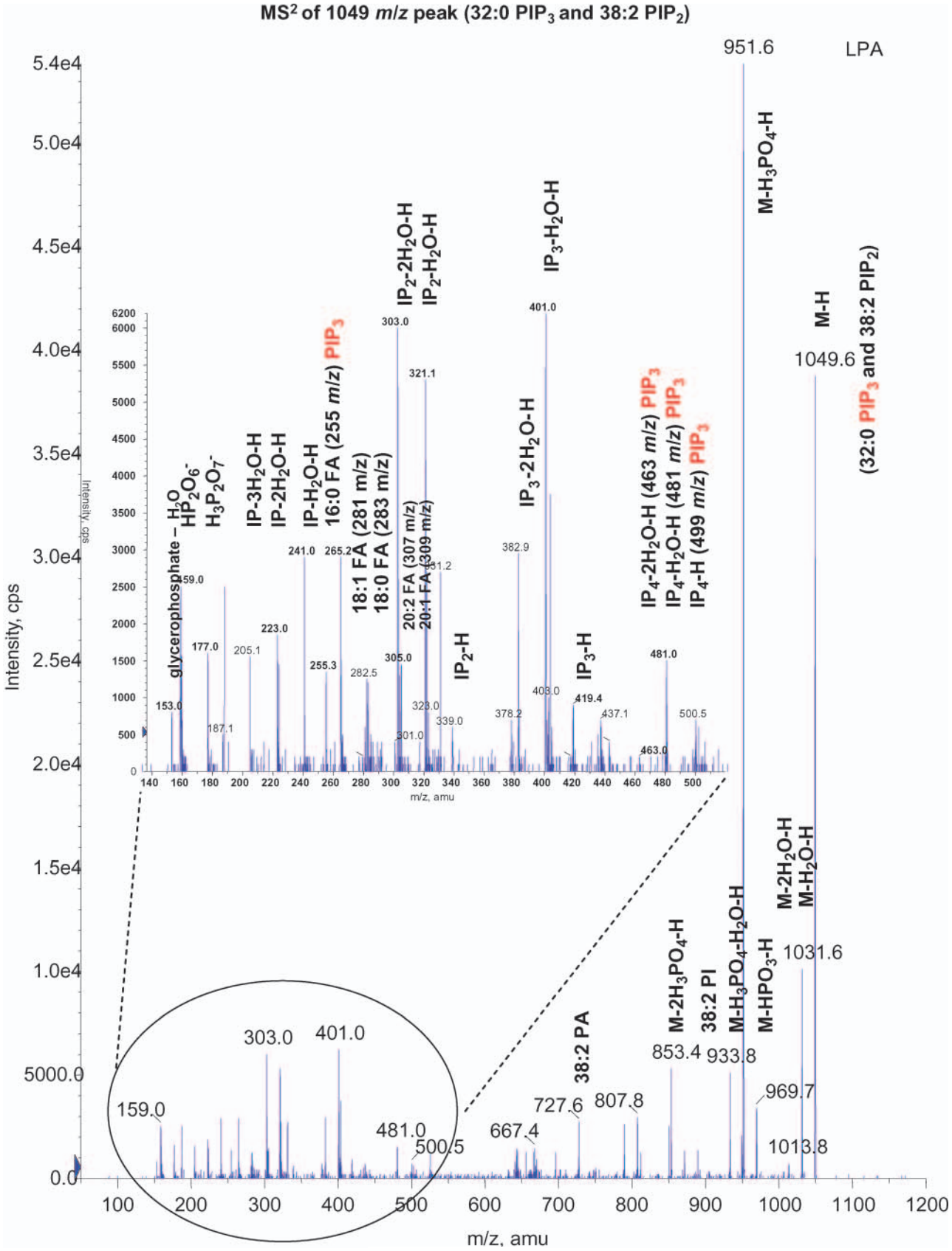

Fig. 2. RAW 264.7 cells stimulated with 2.5μM lysophosphatidic acid (LPA) for 30 s show accumulation of 32:0 phosphatidylinositol trisphosphate (PIP_3). Fragments highlighted in red can be attributed uniquely to PIP_3 species, allowing unambiguous identification. IP_4, inositol-1,3,4,5-tetrakisphosphate.

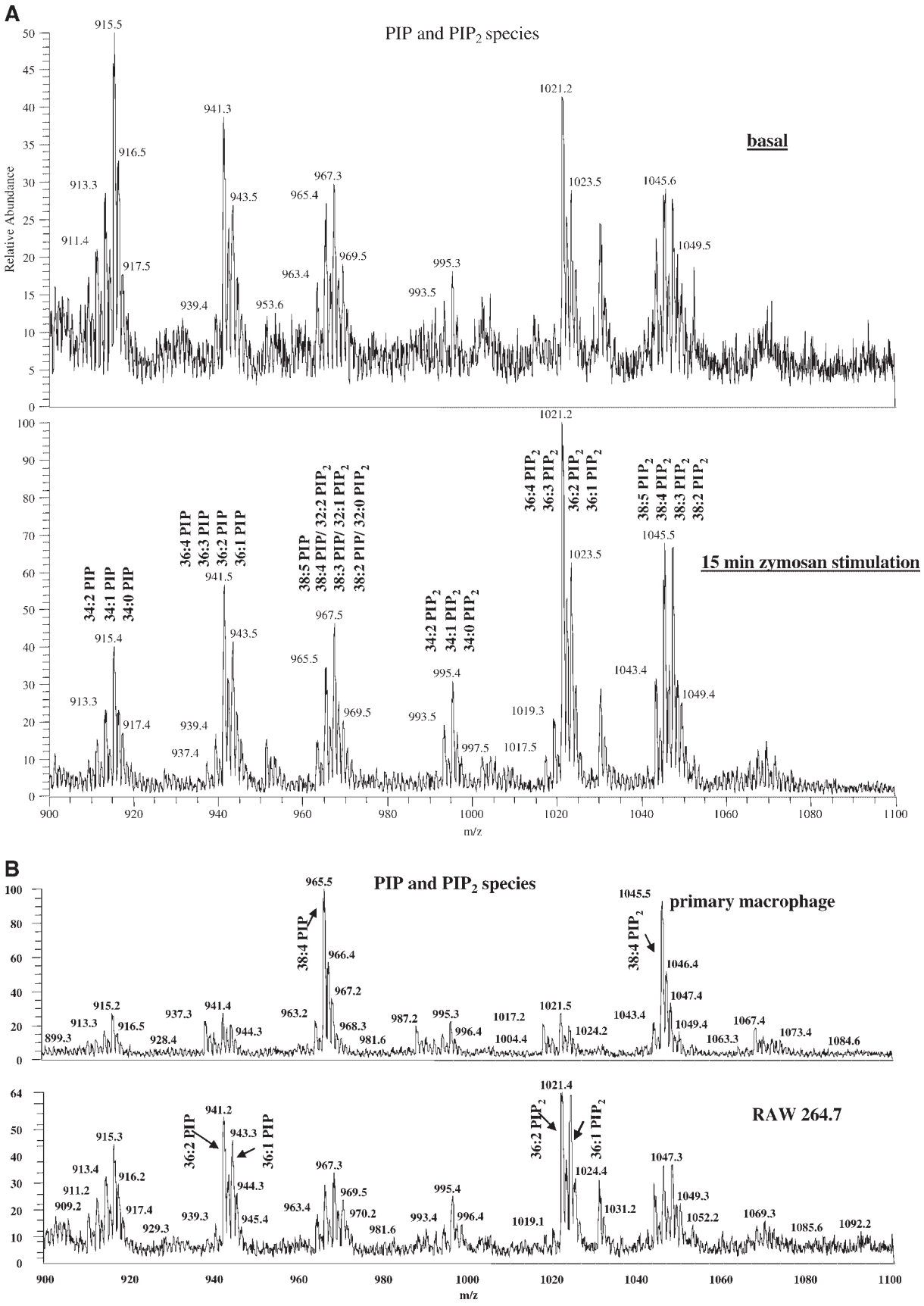

Fig. 3. Electrospray ionization mass spectrometry for PIP and phosphatidylinositol bisphosphate (PIP_2) compounds in basal and ligand-stimulated cells. A: When treated with 50 μg/ml zymosan for 15 min, RAW 264.7 cells showed a dramatic increase in PIP and PIP_2 levels compared with basal conditions. B: Unstimulated primary murine macrophages and RAW 264.7 phosphatidylinositol phosphate compositions are markedly different.

TABLE 2. PIP_3 compounds detected after LPA, C5a, zymosan, or MCF stimulation

Ligand	Concentration	Time	Detected PIP_3 Species
Primary murine macrophages			
LPA	2.5 μM	45 s	1,049, 1,075, 1,101, 1,125 (32:0, 34:1, 36:2, 38:4)
C5a	10 nM	45 s	1,101 (36:2)
Zymosan	125 μg/ml	10 min	1,075, 1,101, 1,125, 1,127 (34:1, 36:2, 38:4, 38:3)
RAW 264.7 cells			
LPA	2.5 μM	30 s	1,049, 1,101 (32:0, 36:2)
C5a	10 nM	45 s	1,049, 1,075, 1,101, 1,103, 1,125 (32:0, 34:1, 36:2, 36:1, 38:4)
Zymosan	125 μg/ml	10 min	1,049, 1,075, 1,101, 1,103 (32:0, 34:1, 36:2, 36:1)
MCF	400 pM	3 min	1,075, 1,101, 1,103, 1,125, 1,127 (34:1, 36:2, 36:1, 38:4, 38:3)

Parallel experiments were performed with different strains of RAW 264.7 cell lines obtained from various sources, including the Alliance for Cellular Signaling and LIPID MAPS projects. Experiments with LPA, C5a, and zymosan were performed with all cell lines. Macrophage colony-stimulating factor (MCF) stimulation was carried out in LIPID MAPS RAW 264.7 cells only.

As shown in **Fig. 3A**, agonist (zymosan) challenge of RAW 264.7 cells led to increased levels of a wide variety of PIP and PIP_2 species compared with basal levels. All of the polyphosphoinositides identified in basal RAW 264.7 cells were also detected in primary macrophages. In basal samples, the two systems differed in the levels of 36:2, 36:1, and 38:4 series phosphatidylinositol, PIP, and PIP_2 compounds. Primary macrophages have conspicuously higher levels of 38:4 series phosphoinositides compared with RAW 264.7 cells (Fig. 3B).

As shown in **Table 2**, different PIP_3 species were produced as a result of distinct ligand stimulation, suggesting differential mechanisms of enzyme activation or selective substrate availability triggered by the specific agonist. Although stimulation with 2.5 μM LPA elicited 32:0 and 36:2 PIP_3 species, treatment with 10 nM C5a, 400 pM MCF, or 125 μg/ml zymosan produced a wider range of molecular species. The stimulation times were selected by identifying the optimal time points for maximum PIP_3 activity measured by the peak of serine/threonine kinase phosphorylation and Ca^{2+} release (data not shown). We observed some differences between the molecular species generated in RAW 264.7 cells and primary macrophages for a given ligand, but in general there was significant conservation in the PIP_3 species generated. Interestingly, no inter-cell-type differences in PIP_3 content were observed among the three different sources of RAW 264.7 cells after LPA or C5a stimulation. This suggests that primary macrophage lipid signaling pathways are well conserved in the various RAW 264.7 cell strains available.

Using lipidomic analysis, we unequivocally identified ~75 phosphatidylinositol and phosphatidylinositol phosphate compounds (**Table 3**) according to their acyl composition. We have observed that the phosphatidylinositol phosphate "fingerprint" can vary greatly between different cell types. Additionally, results suggest that the PIP_3 species formed after cell stimulation are ligand-dependent. This finding implies that PIP_3 may not function as a single entity in cells. Rather, there appear to be eight or more species in the macrophage alone, functioning as a lipid second messenger. Whether these distinct species have differential binding preferences to downstream effector molecules remains to be determined. However, this virtual lipid array of signaling polyphosphoinositides allows us to begin the molecular dissection of various cellular outcomes regulated by differential molecular species of PIP_3 and to establish substrate-product relationships between essential signaling lipid pools that are associated with a specific cell surface receptor in a way that has not previously been possible.

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TABLE 3. Summary table of acyl chain composition and *m/z* values of phosphatidylinositol (PI), PIP, PIP_2, and PIP_3 species identified in RAW 264.7 macrophage cells

	32:0	32:1	32:2	34:0	34:1	34:2	36:0	36:1	36:2	36:3	36:4	38:0	38:1	38:2	38:3	38:4	38:5	40:4	40:5	40:6
PI	**809**	**807**	**805**	**837**	**835**	**833**	**865**	**863**	**861**	**859**	**857**	893	891	**889**	**887**	**885**	**883**	**913**	**911**	**909**
PIP	889	887	**885**	**917**	**915**	**913**	945	**943**	**941**	**939**	**937**	973	971	**969**	**967**	**965**	**963**	993	991	989
PIP_2	**969**	**967**	**965**	**997**	**995**	**993**	1,025	**1,023**	**1,021**	**1,019**	**1,017**	1,053	1,051	**1,049**	**1,047**	**1,045**	**1,043**	1,073	1,071	1,069
PIP_3	**1,049**	**1,047**	1,045	1,077	**1,075**	1,073	1,105	**1,103**	**1,101**	1,099	1,097	1,133	1,131	1,129	**1,127**	**1,125**	1,123	1,153	1,151	1,149

Boldface values indicate identified peaks.

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