Sphingosylphosphorylcholine Activation of Mitogen-activated Protein Kinase in Swiss 3T3 Cells Requires Protein Kinase C and a Pertussis Toxin-sensitive G Protein*

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Sphingosylphosphorylcholine (SPC) is a potent mitogen for Swiss 3T3 cells, but the signaling mechanisms involved are poorly characterized. Here, we report that addition of SPC induces a rapid and transient activation of p42 mitogen-activated protein kinase (p42MAPK) in these cells. SPC-induced p42MAPK activation peaked at 5 min and was undetectable after 30 min of incubation with SPC. The effect of SPC on p42MAPK activation was comparable to that induced by bombesin and platelet-derived growth factor. As SPC strongly induced phosphorylation of the major protein kinase C (PKC) substrate 80K/MARCKS in either intact or permeabilized cells, we examined whether PKC could be involved in SPC-induced p42MAPK activation. Here, we demonstrate that p42MAPK activation by SPC was dependent on PKC activity as shown by inhibition of PKC with the bisindolylmaleimide GF 109203X or down-regulation of PKC by prolonged treatment of Swiss 3T3 cells with phorbol esters. Activation of both PKC and p42MAPK by SPC was markedly inhibited by treatment with pertussis toxin, implicating a G protein(s) of the G1/G0 subfamily in the action of SPC. SPC-induced rapid activation of a downstream target of p42MAPK, p90 ribosomal S6 kinase (p90rsk), also required PKC and a pertussis toxin-sensitive G protein. In addition, SPC-induced mitogenesis was dependent on a G protein in Swiss 3T3 cells. SPC also induced p42MAPK activation and DNA synthesis in secondary cultures of mouse embryonic fibroblasts through a pertussis toxin-sensitive pathway. As G-proteins link many cell surface receptors to effector proteins, we hypothesize, therefore, that SPC could bind to a receptor that mediates at least some of its biological effects in Swiss 3T3 cells and mouse embryonic fibroblasts.

Lipid-derived messengers are implicated in intercellular communication and intracellular signaling, and the mechanisms involved are attracting increasing interest (1). Sphingolipids and their breakdown products have emerged as active mediators involved in the regulation of cell growth, differentiation, transformation, and cell-cell contact (reviewed in Refs. 2 and 3). Lysosphingolipids such as sphingosylphosphorylcholine (SPC) are potential derivatives of sphingolipids. SPC has been demonstrated in normal mouse cerebrum and human meningiomas (4) as well as in tissues of patients with Niemann Pick disease, a lipid storage disorder (5). Furthermore, SPC has been identified as a growth-promoting factor for Swiss 3T3 cells (6, 7), but the molecular basis of this mitogenic effect is poorly understood.

The best characterized early cellular response induced by SPC is the direct induction of Ca$$^{2+}$$ mobilization from internal stores in a variety of cell lines thereby acting as a potential second messenger (7–10). Recent data from our laboratory demonstrated that Ca$$^{2+}$$ mobilization in the absence of inositol phosphate production can stimulate DNA synthesis in Swiss 3T3 cells only in synergistic combination with other growth-promoting agents (11). In contrast, SPC, like bombesin and PDGF, can induce a mitogenic response in these cells in the absence of any other growth factor (Refs. 6 and 7 and this paper). It is increasingly recognized that the initiation of DNA synthesis is triggered by multiple signal transduction pathways that act synergistically in mitogenic stimulation (12–14). We therefore reasoned that the ability of SPC to act as a sole mitogen may be due to the stimulation of other signaling pathways in addition to direct Ca$$^{2+}$$ mobilization.

Mitogen-activated protein kinases (MAPKs), also known as extracellular signal-related kinases, are a family of protein-serine/threonine kinases that play a role as important intermediates in signal transduction pathways. MAPKs are stimulated by a variety of polypeptide growth factors signaling via tyrosine kinase receptors that activate p21$$^{ras}$$ (15, 16). The pathway activated by these agents is a protein kinase cascade, which involves c-Raf-1, MEK, p44MAPK, and p42MAPK (15, 16). Activation of seven transmembrane receptors also leads to p44MAPK and p42MAPK activation, but the mechanism(s) involved are less clear, though both Raf-1 activation and PKC stimulation have been implicated (17–19). Activated MAPKs directly phosphorylate and activate various enzymes, including the 90-kDa S6 protein kinase named p90rsk (20). Both, MAPK and p90rsk regulate gene expression by transcription factor phosphorylation and have been strongly implicated in the stimulation of cell proliferation (15, 20, 21).

Here, we demonstrate that addition of SPC to quiescent cultures of Swiss 3T3 cells leads to rapid and transient activation of p42MAPK and p90rsk through a novel, pertussis toxin-sensitive and PKC-dependent signal transduction pathway that can be distinguished from that utilized by other single mitogens for these cells, such as bombesin and PDGF.
Experimental Procedures

Cell Culture—Stock cultures of Swiss 3T3 fibroblasts were maintained in DMEM supplemented with 10% fetal bovine serum in a humidified atmosphere containing 10% CO2 and 90% air at 37°C. For experimental purposes, cells were plated either in 35-mm Nunc Petri dishes at 10^4 cells/dish or in 100-mm dishes at 6 × 10^5 cells/dish in DMEM containing 10% fetal bovine serum and used after 6–8 days when the cells were confluent and quiescent. Secondary passage MEFs were seeded at 1 × 10^5 cells/35-mm Nunc Petri dishes at 10 cells/cm². After 40 h of incubation at 37°C, cultures were washed twice with PBS and incubated in 50% trichloroacetic acid at 4°C for 30 min to remove acid-soluble radioactivity, washed with ethanol, and solubilized in 1 ml of 2% Na2CO3, 0.1 M NaOH, 1% SDS. The acid-insoluble radioactivity was determined by Cerenkov counting in 6 ml of Ultima Gold (Packard).

For detection of BrdUrd incorporation into cellular DNA—Confluent and quiescent cultures of Swiss 3T3 cells were washed twice with DMEM and incubated with DMEM/Waymouth's medium (1:1 v/v) containing [3H]thymidine (0.25 μCi/ml, 1 μM BrdUrd, and various additions as described in the figure legends). After 40 h of incubation at 37°C, cultures were washed twice with PBS and incubated in 50% trichloroacetic acid at 4°C for 30 min to remove acid-soluble radioactivity, washed with ethanol, and solubilized in 1 ml of 2% Na2CO3, 0.1 M NaOH, 1% SDS. The acid-insoluble radioactivity was determined by Cerenkov counting in 6 ml of Ultima Gold (Packard).

For detection of BrdUrd incorporated into cellular DNA, confluent and quiescent cultures of Swiss 3T3 cells were washed twice with DMEM and incubated with DMEM/Waymouth's medium (1:1 v/v) containing 10 μM BrdUrd and various additions as described in the figure legends. After 40 h of incubation at 37°C, cultures were washed twice with PBS, fixed in 70% ethanol for 20 min, and incubated with anti-BrdUrd monoclonal antibody followed by labeling the monoclonal antibody with anti-rabbit IgG (goat) antibody. Cells were examined using a Zeiss Axioskop immunofluorescence microscope, and data are expressed as the percentage of GFP-BrdUrd-labeled nuclei.

2p Labeling of Cells and Analysis of 80K/MARK5s Phosphorylation—Quiescent and confluent cultures of Swiss 3T3 cells in 35-mm dishes were washed twice in phosphate-free DMEM and incubated at 37°C with this medium containing 50 μCi/ml of carrier-free [32P]Pi. After 12 h, various factors were added for the indicated times. The cells were subsequently lysed, and the lysates were immunoprecipitated with specific anti-80K/MARK5 antibody (22). The immunoprecipitates were further analyzed by SDS-PAGE prior to autoradiography.

Phosphorylation in Permeabilized Cells—Quiescent and confluent Swiss 3T3 cells in 35-mm dishes were washed twice in DMEM and twice with a KCl salt solution at 37°C immediately before the onset of the experiment. The KCl salt solution contained 120 mM KCl, 30 mM NaCl, 1 mM MgCl₂, 1 mM K₂HPO₄, 10 mM sodium-PIPES (pH 7.0), 1 mM EGTA, and 0.037 mM CaCl₂ to give a final free Ca²⁺ concentration of 15 μM. Phosphorylation was initiated by replacing the salt solution from the last wash with 1 ml of permeabilization medium containing 40 μM digitonin and 10 μM [γ-32P]ATP in 2% KCl salt solution. SPC and bombesin were added in the presence or absence of GDP as described in each experiment, and the cells were incubated at 37°C for 1 min. Samples were solubilized in 0.15 ml of 2 × SDS-PAGE sample buffer (200 mM Tris/HCl, 6% SDS, 2 M EDTA, 4% 2-mercaptoethanol, 10% glycerol, pH 6.8), boiled for 10 min, and subsequently analyzed by one-dimensional SDS-gel electrophoresis.

Down-regulation of PKC—Phorbol ester-sensitive PKC isoforms were down-regulated in Swiss 3T3 cells by prolonged pretreatment with PDB (23–26). In the present study, confluent and quiescent cultures were pretreated with 800 nM PDB for 48 h in conditioned medium, which was depleted of growth-promoting activity.

p42/44 MAPK Shift Assays and Western Blotting—Quiescent cultures of Swiss 3T3 cells were treated with factors as indicated, washed once in 2 × SDS-PAGE sample buffer and analyzed by SDS-PAGE. After SDS-PAGE, proteins were transferred to Immobilon membranes (27). Membranes were blocked using 5% non-fat dried milk in PBS, pH 7.2, and incubated for 1 h at 22°C with a polyclonal p42/44 MAPK antisera (1:1000) in PBS containing 3% non-fat dried milk. Immunoreactive bands were visualized by autoradiography using [125I]-labeled protein A following SDS-PAGE and autoradiography.

Immune Complex Assay for p42/44 MAPK and p90Rsk Activation—Quiescent and confluent Swiss 3T3 cells were treated with factors as described in the figure legends and lysed at 4°C. Lysates were clarified by centrifugation at 15,000 × g for 10 min at 4°C. Immunoprecipitation was performed using a polyclonal anti-p42/44 MAPK or a polyclonal anti-p90Rsk antibody, incubating the samples on a rotating wheel at 2°C. Proteins were added to 40 μl of 1:1 slurry of protein A-Sepharose, and precipitates were collected by centrifugation and washed twice in lysis buffer and twice in kinase buffer (15 mM Tris/HCl, pH 7.4, 15 mM MgCl₂). The kinase reaction was performed by resuspending the pellet in 25 μl of kinase assay mixture containing kinase buffer, 100 μM ATP, 100 μCi/ml of [γ-32P]ATP, 200 μM microcystin LR, and either 1 mg/ml myelin basic protein peptide (APRTPGGRR) or 56 peptide (RRLRSSLR) for the assays of p42/44 MAPK and p90Rsk, respectively. Incubations were performed for 10 min (linear assay conditions) at 30°C and terminated by spotting 20 μl of the supernatant onto P81 phosphocellulose paper (Whatman). Filters were washed four times for 5 min in 0.5% orthophosphoric acid, immersed in acetone, and dried before Cerenkov counting. The average radioactivity of two blank samples containing no immune complex was subtracted from the result of each sample. Results are expressed as cpm/1.5 × 10⁶ cells. The specific activity of [γ-32P]ATP used was 900–1200 cpm/pmol.

Materials—SPC, PDB, bombesin, and cytochalasin D were obtained from Sigma. The PKC inhibitor GF 109203X, thapsigargin, and microcystin LR were obtained from Calbiochem-Novabiochem Ltd. (Nottingham, UK). PDGF (BB homodimer), 125I-labeled protein A (15 μCi/mg), [γ-32P]ATP (370 MBq/mmol), carrier-free [32P]Pi (370 MBq/mmol), and [3H]thymidine were from American. The BrdUrd labeling and detection kit was from Boehringer Mannheim (Germany). The polyclonal anti-p42/44 MAPK antibody raised against a COOH-terminal peptide (EE-TARFQPQGYS) and the polyclonal anti-p90Rsk antibody raised against a COOH-terminal peptide (IESLIAQQVRKLPSTTL) were generous gifts from Dr. J. Van Lint (Katholieke Universiteit Leuven, Belgium). The anti-p42/44 MAPK polyclonal antibody was obtained from Santa Cruz Biotechnology Ltd. GST-MEK and GST-MAPK fusion proteins were generous gifts from Professor C. Marshall (Institute of Cancer Research, London). All other reagents were of the purest grade available.

Results

SPC is a Potent Mitogen in Swiss 3T3 Cells—To assess the effect of SPC on DNA synthesis, quiescent cultures of Swiss 3T3 cells were exposed to various concentrations of SPC in the absence or presence of any other exogenously added growth-promoting factor. Cumulative [3H]thymidine incorporation was determined after 40 h of incubation. As shown in Fig. 1, SPC induced a potent, concentration-dependent increase in [3H]thymidine incorporation by Swiss 3T3 cells in serum-free medium. The maximum level of DNA synthesis achieved at 15 μM SPC reached 70% of the DNA synthesis induced by 10% fetal bovine serum (Fig. 1). In contrast, sphingomyelin and lysophosphatidylcholine failed to induce [3H]thymidine incorporation in Swiss 3T3 cells under identical conditions (data not shown). A different bioactive lipid, LPA, which acts as a potent mitogen for Rat1 fibroblasts (28), stimulated [3H]thymidine incorporation in Swiss 3T3 cells at concentrations of 15 μM only when very weakly in Swiss 3T3 cells, reaching 3% of the effect induced by SPC at an identical concentration (data not shown). To substantiate these results, we assessed DNA synthesis in response to SPC and LPA using an immunofluorescence assay for the detection of BrdUrd incorporated into cellular DNA. Addition of SPC at 15 μM induced DNA synthesis in 70% of the cell population, whereas LPA failed to induce BrdUrd incorporation (Fig. 1, inset). These data are in good agreement with those obtained in the [3H]thymidine incorporation assay. Thus, the growth-promoting effect of...
SPC in serum-free medium is comparable to that of PDGF or bombesin (12, 29) and clearly distinguishable from that of LPA in Swiss 3T3 cells.

SPC Induces p42MAPK Activation in Swiss 3T3 Cells—A variety of growth factors stimulate the rapid and transient activation of one or a pair of 42-44-kDa MAPK family members (15). A significant property of MAPK activation is the requirement for both Thr and Tyr phosphorylation within its protein kinase subdomain VIII, resulting in a mobility shift in SDS-PAGE gels (15). To determine whether SPC induces p42MAPK activation, lysates of Swiss 3T3 cells stimulated for various times with 10 μM SPC were analyzed by Western blotting with anti-p42MAPK antibody. SPC induced a striking and transient p42MAPK activation as judged by the appearance of a slower migrating form of p42MAPK. As shown in Fig. 2A (upper panel), the activation was detectable as early as 3 min after addition of SPC, reached a maximum after 5 min, and disappeared 20 min after incubation of the cells with SPC.

The results obtained with the mobility shift assay were substantiated by immune complex assays of p42MAPK activity using a myelin basic protein peptide as a substrate. As shown in Fig. 2A, lower panel, 10 μM SPC induced a marked stimulation of p42MAPK activity, which peaked within 5 min of incubation. p42MAPK activity returned toward basal levels after 30 min of incubation with SPC and did not change afterward for up to 3 h of incubation. This result was in good agreement with the data obtained in the mobility shift assays. SPC induced MAPK activation in a concentration-dependent manner. Half-maximum and maximum stimulation of p42MAPK activity in immune complex assays were achieved at 6 and 10 μM SPC, respectively (Fig. 2B). The level of p42MAPK activity induced by 10 μM SPC was comparable to that promoted by 20 ng/ml PDGF or 10 nM bombesin in Swiss 3T3 cells (Fig. 2B, inset).

Next, we examined the signaling pathways responsible for SPC-mediated p42MAPK activation in Swiss 3T3 cells.

SPC-induced p42MAPK Activity Is Independent of Calcium and the Integrity of the Actin Cytoskeleton—A pathway leading to stimulation of p44⁹⁰raf-1 and p42MAPK in certain cell lines involves the mobilization of intracellular Ca²⁺ (30, 31). As SPC releases Ca²⁺ from intracellular stores (7–10), we examined whether Ca²⁺ mobilization was involved in the activation of p42MAPK by SPC. Quiescent Swiss 3T3 cells were incubated with 30 nM thapsigargin, which depleted Ca²⁺ from intracellular stores and thereby prevented the rapid increase in [Ca²⁺], induced by SPC (results not shown). This treatment did not inhibit the subsequent stimulation of p42MAPK activity by 10 μM SPC as shown either by mobility shift assays (Fig. 3A, upper panel) or by immune complex assays of p42MAPK activity (Fig. 3A, lower panel). Incubation of Swiss 3T3 cells in a medium containing 3 mM EGTA to chelate extracellular Ca²⁺ also failed to inhibit the subsequent stimulation of p42MAPK by SPC (Fig. 3A, upper and lower panels). Therefore, p42MAPK activation by SPC is not dependent on either mobilization or influx of Ca²⁺ in Swiss 3T3 cells.

Recently, it has been reported that MAPK activation in response to integrin-mediated cell adhesion is dependent on the integrity of the actin cytoskeleton (32, 33). This effect is probably mediated by Grb2-SOS association to tyrosine-phosphorylated p125⁵⁰⁵⁰AK (34). As SPC induces p125⁵⁰⁵⁰AK tyrosine phosphorylation as well as dramatic changes in the organization of the actin cytoskeleton (35), we examined a possible link between these events and SPC-induced p42MAPK activation. Treatment with cytochalasin D, at concentrations that completely disrupted the actin cytoskeleton and blocked p125⁵⁰⁵⁰AK tyrosine phosphorylation (35), had no effect on SPC-induced
Therefore, SPC stimulated \( p42\text{MAPK} \) activation by a pathway that could mediate SPC-induced \( p42\text{MAPK} \) activation in Swiss 3T3 cells. Thus, it was important to examine whether PKC through Raf-1-dependent (36–38) and/or -independent (31) pathways. The lysates were immunoprecipitated with a polyclonal antibody, and the activity of this kinase in the immune complex was measured by a sensitive assay based on the sequential activation of MEK and MAPK. SPC induced a rapid increase in the phosphorylation of 80K/MARCKS, which was first detectable after 1 min and reached a maximum 2.3-fold increase after 3 min of incubation (Fig. 4, inset). Interestingly, the level of \( p74^{raf-1} \) activity measured in SPC-treated cells reached only 15% of that induced by addition of 20 ng/ml PDGF (Fig. 4). PDGF at a low concentration of 0.5 ng/ml induced a level of \( p74^{raf-1} \) activity comparable to that in response to SPC. However, at this concentration, PDGF only weakly induced MAPK activation (data not shown). As 10 \( \mu \text{M} \) SPC was as potent as 20 ng/ml PDGF in stimulating \( p42\text{MAPK} \) activity (Fig. 2B), it therefore seemed unlikely that \( p74^{raf-1} \) was the major mediator of SPC-induced \( p42\text{MAPK} \) activation in Swiss 3T3 cells.

SPC Induces PKC Activation by a Pertussis Toxin-sensitive Pathway—PKC has been shown to regulate the MAPK cascade through Raf-1-dependent (36–38) and/or -independent (31) pathways. Thus, it was important to examine whether PKC could mediate SPC-induced \( p42\text{MAPK} \) activation in Swiss 3T3 cells. As activation of PKC by SPC has not been demonstrated, we first examined whether SPC could stimulate PKC activation in intact and permeabilized Swiss 3T3 cells.

A rapid increase in the phosphorylation of \( \alpha \)-tubulin 80K/MARCKS has been shown to reflect the activation of phorbol ester-sensitive PKC isoforms in intact Swiss 3T3 fibroblasts (39–41). As shown in Fig. 5A, left panel, 10 \( \mu \text{M} \) SPC induced a rapid and sustained increase in the phosphorylation of 80K/MARCKS in \( \alpha^{32\text{P}} \)-labeled Swiss 3T3 cells, which was first detectable 1 min after addition of SPC and peaked within 3 min of incubation. Pretreatment of the cells with the bisindolylmaleimide GF 109203X, a selective inhibitor of the PKC isoforms expressed in Swiss 3T3 cells (26, 42), abolished PDB-induced 80K/MARCKS phosphorylation and markedly reduced SPC-induced 80K/MARCKS phosphorylation (Fig. 5A, right panel), substantiating the role of PKC in the action of SPC.

To corroborate these results, we examined the effect of SPC on 80K/MARCKS phosphorylation in digitonin permeabilized Swiss 3T3 cells. This procedure allows direct access to intracellular compartments while leaving PKC-mediated phosphorylation of 80K/MARCKS unaffected (43). As shown in Fig. 5B, SPC at 10 \( \mu \text{M} \) induced a 3.5 ± 0.6-fold stimulation (\( n = 4 \)) of the phosphorylation of 80K/MARCKS.
werepretreatedwith 3.5 μCi/ml [32P]Pi. After 12 h of incubation at 37 °C, cells were treated with 10 μM SPC for various times (left panel). In parallel experiments, cells were pretreated with 3.5 μM GF 109203X (GF) for 1 h (+) or received an equivalent amount of solvent (−) and were then challenged for 10 min with 200 nM PDB or 10 μM SPC, as indicated (right panel). Cells were subsequently lysed, and lysates were immunoprecipitated with anti-80K/MARCKS antibody and further analyzed by SDS-PAGE prior to autoradiography. The results shown are representative of three independent experiments. The position of 80K/MARCKS is indicated by an arrow. B, effect of GDPβS on the stimulation of 80K/MARCKS phosphorylation by SPC. Quiescent cells were incubated for 1 min in permeabilization medium containing 10 μCi/ml [32P]ATP and 50 μM GDPβS, without (−) or with 10 μM SPC or 6 nM bombesin (Bom), in the absence (−) or presence (+) of 100 μM GDPβS. The result shown is representative of four independent experiments. C, SPC stimulates activation of 80K/MARCKS by a pertussis toxin-sensitive pathway. Swiss 3T3 cells in 35-mm dishes were incubated in phosphate-free DMEM with 50 μCi/ml [32P]Pi. After 12 h of incubation at 37 °C, cells were treated with 30 ng/ml pertussis-toxin (PTX) for another 3 h (+). Control cells received an equivalent amount of solvent (+). Subsequently, the cells were stimulated with 200 nM PDB, 10 μM SPC, or 10 nM bombesin (Bom) for 10 min and lysed. The lysates were immunoprecipitated with anti-80K/MARCKS antibody and further analyzed by SDS-PAGE prior to autoradiography. The results shown are representative of three independent experiments. The position of 80K/MARCKS is indicated by an arrow.

phosphorylation of 80K/MARCKS in permeabilized Swiss 3T3 cells. Interestingly, SPC-induced 80K/MARCKS phosphorylation was prevented by GDPβS, which inhibits G protein activity. This result closely resembled the effect of GDPβS on 80K/MARCKS phosphorylation in response to bombesin, which is shown for comparison (Fig. 5B). GDPβS inhibited 80K/MARCKS phosphorylation by SPC in a concentration-dependent manner with a maximum inhibition occurring at 100 μM GDPβS (data not shown). Thus, the data suggest that a G protein is involved in the signaling pathway leading to PKC activation in response to SPC.

To characterize the nature of this G protein, quiescent Swiss 3T3 cells were labeled with [32P]Pi and treated with 30 ng/ml pertussis toxin for 3 h prior to stimulation with 10 μM SPC. Pertussis toxin ADP-ribosylates and thereby inactivates G proteins of the G_{i/0} subfamily (44). As shown in Fig. 5C, SPC-induced 80K/MARCKS phosphorylation was markedly inhibited by 52 ± 1.6% by pertussis-toxin (n = 4). In contrast, PDB or bombesin-induced 80K/MARCKS phosphorylation was not affected by pertussis toxin. These data demonstrate that a G protein of the G_{i/0} subfamily mediates SPC-induced PKC activation.

SPC induces p42MAPK and p90rsk Activity through PKC and G_{i/0}. Having established that SPC could induce PKC activation, we next assessed whether PKC was required for the stimulation of p42MAPK in response to SPC. Confluent and quiescent Swiss 3T3 cells were incubated with or without GF 109203X prior to stimulation with either 200 nM PDB or 10 μM SPC for 5 min. As shown in Fig. 6A, upper panel, the mobility shift of p42MAPK in response to PDB was completely prevented and, in response to SPC, markedly decreased by pretreatment of the cells with GF 109203X. These results were substantiated by immune complex assays of p42MAPK activity. As shown in Fig. 6A, lower panel, both PDB and SPC induced a striking increase in p42MAPK activity. PDB-induced p42MAPK activity was completely abolished, and SPC-induced p42MAPK activation was largely reduced by 87% in cells pretreated with GF 109203X. Addition of GF 109203X at 3.5 μM did not inhibit p42MAPK activity when added directly to the incubation mixture immediately prior to the start of the kinase reaction (data not shown). Furthermore, when PKC activity was down-regulated by pretreatment of cells with PDB, SPC-induced p42MAPK ac-
MAPK Activation by SPC

SCF induces activation of MAPK through a pertussis toxin-sensitive pathway. Upper panel, confluent and quiescent Swiss 3T3 cells were incubated with various concentrations of pertussis toxin (PTX) for 3 h or received an equivalent amount of solvent (−). Cells were then stimulated with 10 µM SPC for 5 min and lysed, and the lysates were subjected to SDS-PAGE followed by Western blotting with anti-p42MAPK antibody. Shown is a representative of four independent experiments. Lower panel, in parallel experiments immune complex assays for p42MAPK activity were performed as described under “Experimental Procedures.” Cells were treated with 30 ng/ml pertussis toxin (PTX, +, hatched bars) for 3 h or received an equivalent amount of solvent (−, filled bars) and then stimulated for 5 min with 10 µM SPC or 10 nM bombesin (Bom) as indicated. Results are expressed as cpm/1.5 × 10^6 cells, and shown are the mean values ± S.E. of three independent experiments each performed in duplicate.

In conclusion, SPC stimulates MAPK and p90rsk activity, predominantly by a signaling pathway involving a pertussis toxin-sensitive G protein and PKC activation.

SCF-induced DNA Synthesis Is Partially Dependent on Gi—MAPK and p90rsk have both been implicated in the stimulation of cell proliferation (15, 20). SPC-induced activation of MAPK and p90rsk involves a GxiGi protein. Thus, if these pathways were important for the mitogenic effect of SPC, a Gi protein should also participate in SPC-induced DNA synthesis. To assess an involvement of Gi proteins in DNA synthesis in response to SPC, quiescent cultures of Swiss 3T3 cells were exposed to various concentrations of SPC in the absence or presence of 30 ng/ml pertussis toxin. As shown in Table I, treatment of the cells with pertussis toxin inhibited SPC-induced DNA synthesis at all concentrations of SPC examined by about 50%.

SCF Also Stimulates DNA Synthesis and p42MAPK Activation in MEF—In view of the preceding results, it was important to verify that the stimulatory effects of SPC on DNA synthesis, p42MAPK, and p74raf-1 are not confined to Swiss 3T3 cells. As shown in Fig. 9A, SPC also acted as a single mitogen in secondary cultures of MEF reaching 26% of the level of [3H]thymidine incorporation induced by 10% fetal bovine serum. Interestingly, treatment of these cells with pertussis toxin reduced SPC-induced DNA synthesis by about 50% (Fig. 9A). Furthermore, SPC potently induced p42MAPK activation in immune

![Graph](https://example.com/image1.png)

Fig. 7. SPC induces activation of MAPK through a pertussis toxin-sensitive pathway. Upper panel, confluent and quiescent Swiss 3T3 cells were incubated with various concentrations of pertussis toxin (PTX) for 3 h or received an equivalent amount of solvent (−). Cells were then stimulated with 10 µM SPC for 5 min and lysed, and the lysates were subjected to SDS-PAGE followed by Western blotting with anti-p42MAPK antibody. Shown is a representative of four independent experiments. Lower panel, in parallel experiments immune complex assays for p42MAPK activity were performed as described under “Experimental Procedures.” Cells were treated with 30 ng/ml pertussis toxin (PTX, +, hatched bars) for 3 h or received an equivalent amount of solvent (−, filled bars) and then stimulated for 5 min with 10 µM SPC or 10 nM bombesin (Bom) as indicated. Results are expressed as cpm/1.5 × 10^6 cells, and shown are the mean values ± S.E. of three independent experiments each performed in duplicate.

![Graph](https://example.com/image2.png)

Fig. 8. SPC stimulates p90rsk activity through a PKC-dependent and pertussis toxin-sensitive pathway. A, quiescent Swiss 3T3 cells were stimulated with 10 µM SPC for various times. Cells were subsequently lysed and further processed as described under “Experimental Procedures.” Results are expressed as cpm × 10^-3/1.5 × 10^6 cells. Shown are the mean values ± S.E. of three independent experiments each performed in duplicate. Where no error bar is shown, it lies within the dimensions of the symbol. B, quiescent Swiss 3T3 cells were incubated with 3.5 µM GF 109203X (GF, −) for 1 h or 30 ng/ml pertussis toxin (PTX, +) for 3 h, or they received an equivalent amount of solvent (−). Subsequently, the cells were stimulated with 10 µM SPC for 5 min, lysed, and further processed as described under “Experimental Procedures.” Results are expressed as cpm × 10^-3/1.5 × 10^6 cells, and shown are the mean values ± S.E. of three independent experiments each performed in duplicate.

To examine whether SPC could also stimulate p90rsk activity, lysates of quiescent Swiss 3T3 cells treated with 10 µM SPC for various times were immunoprecipitated with a polyclonal anti-p90rsk antibody and, the immunoprecipitates were further analyzed by an immune complex kinase assay. As shown in Fig. 8A, SPC rapidly stimulated p90rsk activity, reaching a maximum after 5 min of incubation. Pretreatment of Swiss 3T3 cells with either GF 109203X or pertussis toxin markedly reduced p90rsk activation in response to 10 µM SPC (Fig. 8B).

In conclusion, SPC stimulates MAPK and p90rsk activation was reduced by 87% (Fig. 6B). Thus, SPC induced p42MAPK activation by a pathway dependent on PKC activity in Swiss 3T3 cells.

As shown above, SPC stimulated PKC activation via a pertussis toxin-sensitive G protein, and, in turn, MAPK activation was dependent on PKC. Therefore, we reasoned that SPC-induced p42MAPK activation should also be dependent on G<sub>i</sub>. To examine this prediction, quiescent Swiss 3T3 cells were incubated with various concentrations of pertussis toxin for 3 h and then treated with 10 µM SPC for 5 min. As shown in Fig. 7, upper panel, pertussis toxin prevented a subsequent activation of p42MAPK by SPC in a concentration-dependent manner. Stimulation of p42MAPK by SPC was attenuated at a concentration of pertussis toxin as low as 0.3 ng/ml, and a maximum inhibition of the MAPK mobility shift was achieved at 30 ng/ml.

The inhibitory effect of pertussis toxin was selective as shown by immune complex assays of p42MAPK activity. Pretreatment of the cells for 3 h with 30 ng/ml pertussis toxin prior to stimulation did not inhibit bombesin or PDB-stimulated p42MAPK activity but reduced SPC-induced p42MAPK activity by 75% (Fig. 7, lower panel and data not shown). These results indicate that SPC stimulates MAPK by a pertussis toxin-sensitive pathway.

One of the major downstream targets of MAPK is p90rsk (20). To examine whether SPC could also stimulate p90rsk activity, lysates of quiescent Swiss 3T3 cells treated with 10 µM SPC for various times were immunoprecipitated with a polyclonal anti-p90rsk antibody and, the immunoprecipitates were further analyzed by an immune complex kinase assay. As shown in Fig. 8A, SPC rapidly stimulated p90rsk activity, reaching a maximum after 5 min of incubation. Pretreatment of Swiss 3T3 cells with either GF 109203X or pertussis toxin markedly reduced p90rsk activation in response to 10 µM SPC (Fig. 8B).

In conclusion, SPC stimulates MAPK and p90rsk activation...
complex assays in MEF (Fig. 9B). However, as in Swiss 3T3 cells, SPC only weakly stimulated p74\textsuperscript{ras} activity, reaching only 14% of that obtained in response to PDGF (Fig. 9C). We found that SPC-induced p42\textsuperscript{MAPK} activation in MEF was similarly dependent on PKC and a G\textsubscript{i} protein, as pretreatment of these cells with either GF 109203X or pertussis toxin inhibited SPC-induced p42\textsuperscript{MAPK} activation by 67 and 85%, respectively (Fig. 9D).

**DISCUSSION**

The results presented here demonstrate, for the first time, that SPC induces a rapid and striking activation of p42\textsuperscript{MAPK}, p90\textsuperscript{rsk}, and PKC. Our results reveal that SPC-induced activation of the MAPK cascade has unique features in terms of its kinetics and the signaling pathways that mediate this process in Swiss 3T3 cells as well as in secondary cultures of MEF.

The time course of the p42\textsuperscript{MAPK} activation by extracellular stimuli has been the subject of considerable attention. It has been proposed that agents that can act as single mitogens induce persistent stimulation of the MAPK cascade (45). In contrast, our results show that SPC, which induces DNA synthesis in the absence of any other exogenously added growth factor as efficiently as bombesin and PDGF, stimulated a transient rather than persistent activation of p42\textsuperscript{MAPK}. Thus, the SPC results demonstrate that a persistent stimulation of the MAPK cascade is not a necessary prerequisite for the induction of DNA synthesis in Swiss 3T3 cells.

MAPK activation is known to be triggered by a variety of signaling pathways (15). Our studies demonstrate that SPC-induced p42\textsuperscript{MAPK} activation is mediated predominantly by phorbol ester-sensitive PKCs as judged by both inhibition and down-regulation of these enzymes. None of the previous reports on SPC signaling demonstrated stimulation of PKC. Indeed, previous studies using concentrations of SPC 10 times higher than those used in this paper suggested that SPC is an inhibitor of PKC activity (46). In the present study, we found that SPC markedly and rapidly increases the phosphorylation of the major PKC substrate 80K/MARCKS either in intact or in permeabilized cells. Interestingly, recent results demonstrated that this protein is in fact phosphorylated by PKCs \(\alpha, \epsilon, \) and \(\delta\) (39). All of these PKC isoforms are expressed in Swiss 3T3 cells, inhibited by GF 109203X, and down-regulated by prolonged treatment with PDB in these cells (26). Thus, SPC stimulates transient p42\textsuperscript{MAPK} activation through a PKC-dependent signaling pathway.

In the present study, we provide two independent lines of evidence that SPC stimulation of PKC involves an intermediary G protein: a) the stimulation of 80K/MARCKS in permeabilized cells was severely inhibited by the G protein antagonist GDP\textsubscript{B}S and b) the increase in 80K/MARCKS phosphorylation in intact cells was inhibited by prior exposure to pertussis toxin, which ADP-ribosylates and inactivates G\textsubscript{i}. and G\textsubscript{q}. The concentration of pertussis toxin required to block PKC activation has previously been demonstrated to ADP-ribosylate a 40-kDa G protein in intact Swiss 3T3 cells (47). If PKC mediates activation of p42\textsuperscript{MAPK} in response to SPC, treatment of cells with pertussis toxin should be expected to prevent p42\textsuperscript{MAPK} activation. This prediction was verified experimentally. We found that SPC-mediated activation of p42\textsuperscript{MAPK} was profoundly inhibited by prior treatment of Swiss 3T3 cells with pertussis toxin.

A 90-kDa S6 protein kinase, named p90\textsuperscript{rsk}, is directly regulated by MAPKs that phosphorylate and partially reactivate dephosphorylated p90\textsuperscript{rsk} (20). The results presented here demonstrate that SPC induces a transient activation of p90\textsuperscript{rsk} ac-

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**Table I**

| [SPC] (\(\mu\text{M}\)) | [\(^{3}H\)Thymidine incorporation (% of FBS) | + PTX |
|-----------------------|-------------------------------------------|------|
| 0.5                   | 5 ± 0.5                                   | 3.2 ± 0.2 |
| 2.5                   | 12.5 ± 0.8                                | 6.4 ± 0.3 |
| 10                    | 40.6 ± 2                                  | 18.7 ± 2.6 |

**Fig. 9**

**A** Effect of SPC on DNA synthesis, p42\textsuperscript{MAPK}, and p74\textsuperscript{ras} activity in secondary cultures of MEF. A, SPC stimulates DNA synthesis in MEF. Confluent secondary cultures of MEF in 35-mm dishes were stimulated with 10 \(\mu\text{M}\) SPC in the presence (+) or absence (−) of 30 ng/ml pertussis-toxin (PTX). [\(^{3}H\)Thymidine incorporation was measured as described under “Experimental Procedures.” Results are expressed as cpm \(\times 10^{3}\) of FBS. Shown are the mean values of two independent experiments each performed in duplicate. B, SPC induces p42\textsuperscript{MAPK} activation in MEF. Confluent secondary cultures of MEF were incubated with 10 \(\mu\text{M}\) SPC or 20 ng/ml PDGF for 5 min, and immune complex assays for p42\textsuperscript{MAPK} activation were performed as described under “Experimental Procedures.” Results are expressed as cpm \(\times 10^{-3}\) of FBS of 30 ng/ml pertussis-toxin (PTX) for 1 h or 30 ng/ml pertussis toxin (PTX) for 3 h; or they received an equivalent amount of solvent (−). Subsequently, the cells were stimulated with 10 \(\mu\text{M}\) SPC for 5 min, lysed, and further processed as described under “Experimental Procedures.” Shown are the mean values of two independent experiments each performed in duplicates. C, SPC induces p74\textsuperscript{ras} activity in MEF. Confluent secondary cultures of MEF were treated with 10 \(\mu\text{M}\) SPC or 20 ng/ml PDGF for 3 min and lysed; p74\textsuperscript{ras} activity was then assayed as described under “Experimental Procedures.” Data are expressed as cpm \(\times 10^{-3}\) of FBS of 30 ng/ml pertussis-toxin (PTX) for 1 h or 30 ng/ml pertussis toxin (PTX) for 3 h; or they received an equivalent amount of solvent (−). Subsequently, the cells were stimulated with 10 \(\mu\text{M}\) SPC for 5 min, lysed, and further processed as described under “Experimental Procedures.” Shown are the mean values of two independent experiments each performed in duplicates.
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ity. As expected from the results obtained with p42MAPK, SPC stimulation of p90Sk is mediated by PKC via a pertussis toxin-sensitive signal transduction pathway.

As both p42MAPK and p90Sk are localized in the cytoplasm and in the nucleus and possess the potential for directly regulating gene expression by transcription factor phosphorylation (15, 20, 21), these signaling pathways are likely to contribute to SPC-induced mitogenesis in fibroblasts. We demonstrate in this paper that SPC-induced mitogenesis is also dependent on a G protein in Swiss 3T3 cells. These findings are not restricted to Swiss 3T3 cells. We verified that SPC induced p42MAPK activation and DNA synthesis in secondary cultures of MEF through a largely pertussis toxin-sensitive pathway.

The signaling pathways leading to p42MAPK activation in response to SPC can be distinguished from those utilized by bombesin and PDGF, which, like SPC, are potent mitogens for Swiss 3T3 cells. Bombesin-induced p42MAPK activation is predominantly mediated by PKC (19) but independent of a G, signaling pathway in Swiss 3T3 cells. PDGF causes accumulation of p21ras-GTP, which then initiates activation of a kinase cascade comprising p74raf, MEK, and MAPKs (15). In agreement with these reports, we found that PDGF induces a marked increase in p74raf and p42MAPK activity in Swiss 3T3 cells. In contrast, SPC is as potent as PDGF in stimulating p42MAPK activity but elicits only a small increase in p74raf activation. Furthermore, the stimulation of the MAPK cascade by PDGF is entirely resistant to pertussis toxin treatment (data not shown).

Stimulation of p42MAPK activation by SPC is also different from that induced in response to integrins. In contrast to integrin signaling (32), SPC-induced p42MAPK activation is independent of the integrity of the actin cytoskeleton. However, SPC-induced tyrosine phosphorylation of p125FAK and changes in the organization of the actin cytoskeleton were strongly inhibited by cytochalasin D (35). Furthermore, the signaling mechanism by which SPC induces MAPK activation appears to differ from that utilized by other ligands that act on G,-coupled receptors such as thrombin and LPA, which stimulate MAPK activation independently of PKC (48, 49).

It is known that SPC directly releases Ca2 + from inositol 1,4,5-trisphosphate-sensitive stores in a variety of permeabilized cell preparations (8). Consequently, it has been proposed that SPC acts intracellularly, perhaps as a direct mediator of the effects of sphingosine and sphingosine-1-phosphate (9, 10). It is known that SPC directly releases Ca2 + (32), which may provide an attractive agonist to explore the molecular mechanisms of SPC-induced mitogenesis in fibroblasts. We demonstrate in this paper that SPC-induced mitogenesis is also dependent on a Gi protein in Swiss 3T3 cells. These findings are not restricted for a different physiological ligand. In view of the possibility of the mitogenic effects induced by SPC in serum-free medium and the striking early responses shown in the present and in the accompanying paper (35), we conclude that SPC provides an attractive agonist to explore the molecular mechanisms underlying the regulation of cytoskeletal architecture and cell proliferation.

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