Aggregation of receptors specific for the constant region of immunoglobulin G activates a repertoire of monocyte responses that can lead ultimately to targeted cell killing via antibody-directed cellular cytotoxicity. The high affinity receptor, FcyRI, contains no recognized signaling motif in its cytoplasmic tail but rather utilizes the \( \gamma \)-chain of FcRI as an accessory molecule to recruit tyrosine kinases for signal transduction. We show here that, in a human monocyte cell line primed with interferon-\( \gamma \), FcyRI mobilizes intracellular calcium stores using a novel pathway that involves tyrosine kinase coupling to phospholipase D and resultant downstream activation of sphingosine kinase. Moreover, FcyRI is not coupled to phospholipase C; hence, calcium release from intracellular stores occurred in the absence of any measurable rise in inositol trisphosphate. Finally, as this novel activation pathway is also shown to be responsible for mediating the vesicular trafficking of internalized immune complexes for degradation, it is likely to play a key role in controlling intracellular events triggered by FcyRI.

The macrophage-specific receptor (FcyRI) for the constant region (Fc) of IgG plays a central role in the clearance of immune complexes (1, 2). FcyRI belongs to a family of receptors for IgG that are distinguished by the affinity for ligand. While FcyRI is a high affinity IgG receptor, FcyRII and FcyRIII are both low affinity IgG receptors (reviewed in Refs. 1 and 2). Aggregation of FcyRI activates macrophages to undergo a repertoire of responses that can ultimately lead to cell killing through the process of antibody-directed cellular cytotoxicity, a critically important feature in the body’s defense against virus-infected cells and in cancer surveillance (3, 4). Immune complex aggregation of FcyRI initiates signal transduction events, which include protein tyrosine phosphorylation (5, 6) and tyrosine kinase-dependent calcium transients (7, 8). However, the cDNA for FcRI predicts an integral type I glycoprotein in which, unlike FcRIIA, the cytoplasmic tail contains no recognized signaling motifs (9). FcyRI has been shown to associate noncovalently with the signal-transducing \( \gamma \)-chain (10), which contains an immunoreceptor tyrosine activation motif (11, 12) in its cytoplasmic tail, and this association is thought to allow aggregated FcyRI to recruit and activate soluble tyrosine kinases (13). The \( \gamma \)-chain was originally identified in mast cells as a component of the high affinity IgE receptor, FcRI, but has subsequently been found in macrophages in the absence of the \( \alpha \)-chain of FcRI (14). Thus, although expressed in different cell types, the ligand recognition subunits (\( \alpha \)-chains) of FcRI and FcRI are able to use the same signal-transducing molecule. Recently, FcRI has been shown to mobilize calcium transients in a mast cell line through the activation of a novel pathway involving sphingosine kinase (15). However, the precise details of the signaling pathway and its relationship to tyrosine kinase activation are as yet unclear.

In this study, we demonstrate that FcyRI mobilizes calcium from intracellular stores by activating sphingosine kinase in the absence of phospholipase C activation and resultant generation of inositol 1,4,5-trisphosphate (InsP\(_3\)). We also show that FcyRI-stimulated activation of sphingosine kinase is downstream of phospholipase D activation and that both these enzymes are dependent on tyrosine kinase activation. Moreover, activation of this pathway is necessary and sufficient to account for intracellular calcium mobilization after FcyRI aggregation in cytokine-primed U937 cells and for efficient vesicular trafficking of internalized immune complexes for degradation.

**MATERIALS AND METHODS**

**Receptor Aggregation—**U937 cells, a human monocyte cell line (16), treated with 200 ng/ml interferon-\( \gamma \) for 18 h were used for all experiments (8, 17). For the biochemical assays, approximately 3 \( \times \) 10\(^6\) cells were harvested and incubated with 1 \( \mu \)g human monomeric IgG (Suretec) to occupy surface FcRI. Unbound IgG was removed by dilution and centrifugation of the cells. The cells were resuspended in ice-cold Hepes-buffered saline (HBS), and cross-linking antibody (goat anti-human IgG, 1:100 dilution) was added. The cells were then warmed to 37 °C and harvested at specified times for biochemical assays. Where the low affinity receptor was specifically aggregated using anti-FcRIIa, the cells were loaded with the monoclonal antibody 2e1 (1 \( \mu \)g) (Serotec) in the presence of saturating concentrations (3 \( \mu \)M) of human IgG4 (to block binding of the Fc portion of 2e1 to FcRI). After removal of excess antibody, anti-FcRIIA was aggregated by the addition of goat anti-mouse IgG F(ab) (1:100 dilution).

**Measurement of Sphingosine Kinase**—Sphingosine kinase was assayed as described by Olivera et al. (18). Briefly, reactions were terminated at the times specified in the figures by the addition of ice-cold phosphate-buffered saline (PBS). After centrifugation, the cells were resuspended in ice-cold 0.1 M phosphate buffer (pH 7.4) containing 20% glycerol, 1 mm mercaptoethanol, 1 mm EDTA, phosphatase inhibitors and DTT. The supernatant proteins were assayed for the incorporation of \(^3\)H into sphingosine by the addition of 1 \( \mu \)g/ml of \(^3\)HSphingosine and radioactive labelling was stopped by the addition of trichloroacetic acid and centrifugation. The radioactive supernatant was then analyzed by thin-layer chromatography on silica gel plates in chloroform-methanol-water (65:25:4, v/v/v) with choline and sphingosine as markers.

**Calcium Transient Measurements**—Calcium transients were measured in a fluorescent cytosolic calcium indicator, fura-2, as described by Gryniewicz et al. (19). Briefly, fura-2 was loaded into the U937 cells by incubation with 1 \( \mu \)g/ml of fura-2 for 10 min at room temperature. The cells were then washed and the fluorescence was measured as described in Materials and Methods.
(20 mM ZnCl₂, 1 mM sodium orthovanadate, and 15 mM sodium fluoride), protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF), and 0.5 mM 4-deoxypyridoxine. Cells were disrupted by freeze thawing and centrifuged at 105,000 × g for 90 min at 4 °C. Supernatants were assayed for sphingosine kinase activity using sphingosine (Sigma) and [γ³²P]ATP (2 μCi, 5 μM) as specified by Olivera et al. (18). After incubation, products were separated by TLC on silica gel G60 using chloroform/methanol/acetic acid/water (90:90:15:6) and visualized by autoradiography. The radioactive spots corresponding to sphingosine phosphate were scraped and counted in a scintillation counter.

Measurement of Sphingosine 1-Phosphate—Sphingosine 1-phosphate concentrations were measured as described by Olivera and Spiegel (19). Briefly, cells were preincubated overnight (15 h) in media containing [³H]serine (20 μCi/ml) to label cellular sphingolipids and free sphingosine pools. Following labeling, the cells were washed in ice-cold RPMI 1640, 10 mM HEPES, 0.1% bovine serum albumin (RHB medium) and resuspended in ice-cold RHB medium containing 0.1 mM l-canaline and the pyridoxal phosphate analog 4-deoxypyridoxine (0.5 mM) to inhibit the pyridoxal-dependent sphingosine-1-phosphate lyase. Cells were then stimulated by the addition of cross-linking antibody and warming

**FIG. 1.** Mobilization of intracellular calcium stores by FcγRI associated with activation of sphingosine kinase and the appearance of sphingosine 1-phosphate. A, cytosolic calcium concentrations in IFN-γ-treated U937 cells following aggregation of FcγRI. The arrow indicates the addition of either 25 μM DHS (dashed line) or vehicle (solid line). Cross-linking antibody (XL) was added to the cuvette at 300 s, and thapsigargin was added at 475 s. Thapsigargin (250 nM) was added to assess the viability of the stores. A typical trace from 5 separate experiments is shown. B, activation of sphingosine kinase by FcγRI aggregation and the effect of tyrosine kinase inhibition. IgG-loaded FcγRI was aggregated by the addition of cross-linking antibody (Cross link [XL]), and sphingosine kinase was assayed in cell extracts at specified time points after aggregation. Results were compared with non-cross-linked controls (No Cross-link Control) and to cells pretreated with genistein (0.37 mM) for 30 min prior to the addition of cross-linking antibody to inactivate tyrosine kinases (XL + genistein). Results shown are the mean ± S.D. for triplicate measurements at each time point. The results shown are typical from three separate experiments. C, effect of varying concentrations of genistein on sphingosine kinase activation by FcγRI aggregation. Sphingosine kinase activity was measured in cells 30 s after aggregating FcγRI in cells pretreated for 30 min with varying concentrations of genistein (0.01, 0.03, 0.1, 0.3, and 1.0 mM) and compared with untreated control cells. Results shown are the mean ± S.D. for triplicate measurements at each concentration. The results shown are typical from three separate experiments. D, increase in sphingosine 1-phosphate concentrations following FcγRI aggregation and effect of tyrosine kinase inhibition. Sphingosine 1-phosphate concentrations were measured in cells following aggregation of FcγRI (Cross link [XL]) and compared with non-cross-linked control cells (No Cross-link Control) and to cells pretreated with genistein (0.37 mM) for 30 min prior to the addition of cross-linking antibody (XL + genistein). Results shown are the mean ± S.D. for triplicate measurements at each time point. The results shown are typical from three separate experiments.
to 37 °C, and the reactions were terminated at selected times. Cells were harvested by centrifugation, and the lipids were extracted and analyzed by TLC on silica gel G60 using chloroform/methanol/acetic acid/water (90:90:15:6). Standard sphingosine 1-phosphate was applied with the samples, and the lipids were visualized using iodine vapors. Bands corresponding to sphingosine were detected using iodine vapors on the plate and quantified by liquid scintillation spectrometry. Results were calculated as a percentage of the total radioactivity incorporated in the lipids. Data presented are the mean ± S.D. of triplicate measurements, and the results shown are representative of three different experiments.

Measurement of Inositol Phosphate and DAG Generation—Inositol phosphates were assayed essentially as described by Harnett and Harnett (20). Briefly, U937 cells were labeled with Na32P with 1 μCi/106 cells) for 15 min at 37 °C. The cells were washed twice and resuspended in 100 μl of PBS, transferred to glass vials, and incubated with the addition of 0.94 ml of chloroform/methanol (1:2) on ice for 10 min. A Bligh-Dyer phase separation was achieved by the addition of 0.31 ml of chloroform and 0.31 ml of water, vortexing, and centrifugation at 20 000 g for 5 min. Levels of [32P]InsP3 or total [32P]Insphosphates (reaction mixture containing 10 mM LiCl) were determined by liquid scintillation counting of fractions eluted following Dowex (formate form) ion exchange chromatography of aliquots of the supernatants. Lipids were separated on TLC plates as a percentage of the total radioactivity incorporated in the lipids. Data presented are the mean ± S.D. of triplicate measurements, and the results shown are representative of three different experiments.

DAG Assay—Mass DAG was measured as described by Briscoe et al. (21). The lower organic phase of Bligh-Dyer extractions was dried in vacuo, and the lipids were solubilized in a Triton X-100/phosphatidylinerine mixture. Briefly, phosphatidylserine (30 μl, supplied as 25 mM stock from Lipid Products) was dried under nitrogen and then phosphate-buffered saline (PBS). The tubes were incubated at 30 °C for 30 min. The reaction was stopped by the addition of 1 ml of chloroform/methanol/HCl (150:300:2). After 10 min, 300 μl of chloroform and 400 μl of H2O were added. The tubes were vortexed and centrifuged at 20 000 g for 5 min to form phase separation and washed once with 1 ml of a synthetic upper phase. The samples were then dried in vacuo and solubilized in 0.2 μl of chloroform/methanol (2:1) (v/v) in 50 μl of PBS. Following labeling, the cells were washed twice with PBS and incubated with the addition of 20 μl of 1 μCi [3H]palmitic acid (5 Ci/mmol) in RPMI 1640 medium containing 0.1% (v/v) fetal calf serum for 16 h. Following centrifugation, the cells were washed in ice-cold HBS and resuspended at 2 × 107 cells/ml, and incubated at 37 °C for 15 min in HBS medium containing butan-1-ol (0.5% final). Specific Fc receptors were cross-linked as described above, and after the times indicated, cells were harvested by centrifugation at 20 000 g for 15 min, and the cell lysates were sonicated in 2.5 ml of 10 mM imidazole buffer, pH 6.6, containing 0.6% (v/v) Triton X-100. Samples were sonicated 20 times for 5 min. The tubes were then centrifuged at 20 000 g for 15 min, and the cell lysates were incubated with an agarose-CLAP precipitation lysis buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml CLAP (1 mg/ml each of chymostatin, leupeptin, antipain, and pepstatin), 1 mM sodium orthophosphate, and 1 mM sodium fluoride for 30 min. Cellular debris was removed by centrifugation at 13 000 rpm for 15 min, and the cell lysates were incubated with an agarose-linked anti-phosphotyrosine monoclonal antibody (clone 4G 10; Upstate Biotechnology, Inc.) at 4 °C overnight. Phosphotyrosine proteins were then harvested by centrifugation of the agarose beads and were then dissociated from the beads by boiling in sample buffer (22) containing 50 mM dithiothreitol for 15 min. Samples were run in a 10% SDS-polyacrylamide gel (23). After electrophoresis, the proteins were transferred to a nitrocellulose membrane (0.2-μm pore size) as described by Tobiw et al. (24). The presence of tyrosine-phosphorylated proteins was then detected by Western blotting with a monoclonal anti-phosphotyrosine monoclonal antibody (clone 4G 10; Upstate Biotechnology). Western blots were developed using the ECL system (Amersham Pharmacia Biotech).

Measurement of Cytosolic Calcium—Cytosolic calcium was measured in cell populations via 37 °C using a Cairn Research Spectrophotometer system (Cairn Research Ltd.). Excitation wavelengths of 340, 360, and 380 nm were provided by a filter wheel rotating at 35 Hz in the light path. Emitted light was filtered by a 485-nm-long pass filter, and samples were averaged to give a data point every 500 ms. The background-corrected 340/380 ratio was calibrated using the method of Gryniewicz et al. (25). Following each experiment, cells were lysed by the addition of 50 μl digitonin in the presence of external 2 μM CaCl2 to give a final R0 value. R0 values were subsequently determined by the addition of 20 mM EGTA (pH 7.4) in the presence of an equimolar concentration of Tris base.

Measurement of Endocytosis and Rate of Trafficking for Degradation—Interferon-γ (IFN-γ)-treated cells were harvested and washed in ice-cold PBS. Cells were then centrifuged and resuspended in cold saline (PBS), 1% bovine serum albumin. The cell membranes were then loaded with 125I-labeled IgG as described previously (17). After removal of nonbound radiolabel by dilution and centrifugation, cross-linking antibody was added, and the cells were warmed to 37 °C for the given times.

Endocytosis—The rate of endocytosis was assessed by measuring the rate of internalization of radiolabeled surface immune complexes. At each time point, the cells were harvested and washed in ice-cold PBS (pH 7.4), and this was counted in a Packard γ-counter to provide a measure of the total counts bound to the cell surface. To measure the proportion of radiolabeled immune complexes internalized after incubation at 37 °C, any surface-bound radiolabeled immune complexes can be stripped from the cell by incubating the cells in ice-cold acidified PBS (pH 2.0) for 5 min to strip off cell surface radiolabeled immune complexes (17). The cells were then centrifuged, and the pellets were counted in a Packard γ-counter to yield the counts that had been internalized, or the cell-associated counts. The cell-associated counts for each time point were then expressed as the percentage of total counts bound at time 0 to provide a measure of the rate of internalization of the immune complexes.

Degradation—After warming the cells to 37 °C for long time intervals, the proportion of cell-associated counts was observed to fall. To determine whether this reduction in cell-associated counts represented the degradation of the immune complexes by the cells or the degradation of the immune complexes on the cell surface, the cell incubation was examined for the presence of trichloroacetic acid-soluble radiolabel indicating that the radiolabeled IgG had been degraded. Thus, cells were also harvested at the same time points to measure the rate of degradation of the internalized counts. Cells were centrifuged, the supernatants were harvested, and trichloroacetic acid was then added to these supernatants. After incubation on ice for 60
Phospholipase D and not phospholipase C is activated by aggregation of FcγRI in IFN-γ-primed cells. A, total InsPs accumulation over 20 min following aggregation of Fcγ receptors. Inositol phosphate accumulation over 20 min was measured following the coupling to Phospholipase D and Sphingosine Kinase.

Fig. 2. Phospholipase D and not phospholipase C is activated by aggregation of FcγRI in IFN-γ-primed cells. A, total InsPs accumulation over 20 min following aggregation of Fcγ receptors. Inositol phosphate accumulation over 20 min was measured following the coupling to Phospholipase D and Sphingosine Kinase.
min, the samples were centrifuged at 12,000 × g at 4 °C, and the supernatants were counted to provide a measure of the trichloroacetic acid-soluble counts in the supernatant. The results were expressed as a percentage of the initial cell-associated counts at time 0.

The results shown are the mean ± S.D. of triplicate measurements and are representative of three different experiments.

RESULTS

Aggregation of FcyRI Activates Sphingosine Kinase in a Tyrosine Kinase-dependent Manner—In IFN-γ-primed U937 cells, aggregation of FcyRI with surface-bound immune complexes results in calcium transients in the form of a single spike (see Ref. 8 and Fig. 1A). The FcyRI-associated accessory transducing molecule, γ-chain, has recently been reported to mobilize calcium via activation of sphingosine kinase when coupled to the high affinity IgE receptor, FcεRI (15). Thus, to compare the nature of this FcyRI-calium response to that of FcεRI, the effect of DL-threo-dihydrosphingosine (DHS) on the release of calcium from intracellular stores was determined. Pretreatment of cells with 25 μM DHS completely abolished the FcyRI-mediated rise in cytosolic calcium, indicating that intracellular calcium stores are mobilized in these cells in a similar fashion to that observed for FcεRI in mast cells (15). The calcium stores were intact in cells treated with DHS, since the subsequent addition of thapsigargin (250 nm) resulted in a prompt increase in cytosolic calcium, thereby demonstrating that the failure to observe a rise in calcium following aggregation of FcyRI in cells pretreated with DHS was not secondary to depletion of intracellular calcium stores.

Since DHS acts as a competitive inhibitor of sphingosine kinase, the activity of this enzyme after FcyRI aggregation was next assessed. Aggregation of FcyRI stimulated a prompt increase in sphingosine kinase activity, which was detectable within 30 s (Fig. 1B). Sphingosine kinase activation by FcyRI aggregation in these cytokine-primed cells was dependent on tyrosine kinase activation, since treatment of the cells with genistein (0.37 mM) completely abolished the response (Fig. 1B). Pretreatment with genistein at a lower concentration (0.1 mM) also completely inhibited FcyRI activation of sphingosine kinase (Fig. 1C), although concentrations below this only resulted in partial inhibition (Fig. 1C).

In parallel with the activation of sphingosine kinase, FcyRI aggregation resulted in a prompt increase in the concentration of sphingosine 1-phosphate in these cells (Fig. 1D). The concentration of sphingosine 1-phosphate peaked 30 s after receptor aggregation, and although levels fell gradually thereafter, concentrations remained elevated above control values 5 min after receptor aggregation. Pretreatment of cells with genistein (0.37 mM) completely abolished the FcyRI-mediated increase in sphingosine 1-phosphate generation.

Aggregation of FcyRI Activates Phospholipase D and Not Phospholipase C in a Tyrosine Kinase-dependent Manner—Since immune complex aggregation of FcyRI has previously been reported to lead to tyrosine phosphorylation of phospholipase Cγ1 (5) with presumed generation of InsP3 and DAG, the role of this phospholipid signaling pathway in mediating the cytosolic calcium response was also investigated. Surprisingly, no increase in InsP3 could be detected (data not shown). Since InsP3 generation can be transient in nature, the accumulation of total inositol phosphates (InsPs) was measured to ensure that any small transient InsPs signals did not go undetected. No accumulation of total InsPs over 20 min could be detected in IFN-γ-primed U937 cells after aggregation of FcyRI (Fig. 2A).

Phospholipase C signaling was, however, functional in these cells, since aggregation of a related immune receptor, the low affinity IgG receptor (FcγRIIa), using monoclonal antibodies resulted in an easily measurable accumulation of InsP3 (data not shown) and total InsPs (Fig. 2A). Unlike FcyRI, the low affinity receptor possesses an integral, albeit unconventional, immunoreceptor tyrosine activation motif in its cytoplasmic tail; the tyrosine residues are separated by an unusually long intervening sequence (26). Taken together, these data indicate that the high affinity receptor, FcεRI, mobilizes calcium stores through a novel pathway that, unlike the low affinity receptor (FcγRIIa), does not involve InsP3.

Interestingly, despite the lack of generation of InsPs over 20 min, mass DAG concentrations were elevated following aggregation of FcyRI (Fig. 2B). Thus, in an attempt to delineate alternative lipid signaling pathways involved in mediating the response to FcεRI, DAG was measured in the presence of 0.3% butan-1-ol to block the generation of DAG derived from PtdOH resulting from PLD activation (21). Under these conditions, the primary alcohol, butan-1-ol, traps the phosphatidyl-moiety generated by PLD-mediated hydrolysis of phosphatidylcholine as PtdBu; PtdBu is not a substrate for the enzyme, phosphatidic acid phosphohydrolase, that converts PtdOH to DAG (21). FcεRI-coupled DAG was indeed shown to be derived from Pt-DOH generated by phospholipase D activation, since pretreating cells with 0.3% butan-1-ol completely abolished the receptor-stimulated rise in mass levels of DAG (Fig. 2B).

Activation of phosphatidylcholine-specific phospholipase D (PtdCho-PLD) following aggregation of FcyRI was demonstrated by the definitive transphosphatidylation assay (21). These experiments showed that aggregation of Fcγ receptors in IFN-γ-primed, [3H]palmitate-labeled cells stimulated activation of PtdCho-PLD, as evidenced by substantial generation of [3H]PtdBu, in the presence of butan-1-ol, over a 30-min time course (Fig. 2C). Higher concentrations of butan-1-ol (1%) resulted in no further increase in measured [3H]PtdBu compared with cells incubated with 0.3% butan-1-ol, indicating that all of the phosphatidyl moiety generated by PtdCho-PLD aggregation of human monomeric IgG (FcγRI XL) in IFN-γ-treated U937 cells in the presence of lithium chloride (10 mM) and compared with accumulation following the specific aggregation of FcγRIIa (FcγRIIa XL) in the cells treated in an identical way. Results shown are the mean ± S.D. for triplicate measurements at each time point. The results shown are typical from three separate experiments. B, accumulation of DAG over 20 min following aggregation of Fcγ receptors. DAG was measured in cells 20 min after receptor cross-linking in cells preincubated with 0.3% butan-1-ol (XL + butan-1-ol) and compared with untreated cells (XL). The effect of butan-1-ol on basal levels was also measured (Control and Control + butan-1-ol). Results shown are the mean ± S.D. for triplicate measurements at each time point. The results shown are typical from three separate experiments. C, activation of PtdCho-PLD following aggregation of Fcγ receptors. The time course of accumulation of [3H]PtdBu was measured, using the PtdCho-transphosphatidylation assay. The accumulation of [3H]PtdBu in cells following the addition of the cross-linking antibody (XL) was compared with cells loaded with monomeric IgG but with the cross-linking antibody omitted (No XL). Cells were harvested at 5, 10, 20, and 30 min. Results shown are the mean ± S.D. for triplicate measurements at each time point. The results shown are typical from three separate experiments. D, effect of varying concentrations of butan-1-ol and butan-2-ol on measurement of [3H]PtdBu. Cells were preincubated with either butan-1-ol (0.03, 0.2, 0.3, and 1.0%) or butan-2-ol over the same range, and the total [3H]PtdBu accumulation over 20 min following the addition of the cross-linking antibody was measured. Results shown are the mean ± S.D. for triplicate measurements at each time point. The results shown are typical from three separate experiments. E, activation of PtdCho-PLD following aggregation of Fcγ receptors. The accumulation of [3H]PtdBu was measured, using the PtdCho-transphosphatidylation assay, in cells 20 min after aggregation of FcγRI. The activity was compared with cells pretreated with genistein for 30 min, and the effect of varying concentrations of genistein (0.01, 0.03, 0.1, 0.3, and 1.0 mM) was measured. Results shown are the mean ± S.D. for triplicate measurements at each time point. The results shown are typical from three separate experiments.
FIG. 3. Sphingosine kinase activation is downstream of PtdCho-PLD activation. A, sphingosine kinase activity following FcγRI aggregation in cells treated with butan-1-ol. Cells were preincubated with 0.3% butan-1-ol and harvested at given times after aggregation of FcγRI.
is trapped at the lower concentration of primary alcohol. Lower concentrations of butan-1-ol (0.1%) resulted in less measurable \(^{3}H\)PtdBut. Thus, the optimal concentration of butan-1-ol to trap the phosphatidyl moiety is 0.3% (Fig. 2D). The specificity of the measurement was confirmed using butan-2-ol, which is unable to trap the phosphatidyl moiety generated by PtdCho-PLD. No \(^{3}H\)PtdBut could be detected in cells preincubated with butan-2-ol even at 1% preincubation.

The increase in PtdCho-PLD activity following receptor aggregation was tyrosine kinase-dependent, since it was completely abolished by treating the cells with genistein. Moreover, the concentration dependence of genistein-mediated inhibition of PtdCho-PLD (Fig. 2E) showed a similar profile to that obtained for sphingosine kinase coupling (Fig. 1C).

Thus, FcyRI is coupled through tyrosine kinases to the activation of PtdCho-PLD and sphingosine kinase.

**Activation of Sphingosine Kinase Is Downstream of Phospholipase D Activation**—To assess the relative relationship of activation of PtdCho-PLD and sphingosine kinase, studies were initially undertaken to explore comparative kinetics of activation and use of selective inhibitors. However, comparison of the relative kinetics was complicated by the difference in the assay characteristics for measuring sphingosine kinase and phospholipase D. Thus, sphingosine kinase is measured as an in vitro kinetic kinase assay, whereas the assay for phospholipase D relies on the accumulation of a nonhydrolyzable product. The difference in assay characteristics, therefore, precluded definitive early comparative time course analysis. The relationship of phospholipase D and sphingosine kinase activation was therefore addressed by examining selective inhibitors of the two enzymes.

To determine whether sphingosine kinase activation was upstream or downstream of phospholipase D, cells were preincubated with butan-1-ol or butan-2-ol for 20 min before aggregation of FcyRI, and the resultant sphingosine kinase activity was compared with that of control pretreated cells. Pretreating cells with butan-1-ol (0.3%) completely abolished the normal sphingosine kinase response to FcyRI aggregation (Fig. 3A). Consistent with these results, the rise in sphingosine 1-phosphate observed after aggregation of FcyRI was also blocked by pretreating cells with butan-1-ol (0.3%) (Fig. 3B).

These data suggested that activation of sphingosine kinase is dependent on the activation of phospholipase D and generation of PtdOH. To assess this in detail, the effect on peak (30 s after receptor aggregation) sphingosine kinase activity of preincubating cells with varying concentrations of butan-1-ol previously shown to influence phospholipase D was examined, and the effects of these concentrations were compared with the same concentrations of butan-2-ol, which does not influence phospholipase D (Fig. 2D). Peak activity of sphingosine kinase following FcyRI aggregation was abolished by incubating cells with either 0.3 or 1.0% butan-1-ol but was completely unaffected by preincubation with butan-2-ol even at the highest concentration (Fig. 3C). Incubation of cells with a lower concentration of butan-1-ol (0.1%) partially inhibited peak sphingosine kinase activity. Of interest, 0.1% butan-1-ol resulted in lower concentrations of \(^{3}H\)Ptd-But (Fig. 2D), suggesting that, at this lower concentration, butan-1-ol is only able to trap a proportion of the phosphatidyl moiety generated by phospholipase D and that as a result some phosphatidic acid may be produced.

These data using butan-1-ol indicates that phospholipase D is upstream of sphingosine kinase. Consistent with this observation, DHS, a competitive inhibitor of sphingosine kinase, had no effect whatsoever on phospholipase D activation at all concentrations examined, even up to 100 \(\mu M\) (Fig. 3D). The potency of DHS on sphingosine kinase was measured directly; DHS at concentrations of 30 \(\mu M\) and above completely abolished the peak sphingosine kinase activity observed after FcyRI aggregation; 10 \(\mu M\) DHS inhibited peak sphingosine kinase activity by about 75% (Fig. 3E).

Taken together, these data clearly indicate that activation of sphingosine kinase is secondary to activation of phospholipase D and generation of PtdOH.

**Tyrosine Phosphorylation Is Triggered Promptly by Aggregation of FcyRI and Is Upstream of both Phospholipase D and Sphingosine Kinase**—Tyrosine phosphorylation events were monitored in these cytokine-primed U937 cells after aggregation of FcyRI by immunoprecipitating tyrosine-phosphorylated proteins with a monoclonal antibody to phosphotyrosine. Consistent with other reports (5–7, 27, 28), the addition of cross-linking antibody to form surface immune complexes resulted in the prompt appearance of a large number of tyrosine-phosphorylated proteins. Preincubating cells with either butan-1-ol (0.3%) or DHS (25 \(\mu M\)) did not influence the pattern of tyrosine phosphorylation (Fig. 4), results consistent with our findings that both PtdCho-PLD (Fig. 2C) and sphingosine kinase (Fig. 1C) activation are downstream of tyrosine kinase activation.

**Activation of Phospholipase D Is Necessary for both Mobilization of Intracellular Calcium and for Trafficking of Immune Complexes for Degradation**—The release of intracellular stores of calcium by aggregation of FcyRI was significantly inhibited by pretreating the cells with 0.3% butan-1-ol (Fig. 5A), thus providing further support for the role of this pathway in mobilizing calcium and the concept that PtdCho-PLD is upstream of sphingosine kinase. The possibility that butan-1-ol affected calcium mobilization through nonspecific effects was ruled out, since the subsequent addition of thapsigargin (25 \(\mu M\)) resulted in a prompt response in cytosolic calcium. In addition, butan-1-ol had no effect on the Ins\(_3\)P\(_3\)-dependent mobilization of calcium following aggregation of the related low affinity receptor, FcyRIIa (Fig. 5A). The difference in release of calcium after thapsigargin is not likely to be significant following manual injection as undertaken here. Although the speed of calcium release by thapsigargin can be influenced by a number of in-

for measurement of sphingosine kinase activity. Results (XL + butan-1-ol) were compared with control cells (Cross Link XL). The results shown are the mean ± S.D. for triplicate measurements at each time point and are typical from three separate experiments. B, sphingosine 1-phosphate concentrations in cells following receptor aggregation in cells treated with 0.3% butan-1-ol (XL + butan-1-ol) compared with control untreated cells (Cross link XL). Results shown are the mean ± S.D. for triplicate measurements at each time point. The results shown are typical from three separate experiments. C, effect of varying concentrations of butan-1-ol or butan-2-ol on peak sphingosine kinase activity after FcyRI aggregation. Sphingosine kinase activity was measured in cells 30 s after aggregating FcyRI in cells pretreated for 30 min with varying concentrations of butan-1-ol (0.03, 0.1, 0.3, and 1.0%) and compared with cells incubated with butan-2-ol over the same concentration range. Results shown are the mean ± S.D. for triplicate measurements at each concentration and are typical from three separate experiments. D, effect of preincubating cells with varying concentrations of DHS on FcyRI-mediated phospholipase D activation. PLD activity was measured using the transphosphatidylating accumulation assay (over 20 min) in cells following the aggregation of FcyRI in cells preincubated with varying concentrations of DHS (1, 3, 10, 30, and 100 \(\mu M\)). Results shown are the mean ± S.D. for triplicate measurements at each concentration and are typical from three separate experiments. E, effect of preincubating cells with varying concentrations of DHS on FcyRI-mediated sphingosine kinase activation. Sphingosine kinase activity was measured in cells 30 s after aggregating FcyRI in cells preincubated with varying concentrations of DHS (1, 3, 10, 30, and 100 \(\mu M\)). Results shown are the mean ± S.D. for triplicate measurements at each concentration. The results shown are typical from three separate experiments.
tracellular factors such as the amount of calcium in the stores, it is well recognized that there is considerable variability between runs for thapsigargin-mediated calcium release, and the largest influence is the rate of addition of thapsigargin and its mixing in the cuvette (29, 30).

As observed previously (17), the internalization of surface-bound immune complexes is very rapid in IFN-γ-treated U937 cells. The cell-associated counts plateau between 15 and 30 min. However, over prolonged incubations, the internalized cell-associated counts were found to diminish gradually in the control cells such that, by 120 min, approximately 50% of the cell-associated counts had been lost. This reduction was entirely matched by the appearance of counts in the culture media of the cells, and these counts were not precipitable by trichloroacetic acid (TCA) (Fig. 5B). The rate of appearance of these trichloroacetic acid-soluble counts in media is an indication of the rate of lysosomal degradation of the radiolabeled immune complexes (31) and is, therefore, a sensitive measure of the rate of intracellular trafficking of internalized immune complexes from endosomes to lysosomes.

Pretreatment of cells with butan-1-ol (0.3%) to inhibit PtdOH generation appeared to reduce to a small extent the initial phase of endocytosis (peak percentage of surface-bound counts internalized by 93 ± 3%; butan-1-ol-treated cells, 77 ± 2%). Nonspecific effects of the alcohol were eliminated, since butan-2-ol (0.3%) had no effect on the rate of endocytosis (peak percentage of surface bound counts internalized butan-
2-ol-treated cells, 90 ± 3%). Following longer time intervals after internalization of immune complexes, the amount of radiolabel trapped inside the cells gradually decreased in the untreated cells and in cells treated with butan-2-ol (0.3%) such that about 50% of the initial internalized radiolabel had been lost from the cells after 2 h of incubation. This loss of cell-associated counts was entirely matched by the appearance in the cell supernatant of radiolabel in a form that was soluble in trichloroacetic acid. Thus, after 2 h, 47 ± 2% of the initial counts in the control cells appear as trichloroacetic acid-soluble counts within the supernatant; this is a measure of trafficking of immune complexes for lysosomal degradation (31). Treatment of cells with butan-1-ol significantly inhibited trafficking of immune complexes for degradation. Thus, the rate of loss of cell-associated (internalized) counts was significantly slowed in cells treated with butan-1-ol (after a 2-h incubation, the percentage of counts remaining internalized for control cells was 43 ± 5%; for butan-1-ol-treated cells, it was 63 ± 3%). In addition, the rate of appearance of trichloroacetic acid-soluble counts in the media over prolonged incubations was significantly slower for cells pretreated with 0.3% butan-1-ol compared with the control untreated cells or cells treated with butan-2-ol (Fig. 5B). Thus, following 120 min of incubation, only 19 ± 0.5% of counts appeared as trichloroacetic acid-soluble counts in the media of cells treated with butan-1-ol in contrast to approximately 45 ± 1% for the control cells and 40 ± 0.4% for cells treated with butan-2-ol.

Consistent with the biochemical data defining the signaling pathway, the inhibition of sphingosine kinase with DHS (25 μM) also significantly inhibited trafficking of immune complexes for lysosomal degradation (Fig. 5B). Thus, activation of this intracellular signaling pathway involving phospholipase D and sphingosine kinase is required for the appropriate trafficking of internalized immune complexes along the degradative pathway.

DISCUSSION

Taken together, these data indicate that FcγRI in cytokine-primed U937 cells is coupled through tyrosine kinase activation to a novel pathway responsible for mobilizing calcium transients through an InsP3-independent route and for trafficking internalized immune complexes for degradation. This novel pathway involves the activation of PtdCho-PLD, in the absence of measurable activation of phospholipase C, and this is upstream of activation of sphingosine kinase, which generates sphingosine 1-phosphate.

Sphingosine 1-phosphate has been proposed previously to play a role in mobilizing calcium from intracellular stores (32–34). However, this proposal has proven highly controversial due to the presence of extracellular G protein-coupled receptors for sphingosine 1-phosphate (35, 36), which are able to mobilize calcium through conventional InsP3 receptor-dependent pathways. The recent cloning of the SCaMPER receptor (37) provides additional evidence that sphingoid derivatives are able to engage intracellular receptors and effect calcium release from stores independently of InsP3 generation. The data presented here provide evidence for specific immune receptor triggering of this pathway in myeloid cells. Thus, aggregation of FcγRI resulted in the rapid activation of sphingosine kinase and consequent cellular increases in sphingosine 1-phosphate concentrations. In these same cells, neither product of phospholipase C activation could be detected; no accumulation of total InsPs could be measured even in the presence of lithium chloride to prevent breakdown. Moreover, the observed increase in DAG could be completely blocked by pretreatment of cells with butanol, indicating PtdCho-PLD rather than phospholipase C activation as the source of the DAG. In contrast, aggregation of an alternative immune receptor, FcγRIIa, on these cells, resulted in increases in both phospholipase C-dependent DAG and inositol phosphate generation, indicating that this pathway is intact and functional in these cells and that the assays used were potentially able to detect any such receptor-triggered changes.

Taken together, the data presented here suggests that the high affinity receptor, FcγRI, mobilizes intracellular calcium through this sphingosine kinase-dependent, InsP3-independent pathway. In this respect, FcγRI is behaving like the high affinity IgE receptor, FcεRI, in mast cells (15). Of interest, both these receptors use the same signal-transducing molecule (γ-chain) (10) to recruit soluble tyrosine kinases to mediate cellular activation. However, the mechanism of coupling of tyrosine kinases to sphingosine kinase activation following FcεRI aggregation in mast cells was unclear (15). Here, we demonstrate that PtdCho-PLD is activated following aggregation of FcγRI in myeloid cells and that sphingosine kinase activation is dependent on PtdCho-PLD activation. The immediate product of PtdCho-PLD is phosphatidic acid, and this is subsequently converted to DAG through the action of phosphatidic acid phosphohydrolase. Previous studies have shown that sphingosine kinase is activated by phosphatidic acid (38) and not by DAG (38), a product of both phospholipase D and phospholipase C. Our finding that sphingosine kinase is downstream of PtdCho-PLD is, therefore, consistent with this in vitro work. Moreover, both components of this novel FcγRI-coupled intracellular signaling pathway involving the sequential activation of PtdCho-PLD and sphingosine kinase depend on tyrosine kinase activation. This finding is consistent with previous in vitro studies demonstrating that v-Src can activate PLD (39).

Aggregation of FcγRI in myeloid cells triggers a number of effector functions. The novel intracellular signaling pathway demonstrated here appears to be functionally interactive/associated with these. Thus, previous studies have implicated phosphatidic acid in modulating neutrophil function, in particular by influencing the respiratory burst/NADPH oxidase cascade (40). In the study reported here, inhibiting this pathway at either the PtdCho-PLD or sphingosine kinase level reduced or abolished the ability of this receptor to mobilize calcium from intracellular stores. In addition, the inhibition of PtdCho-PLD significantly slowed the rate of trafficking of internalized immune complexes for degradation. Of interest, ADP-ribosylation factor plays a major role in regulating vesicular trafficking, and this small molecular weight G protein has also been demonstrated to regulate phospholipase D activity (41). The finding that FcγRI is coupled to the release of intracellular calcium stores and vesicular trafficking via a novel pathway that does not use InsP3 has profound implications for the development of strategies for therapeutic intervention against differential myeloid responses to immune complexes.

Acknowledgments—We thank Sandra Seatter and T. McShane for technical support and Dr. Stewart Sage for use of the Cairn spectrophotometer and for helpful discussions. We are grateful to Dr. W. Cusheley and Professor F. Y. Liew for helpful suggestions in preparing this paper. We thank Bender Wein for the IFN-γ.

REFERENCES
1. van de Winkel, J. G. J., and Anderson, C. L. (1991) J. Leukocyte Biol. 49, 511–524
2. Ravetch, J. V., and Kinet, J.-P. (1991) Annu. Rev. Immunol. 9, 457–492
3. Fanger, M. W., Shen, L., Graziano, R. F., and Guyre, P. M. (1989) Immunol. Today 10, 92–99
4. Ely, P., Wallace, P. K., Givan, A. L., Guyre, P. M., and Fanger, M. W. (1996) Blood 87, 3813–3821
5. Lin, C.-T., Shen, Z., Boros, P., and Unkeless, J. C. (1994) J. Clin. Immunol. 14, 1–13
6. Rankin, B. M., Yokum, S. A., Mittler, R. S., and Kiener, P. A. (1993) J. Immunol. 150, 665–616
8. Davis, W., Sage, S. O., and Allen, J. M. (1994) Cell Calcium 16, 29–36
9. Allen, J. M., and Seed, B. (1989) Science 243, 378–381
10. Ra, C., Jourlin, M.-H., Blank, J., and Kinet, J.-P. (1989) Nature 341, 752–754
11. Reth, M. (1989) Nature 338, 383–384
12. Cambier, J. C. (1995) J. Immunol. 155, 3281–3285
13. Duchemin, A.-M., and Anderson, C. L. (1997) J. Immunol. 158, 865–871
14. Blank, U., Ra, C., Miller, L., White, K., Metzger, H., and Kinet, J.-P. (1989) Nature 337, 187–189
15. Choi, O. H., Kim J.-H., and Kinet, J.-P. (1996) Nature 380, 634–636
16. Harris, P., and Ralph, P. (1985) J. Leukocyte Biol. 37, 407–422
17. Harrison, P. T., Davis, W., Norman, J. C., Hockaday, A. R., and Allen, J. M. (1994) J. Biol. Chem. 269, 24396–24402
18. Olivera, A., Rosenthal, J., and Spiegel, S. (1994) Anal. Biochem. 223, 306–312
19. Olivera, A., and Spiegel, S. (1993) Nature 365, 557–560
20. Harrett, W., and Harrett, M. M. (1993) J. Immunol. 151, 4829–4837
21. Briscoe, C. P., Plevin, R., and Wakelam, M. J. O. (1994) Biochem. J. 298, 61–67
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., p. 18.53, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
23. Lecomber, U. K. (1970) Nature 227, 680–685
24. Towbi, H., Stachelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
25. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3449
26. Stengelin, S., Stamenkovic, I., and Seed, B. (1988) EMBO J. 7, 1053–1059
27. Duchemin, A.-M., Ernst, I. K., and Anderson, C. L. (1994) J. Biol. Chem. 269, 12111–12117
28. Schöll, P. R., Ahern, D., and Geha, R. S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8847–8850
29. Heemskerk, J. W. M., Vis, P., Feijge, A. H., Hoyland, J., Mason, W. T., and Sage, S. O. (1993) J. Biol. Chem. 268, 356–363
30. Sargeant, P., Clarkson, W. D., Sage, S. O., and Heemskerk, J. W. M. (1992) Cell Calcium 13, 553–584
31. Mettinen, H. M., Rose, J. K., and Melman, I. (1989) Cell 58, 317–327
32. Ghosh, T. K., Biau, J., and Gill, D. L. (1990) Science 248, 1653–1656
33. Mattie, M., Brooker, G., and Spiegel, S. (1994) J. Biol. Chem. 269, 3181–3188
34. Olivera, A., Zhang, H., Carlsson, R. O., Mattie, M. E., Schmidt, R. R., and Spiegel, S. (1994) J. Biol. Chem. 269, 17924–17930
35. Wu, J., Spiegel, S., and Sturgill, T. W. (1995) J. Biol. Chem. 270, 11484–11488
36. Postma, F. R., Jalink, K., Hengeveld, T., and Molenaar, W. H. (1996) EMBO J. 15, 2388–2396
37. Mao, C., Kim, S. H., Almenoff, J. S., Rudner, X. L., Kearney, D. M., and Kindman, L. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1993–1996
38. Olivera, A., Rosenthal, J., and Spiegel, S. (1996) J. Cell. Biochem. 60, 529–537
39. Jiang, H., Luo, J. Q., Urano, T., Frankel, P., Foster, D. A., and Feig, L. A. (1995) Nature 378, 409–412
40. Qualliotine-Mann, D., Agwu, D. E., Ellenburg, M. D., McCall, C. E., and McPhail, L. C. (1993) J. Biol. Chem. 268, 23843–23849
41. Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C., and Sternweis, P. C. (1993) Cell 75, 1137–1144

**FcγRI Coupling to Phospholipase D and Sphingosine Kinase**
FcγRI Coupling to Phospholipase D Initiates Sphingosine Kinase-mediated Calcium Mobilization and Vesicular Trafficking
Alirio Melendez, R. Andres Floto, David J. Gillooly, Margaret M. Harnett and Janet M. Allen

J. Biol. Chem. 1998, 273:9393-9402.
doi: 10.1074/jbc.273.16.9393

Access the most updated version of this article at http://www.jbc.org/content/273/16/9393

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 19 of which can be accessed free at http://www.jbc.org/content/273/16/9393.full.html#ref-list-1