Identification of a Compound That Directly Stimulates Phospholipase C Activity

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ABSTRACT
Phosphoinositide-specific phospholipase C (PLC) plays a pivotal role in the signal transduction of various cellular responses. However, although it is undeniably important that modulators of PLC activity be identified, no direct PLC activity modulator has been identified until now. In this study, by screening more than 10,000 different compounds in human neutrophils, we identified a compound that strongly enhances superoxide-generating activity, which is well known to be PLC-dependent. The active compound 2,4,6-trimethyl-N-(meta-3-trifluoromethyl-phenyl)-benzenesulfonamide (m-3M3FBS) stimulated a transient intracellular calcium concentration ([Ca2+]i) increase in neutrophils. Moreover, m-3M3FBS stimulated the formation of inositol phosphates in U937 cells, indicating that it stimulates PLC activity. The compound showed no cell-type specificity in terms of [Ca2+]i increase in the various cell lines including leukocytes, fibroblasts, and neuronal cells. We also ruled out the possible involvement of heterotrimeric G proteins in m-3M3FBS–stimulated signaling by confirming the following: 1) pertussis toxin does not inhibit m-3M3FBS–induced [Ca2+]i increase; 2) m-3M3FBS does not stimulate cyclic AMP generation; and 3) the inhibition of Gαs by the regulator of G protein-signaling 2 does not affect the m-3M3FBS–induced [Ca2+]i increase. We also observed that m-3M3FBS stimulated PLC activity in vitro. The purified isoforms of PLC that were tested (i.e., β2, β3, γ1, γ2, and δ1) were activated by m-3M3FBS and showed no isoform specificity. Taken together, these results demonstrate that m-3M3FBS modulates neutrophil functions by directly activating PLC. Because m-3M3FBS is the first compound known to directly activate PLC, it should prove useful in the study of the basic molecular mechanisms of PLC activation and PLC-mediated cell signaling.

Phosphoinositide (PI) hydrolysis is one of the important early signals associated with the stimulation of leukocytes by diverse extracellular stimuli (Rhee, 2001). Phospholipase C (PLC) hydrolyzes phosphatidylinositol bisphosphate (PIP2) into inositol-1,4,5-triphosphates and diacylglycerol, which mediate intracellular calcium release or the activation of protein kinase C, respectively (Noh et al., 1995; Rhee, 2001). Intracellular calcium concentration ([Ca2+]i) increase and protein kinase C activation subsequently induce diverse intracellular signaling, such as the activation of phospholipase A2, phospholipase D, or mitogen-activated protein kinases. Finally, these intracellular signals result in the modulation of various cellular responses, including superoxide generation, secretion, and proliferation in leukocytic cells (Bae et al., 1999; Kim et al., 1999; McLaughlin and De Vries, 2001). Eleven isoforms of PLC are known (Rhee, 2001). Whereas the β isoforms are known to modulate GTP-binding proteins, the γ isoforms have been reported to activate the stimulation of growth factor receptors (Noh et al., 1995; Rhee, 2001). Although many extracellular ligands that stimulate cell surface receptors leading to the activation of PLC β or γ have been reported, no direct PLC activity modulator has been identified until now.

Recently many synthetic compounds have been reported to modulate diverse immune responses (Tian et al., 1998; Rosania et al., 1999; Zhang et al., 1999). Synthetic compounds are known to regulate cellular activity by modulating cellular target proteins (Tian et al., 1998; Rosania et al., 1999; Zhang et al., 1999). Whereas some of the compounds bind to cell surface receptors and induce receptor-mediated intracellular signals, others directly modulate intracellular target molecules after penetrating cells (Rosania et al., 1999; Strizki et al., 2001). The

ABBREVIATIONS: PI, phosphoinositide; PLC, phospholipase C; PIP2, phosphatidylinositol bisphosphate; [Ca2+]i, intracellular calcium concentration; m-3M3FBS, 2,4,6-trimethyl-N-(meta-3-trifluoromethyl-phenyl)-benzenesulfonamide; PTX, pertussis toxin; α-3M3FBS, 2,4,6-trimethyl-N-(ortho-3-trifluoromethyl-phenyl)-benzenesulfonamide; RGS, regulators of G protein signaling; WKYMVm, Trp-Lys-Tyr-Met-Val-o-Met-CONH2; PI3-kinase, phosphoinositide 3-kinase; GFP, green fluorescent protein.
Materials and Methods

Materials. Compounds were purchased from the Chembridge Corporation (San Diego, CA). Peripheral blood mononuclear cell separation medium (Histopaque-1077), dioloyl-phosphatidylethanolamine, and tetracycline were purchased from Sigma Chemical (St. Louis, MO). RPMI 1640 and Dulbecco’s modified Eagle’s medium were from Invitrogen (Carlsbad, CA); dextran sedimentation medium and supplemented bovine calf serum were from Hyclone Laboratories (Logan, UT). U-73122 and U-73343 were obtained from Sigma (St. Louis, MO). Hygromycin B and pertussis toxin (PTX) were from Calbiochem (San Diego, CA). [myo-[3H]inositol (18.3 Ci/mmol), [8-3H]adenosine 3’,5’-cyclic phosphate, and phosphatidylinositol-4,5-bisphosphate([inositol-2-[3H])] were from Amersham Biosciences (Piscataway, NJ). The AG 1-X8 resin was purchased from Bio-Rad ( Hercules, CA).

Isolation of Human Neutrophils. Peripheral blood leukocytes were donated by the Ulsan Red Cross Blood Center (Ulsan, Korea). Human neutrophils were isolated by standard dextran sedimentation, by the hypotonic lysis of erythrocytes, and by the use of a lymphocyte-separation medium gradient, as described previously (Baek et al., 2001). Isolated human neutrophils were used promptly.

Cell Culture and the Differentiation of HL60 Cells. Human histiocytic lymphoma cells (U937), human promyelocytic leukemia cells (HL60), NIH Swiss mouse embryo fibroblasts (NIH 3T3), and histiocytic lymphoma cells (U937), human promyelocytic leukemia (Bae et al., 2001). Isolated human neutrophils were used promptly.

In this study, we screened a chemical library consisting of more than 10,000 different species in an effort to find a chemical that can stimulate superoxide generation in human neutrophils. We found that the compound 2,4,6-trimethyl-N-[(meta-3-trifluoromethyl-phenyl)-benzenesulfonamide (m-S3M3FBS) can stimulate human neutrophils and that this stimulation leads to superoxide generation. By studying the action mechanism of m-S3M3FBS, we suggest that the compound stimulates neutrophil activity by directly activating PLC.
proteins added to the reaction mixture were dialyzed overnight against the assay buffer. Incubation was performed for 10 min at 37°C in a 200-μl reaction mixture containing lipid micelles (5 μM [3H]PtdIns-4,5-P2, 20,000 cpm). The reaction was stopped by adding 2 ml of CHCl3/CH3OH/HCl (50:50:0.3, v/v). The inositol trisphosphates were extracted with 0.5 ml of 1 N HCl, and radioactivities in the upper aqueous phase were measured.

Results

Identification of a Synthetic Compound That Strongly Enhances Superoxide Generation in Human Neutrophils. In this study, we screened approximately 10,000 chemicals in an effort to identify chemicals that stimulate superoxide generation in human neutrophils, and we found several chemicals that do so within the concentration range of 20 to 50 μM (data not shown). Among these, a chemical named m-3M3FBS proved to be the most potent in terms of its ability to stimulate the generation of superoxide. Figure 1A shows that m-3M3FBS greatly enhanced superoxide generation within the concentration range of 15 to 50 μM. Interestingly, however, 2,4,6-trimethyl-N-(ortho-3-trifluoromethyl-phenyl)-benzenesulfonamide (o-3M3FBS), which has a similar structure to m-3M3FBS except for the position of the trifluoromethyl-phenyl group, did not affect superoxide generation up to 50 μM (Fig. 1A). Therefore, we used o-3M3FBS as an inactive analog of m-3M3FBS. Figure 1B shows the structures of m-3M3FBS and o-3M3FBS.

m-3M3FBS Stimulates [Ca2+]i Increase in Neutrophils. Many extracellular agonists that stimulate superoxide anion generation in human phagocytic cells also increase [Ca2+]i (Liang and Huang, 1995; Bae et al., 1999). Therefore, we examined the effect of m-3M3FBS on [Ca2+]i in human neutrophils. As shown in Fig. 2A, m-3M3FBS caused an increase in [Ca2+]i as a result of the transfer of calcium through the plasma membrane when Ca2+ levels were at physiological levels extracellularly, whereas the inactive analog, o-3M3FBS, failed to elicit this response. m-3M3FBS stimulated [Ca2+]i release in a concentration-dependent manner, showing the saturated maximal activity at a concentration of 50 μM (data not shown). Moreover, m-3M3FBS also evoked a [Ca2+]i increase in the extracellular calcium-depleted condition. These findings demonstrate that m-3M3FBS induces intracellular calcium increases as a result of both plasma membrane calcium entry and the release of intracellularly stored calcium.

Because intracellular calcium mobilization can be caused by the activation of PLC (Rhee, 2001), we undertook to prevent this compound response by blocking the activation of PLC.
PLC with the membrane-permeable PLC inhibitor U-73122. A 3- to 5-min pretreatment of neutrophils with U-73122 has been documented previously to fully prevent PLC activation upon agonist stimulation (Hershfinkel et al., 2001). As shown in Fig. 2B, pretreatment of neutrophils with 4 μM of U-73122 resulted in the complete inhibition of the calcium signal induced by m-3M3FBS. However, preincubating the same cells with U-73343, an inactive analog of U-73122, had no effect on the m-3M3FBS–evoked response. This result indicates that an increase in [Ca\(^{2+}\)], induced by m-3M3FBS is mediated by PLC activity. Preincubation of neutrophils with U-73122 in the presence of extracellular calcium also completely blocked m-3M3FBS–induced calcium signal (data not shown). The results suggest that intracellular calcium increases caused by plasma membrane calcium entry are mediated by intracellular calcium release.

**m-3M3FBS Stimulates the Formation of Inositol Phosphates in U937 Cells.** From previous data, we expected that m-3M3FBS would influence the activity of PLC. We next examined whether m-3M3FBS could stimulate PLC activation by measuring total inositol phosphate formation in U937 cells. After labeling with \[^{3}H\]inositol (1 μCi/10^6 cells), the cells were treated with m-3M3FBS or o-3M3FBS. As shown in Fig. 3, the accumulation of inositol phosphates after treatment with m-3M3FBS increased gradually, giving a 2.5-fold increase at 50 μM of m-3M3FBS. The concentration-dependence of m-3M3FBS–induced inositol phosphate formation was closely correlated with that of m-3M3FBS–induced superoxide generation (Fig. 1A). In contrast, o-3M3FBS had no effect on PLC activity. This result indicates that m-3M3FBS stimulates PLC activation.

**m-3M3FBS Has No Cell-Type Specificity.** Up to this point, we had observed that m-3M3FBS caused superoxide anion generation in a PLC-dependent manner, and we wondered whether this chemical could affect signaling molecules upstream of PLC. In the case of the PLC \(\beta\) series, ligand–specific receptor and heterotrimeric G proteins generally are located upstream of signaling molecules (Rhee and Bae, 1997; Rhee, 2001). First, we investigated whether m-3M3FBS has a specific receptor. As shown in Fig. 4, m-3M3FBS induced a calcium increase in all cell lines (human neutrophils, HL60, differentiated HL60, U937, NIH 3T3, and PC12) examined, showing no cell-type specificity.

**m-3M3FBS Desensitizes the Calcium Increase Induced by Other Agonists.** We next investigated the capacity of m-3M3FBS to desensitize other extracellular agonists by examining its effect on ATP and a synthetic leukocyte chemoattractant peptide, Trp-Lys-Tyr-Met-Val-N-Met-CONH\(_2\) (WKYMVm), which is known to stimulate PLC enzymes (Seo et al., 1997; Bae et al., 2000). In cross-desensitization experiments, stimulation of the cells with m-3M3FBS significantly reduced cellular responses to ATP and WKYMVm (Fig. 5). However, the administration of m-3M3FBS after stimulating cells with ATP or WKYMVm elicited still further [Ca\(^{2+}\)] increases (Fig. 5). Therefore, the m-3M3FBS–induced [Ca\(^{2+}\)] increase was more potent than that induced by other agonists, demonstrating that m-3M3FBS may more strongly affect the PLC enzyme(s) than other extracellular agonists. These results suggest that the target of m-3M3FBS is a common mediator of cell surface receptors, such as PLC or heterotrimeric G protein.

**m-3M3FBS-Induced Signaling Is Not G Protein-Dependent.** Several extracellular signals, including those caused by many chemoattractants, activate phagocytic cells. However, the administration of m-3M3FBS after stimulating cells with ATP or WKYMVm elicited still further [Ca\(^{2+}\)] increases (Fig. 5). Therefore, the m-3M3FBS–induced [Ca\(^{2+}\)] increase was more potent than that induced by other agonists, demonstrating that m-3M3FBS may more strongly affect the PLC enzyme(s) than other extracellular agonists. These results suggest that the target of m-3M3FBS is a common mediator of cell surface receptors, such as PLC or heterotrimeric G protein.

![Fig. 2. Effect of m-3M3FBS on [Ca\(^{2+}\)], and the inhibition of m-3M3FBS–induced calcium increase by U-73122. A, fura-2–loaded neutrophils were stimulated with m-3M3FBS or o-3M3FBS at 25 μM in extracellular calcium containing 2 mM or extracellular free calcium conditions. The results shown are representative of three independent experiments. B, prepared neutrophils were loaded with fura-2 and then stimulated with 25 μM of m-3M3FBS in the absence or in the presence of 4 μM of U-73122 or U-73343. Changes in the 340/380 nm excitation ratio were monitored and converted into [Ca\(^{2+}\)], levels. Data are presented as means ± S.E.M. of three independent experiments.](image1)

![Fig. 3. Effect of m-3M3FBS on in vivo PLC activity. U937 cells were labeled with [myo-\(^{3}H\)]inositol (1 μCi/10^6 cells) for 24 h at 37°C and then treated with various concentrations of m-3M3FBS or o-3M3FBS. Total inositol phosphates were eluted with a solution containing 1 M ammonium formate and 0.1 M formic acid. Radioactivity of the [\(^{3}H\)]inositol phosphates was determined by counting in a scintillation counter. Data are presented as means ± S.E.M. of five independent experiments.](image2)
produced a [Ca\(^{2+}\)]i increase (Simonds, 1999). We next examined whether finely regulate levels of cAMP within several types of cells could activate Gs proteins by measuring the change in the [Ca\(^{2+}\)]i levels. Data are presented as means ± S.E.M. of three independent experiments.

However, the calcium increase caused by m-3M3FBS, which stimulates superoxide generation in human neutrophils. m-3M3FBS also evoked [Ca\(^{2+}\)]i increases not only in neutrophils but also in several other cells, including neuronal cells and fibroblasts, and therefore, it showed no cell-type specificity. Through a study of its mode of action,
we found that it directly activates many PLC isozymes, again without showing isozyme specificity.

Our experiment demonstrates that stimulation of human neutrophils with \( \text{m-3M3FBS} \) induces \([\text{Ca}^{2+}]_{i}\) increases (Fig. 2A), and that this effect of \( \text{m-3M3FBS} \) is inhibited by U73122, a specific PI-PLC inhibitor (Fig. 2B). The stimulation of U937 cells with \( \text{m-3M3FBS} \) also caused PI hydrolysis (Fig. 3). During experiments designed to investigate the effect of \( \text{m-3M3FBS} \) on PLC activity, we observed that it activated several isozymes of PLC in vitro (Fig. 7, A and B). These results suggest that \( \text{m-3M3FBS} \) activates PLC directly, ruling out a possible non-PLC–dependent mechanism of the compound. Because all isozymes of PLC tested (\( \beta_2, \beta_3, \gamma_1, \gamma_2, \) and \( \delta_1 \)) were activated by \( \text{m-3M3FBS} \) in vitro, it seems that the compound does not have any isoform specificity in terms of the activation of this enzyme (Fig. 7B). Although many different extracellular ligands are known to stimulate PLC activity by binding to their specific cell surface receptors, there has been no report on the direct activation of PLC until now. Generally, the activation of cell surface receptors induces diverse signaling pathways. PLC activation is one of the earliest responses downstream of receptor stimulation (Noh et al., 1995; Rhee, 2001). Several previous reports have suggested that PLC is involved in several important cellular functions, such as proliferation, differentiation, and apoptosis (Noh et al., 1995; Rhee and Bae, 1997; Rhee, 2001). However, the complications of cellular receptor-mediated signaling hinder our understanding of the natures of the signals and of the cellular responses regulated by PLC. Bearing this in mind, the identification of a molecule that can modulate...
PLC activity directly will undoubtedly be helpful for the elucidation of PLC-mediated cellular signaling and physiological responses. Furthermore, no ligand has been identified that stimulates the isoforms of PLC, including PLCα1 and δ2. Because m-3M3FBS could stimulate PLCα1 activity directly, it should be useful for the study of cellular signaling and functional events downstream of the enzyme.

In our in vitro experiments, we observed that m-3M3FBS stimulated the β, γ, and δ isoforms of PLC and that it showed no isoform-specificity (Fig. 7, A and B). Moreover, the primary structures of the several different isoforms of PLC are known (Noh et al., 1995; Rhee, 2001). PLC β and PLC δ have an NH2-terminal PH domain, an EF-hand, X and Y domains known to form the catalytic core, and a COOH-terminal C2 domain. In addition, PLC β has a long C-terminal tail beyond the C2 domain (Noh et al., 1995; Rhee and Bae, 1997; Rhee, 2001). PLC γ has three additional SH domains between the X and Y domains, but no long COOH-terminal tail (Noh et al., 1995; Rhee and Bae, 1997; Rhee, 2001). Our study shows that m-3M3FBS stimulates three subfamilies of the PLC isoforms (PLC β, γ, and δ) (Fig. 7, A and B). This suggests that the compound acts on a common conserved region of these three isoforms, thus ruling out the possible involvement of the SH domains and COOH-terminal tail of PLC β. For the proper activation of the PLC enzyme, calcium has been regarded as an essential requirement (Rhee and Bae, 1997). Calcium is required not only for the functioning of C2 domain that mediates the Ca2+-dependent binding to lipid vesicles, but also for the catalytic activity of the enzyme (Rhee and Bae, 1997). In our study, we found that m-3M3FBS stimulated in vitro PLC activity in the presence or absence of Ca2+ (data not shown). The result suggests that m-3M3FBS may stimulate PLC activity with different mechanism from the calcium ion. Previously, Horstman et al. (1996) demonstrated that the addition of a purified X and Y domain in vitro showed lipase activity. An investigation of the effect of m-3M3FBS on the lipase activity of an X and Y domain mixture will be required to confirm the possible action of m-3M3FBS on the two catalytic cores. Because m-3M3FBS is the first compound that directly stimulates PLC activity, the elucidation of the action mechanism of the compound will give useful information on the basic molecular mechanisms of the activation of PLC enzymes.

In Fig. 7, we demonstrated that all tested PLC isoforms were activated by m-3M3FBS. The result led us to check whether the compound acts specifically on PLC or on other enzymes that recognize phosphoinositols as substrates, such as phosphoinositide-3-kinase (PI3-kinase). For this, we tested the effect of m-3M3FBS on the Akt phosphorylation that is dependent on the PI3-kinase in U937 cells. A concentration of 50 μM m-3M3FBS could not significantly increase the phosphorylation level of Akt (data not shown). This indicates that m-3M3FBS has specificity for PLC but not for PI3-kinase. To check the effect of m-3M3FBS on other phospholipase, we also tested the effect of m-3M3FBS on the in vitro activity of phospholipase D. We observed that the compound did not affect on the activity of phospholipase D (data not shown). The results support our notion that m-3M3FBS acts specifically on PLC.

In conclusion, by screening a chemical library, we identified a small synthetic molecule that potently stimulates superoxide generation. This is the first report of a direct activator of PLC, and we believe that the compound will prove to be a useful agent for the study of PLC-mediated cell signaling.

References


Fig. 7. Effect of m-3M3FBS on the activity of PLC isoforms in vitro. The hydrolyzing activity of PtdIns-4,5-P2 was measured on mixed phospholipid vesicles containing 120 μM phosphatidylethanolamine, 30 μM PtdIns-4,5-P2, and 1 μCi/ml 3H[PtdIns-4,5-P2. Purified PLC β2, substrates, and the chemicals (2.5, 5, 10, 20, and 25 μM of m-3M3FBS or α-3M3FBS) were coincubated (A), and 25 μM of m-3M3FBS or α-3M3FBS and phospholipid substrates were mixed with purified PLC β2, γ1, γ2, or δ1 (B). Reactions were performed for 10 min at 37°C in a 200-μl reaction mixture containing lipid micelles (5 μM [3H]PtdIns-4,5-P2, 20,000 cpm). Reactions were stopped by adding 1 ml of CHCl3/CH3OH/HCl (50:50:0.3, v/v). Inositol trisphosphates were extracted with 0.5 ml of 1 M HCl/5 mM EGTA, and the radioactivity in the upper aqueous phase was measured. A and B, data are presented as means ± S.E.M. of three independent experiments.


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