Calcium-induced Human Keratinocyte Differentiation Requires *src*- and *fyn*-mediated Phosphatidylinositol 3-Kinase–dependent Activation of Phospholipase C- γ 1

Zhongjian Xie, Patrick A. Singleton, Lilly Y.W. Bourguignon, and Daniel D. Bikle

Endocrine Unit, Veterans Affairs Medical Center, Northern California Institute for Research and Education and University of California–San Francisco, San Francisco, CA 94121

Submitted February 10, 2005; Revised April 11, 2005; Accepted April 25, 2005

Monitoring Editor: M. Bishr Omary

We have previously demonstrated that phospholipase C (PLC)- γ 1 is required for calcium-induced human keratinocyte differentiation. In the present study, we investigated whether the activation of PLC- γ 1 by nonreceptor kinases such as *src* and *fyn* plays a role in mediating this process. Our results showed that the combination of dominant negative *src* and *fyn* blocked calcium-stimulated PLC- γ 1 activity and human keratinocyte differentiation, whereas each separately has little effect. However, unlike the activation of PLC- γ 1 by epidermal growth factor, calcium-induced activation of PLC- γ 1 was not a result of direct tyrosine phosphorylation. Therefore, we examined an alternative mechanism, in particular phosphatidylinositol 3,4,5-triphosphate (PIP₃) formed as a product of phosphatidylinositol 3-kinase (PI3K) activity. PIP₃ binds to and activates PLC- γ 1. The combination of dominant negative *src* and *fyn* blocked calcium-induced tyrosine phosphorylation of the regulatory subunit of PI3K, p85 α , and the activity of the catalytic subunit of PI3K. PI3K inhibitors blocked calcium activation of PLC- γ 1 as well as the induction of keratinocyte differentiation markers involucrin and transglutaminase. These data indicate that calcium activates PLC- γ 1 via increased PIP₃ formation mediated by *c-src*- and *fyn*-activated PI3K. This activation is required for calcium-induced human keratinocyte differentiation.

INTRODUCTION

Phospholipase C (PLC) catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). These molecules regulate the mobilization of intracellular Ca²⁺ and protein kinase C activation, respectively (Berridge and Irvine, 1984; Majerus *et al.*, 1986). To date, four types of phosphoinositide-specific PLC isozymes have been identified: PLC- β , PLC- γ , PLC- δ , and PLC- ϵ . Each type contains several subtypes (Rhee *et al.*, 1989). All PLC family members contain X and Y domains that fold together to form the catalytic site (Rhee and Bae, 1997). In the γ -type isozymes, including PLC- γ 1 and PLC- γ 2, the linker region between the X and Y domains is extended and contains two Src homology (SH)2 domains and one SH3 domain (Carpenter and Ji, 1999). These two SH2 domains bind to phosphorylated tyrosine residues of receptor kinases including the epidermal growth factor (EGF) receptor, platelet-derived growth factor receptor, and nerve growth factor receptor, resulting in the tyrosine phosphorylation of PLC- γ isozymes (Carpenter and Ji, 1999). PLC- γ isozymes also are phosphorylated and activated by various nonreceptor tyrosine kinases, including *src*,

fyn, *lck*, *lyn*, and *hck* (Liao *et al.*, 1993; Ozdener *et al.*, 2002; Boudot *et al.*, 2003). Some of these kinases such as *src* and *fyn* are increased during human or mouse keratinocyte differentiation induced by calcium (Zhao *et al.*, 1992; Calautti *et al.*, 1995) and also control mouse keratinocyte cell-cell adhesion (Calautti *et al.*, 1998, 2002).

PLC- γ isozymes also can be activated directly by several lipid-derived second messengers, including phosphatidic acid (Jones and Carpenter, 1993a), arachidonic acid (Hwang *et al.*, 1996; Sekiya *et al.*, 1999), and phosphatidylinositol 3,4,5-triphosphate (PIP₃) (Bae *et al.*, 1998; Falasca *et al.*, 1998; Rameh *et al.*, 1998) in the absence of tyrosine phosphorylation. Phosphatidic acid and arachidonic acid are primarily derived from phosphatidylcholine by the action of phospholipase D and phospholipase A₂, respectively. PIP₃ is primarily derived from PIP₂ by the action of phosphatidylinositol 3-kinase (PI3K). Therefore, activation of PLC- γ isozymes may occur secondarily to the activation of phospholipase D, phospholipase A₂, or PI3K.

Unlike PLC- γ 2 expressed mainly in cells of hematopoietic origin, PLC- γ 1 is found in a variety of cells, including keratinocytes. PLC- γ 1 is required for calcium-induced human keratinocyte differentiation as we demonstrated previously (Xie and Bikle, 1999). In the present study, we investigated the mechanism by which PLC- γ 1 is activated by calcium in human keratinocytes. We found that calcium induces *src* and *fyn* activity, that *src* and *fyn* activity are required for calcium activation of PLC- γ 1, but that PLC- γ 1 activation occurs through a mechanism that does not involve direct tyrosine phosphorylation of PLC- γ 1. Rather, this mechanism involves PI3K activation and PIP₃ generation. Inhibition of this mechanism blocks calcium-induced human keratinocyte differentiation.

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05-02-0109>) on May 4, 2005.

Address correspondence to: Zhongjian Xie (zjxie@itsa.ucsf.edu).

Abbreviations used: DAG, diacylglycerol; EGF, epidermal growth factor; IP₃, inositol 1,4,5-trisphosphate; KGM, keratinocyte growth medium; PI3K, phosphatidylinositol 3-kinase; PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP₃, phosphatidylinositol 3,4,5-bisphosphate; PLC, phospholipase C.

MATERIALS AND METHODS

Cell Culture

Human keratinocytes were isolated from neonatal human foreskins and grown in serum-free keratinocyte growth medium (KGM; Cambrex Bio Science Walkersville, Walkersville, MD) as described previously (Pillai *et al.*, 1988). Briefly, human keratinocytes were isolated from newborn human foreskins by trypsinization (0.25% trypsin, 4°C, overnight), and primary cultures were established in KGM containing 0.07 mM calcium. The second passage human keratinocytes were plated with KGM containing 0.03 mM calcium and used in the subsequent experiments. To initiate differentiation, the calcium concentration was raised to 1.2 mM.

Dominant Negative Constructs Transfection and Selection

The dominant negative *src* and *fyn* constructs were kindly provided by Dr. Filippo G. Giancotti (Memorial Sloan-Kettering Cancer Center, New York, NY). The kinase-inactivating mutation in *fyn* was a replacement of Lys 299 by Met, and in *src* it was a replacement of Lys 295 by Met. These dominant negative constructs have been shown to specifically inactivate *src* and *fyn* activities (Twamley-Stein *et al.*, 1993). The dominant negative *src* and *fyn* inserts were resubcloned into pcDNA 3.1(-) (Invitrogen, Carlsbad, CA). Second passage human keratinocytes were transfected with dominant negative constructs by using TransIt-keratinocyte transfection reagent (Mirus, PanVera, Madison, WI). To enrich transfected cells, the transiently transfected cells were selected by a 2-d incubation with G418 at a concentration of 200 μ g/ml starting 2 d after transfection as described previously (Xie and Bikle, 1999).

Cell Lysate Preparation, Western Analysis, and Immunoprecipitation

Human keratinocytes in cell culture plates were washed twice with phosphate-buffered saline (PBS) and then incubated in PBS containing 1% NP-40 and Complete protease inhibitors (Roche Diagnostics, Indianapolis, IN) or radioimmunoprecipitation assay (RIPA) lysis buffer containing 50 mM HEPES, pH 7.4, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and Complete protease inhibitors (Roche Diagnostics) for 5 min. Cells were scraped into Microfuge tubes, incubated at 4°C for 30 min, and pelleted by centrifugation. The supernatant was collected. The protein concentration of the lysate was measured by a bicinchoninic acid (BCA) protein assay kit (Pierce Chemical, Rockford, IL). Equal amounts of protein were electrophoresed through reducing SDS-PAGE and electroblotted onto polyvinylidene fluoride (PVDF) microporous membranes (Immobilon-P, 0.45 μ m; Millipore, Billerica, MA). After incubation in blocking buffer (100 mM Tris base, 150 mM NaCl, 5% nonfat milk or 2% BSA, and 0.5% Tween 20), the blot was incubated overnight at 4°C with appropriate primary antibodies: monoclonal antibodies against human involucrin (Sigma-Aldrich, St. Louis, MO) at a dilution of 1:2000, monoclonal antibodies against human transglutaminase (Biomedical Technologies, Stoughton, MA) at a dilution of 1:200, polyclonal antibodies against PLC- γ 1 (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200, polyclonal antibodies against phospho-PLC- γ 1 (Tyr 783; Santa Cruz Biotechnology) at a dilution of 1:200, polyclonal antibodies against phospho-PLC- γ 1 (Tyr 1254; Santa Cruz Biotechnology) at a dilution of 1:200, monoclonal antibodies against phosphotyrosine-containing proteins (PY99; Santa Cruz Biotechnology) at a dilution of 1:200, polyclonal antibodies against *src* and *fyn* (Santa Cruz Biotechnology) at a dilution of 1:200, polyclonal antibodies against Akt and phosphorylated Akt (Cell Signaling Technology, Beverly, MA) at a dilution of 1:1000, and polyclonal antibodies against PI3K p85 α (Santa Cruz Biotechnology) at a dilution of 1:200. After washes in the blocking buffer, the membranes were incubated for 1 h with the appropriate anti-IgG secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences, Piscataway, NJ) in the blocking buffer. After a second series of washes, bound antibody complexes were visualized using the SuperSignal ULTRA chemiluminescent kit (Pierce Chemical) and subsequent exposure to x-ray film. In some experiments, equal amounts of protein were incubated with a specific antibody at 4°C for 1 h and then with UltraLink immobilized protein G (Pierce Chemical) at 4°C overnight. Primary antibodies included monoclonal antibodies against phosphotyrosine (p-Tyr; Santa Cruz Biotechnology) and polyclonal antibodies against PLC- γ 1 (Santa Cruz Biotechnology). The lysate-antibody-agarose beads mixture was washed four times with PBS and then analyzed by Western analysis as described above.

PLC- γ 1 Activity Assay

PLC- γ 1 activity was determined by measuring accumulation of IP₃ according to the experimental procedure described recently (Bourguignon *et al.*, 2004). Cells in 150-mm dishes were washed with PBS containing 0.1% sodium orthovanadate and 0.1% NaF and then incubated with RIPA lysis buffer or 1% NP-40 containing 0.1% sodium orthovanadate, 0.1% NaF, and Complete protease inhibitors (Roche Diagnostics) for 5 min. Cells were scraped into Microfuge tubes and incubated at 4°C on a rotator for 1 h. The supernatant collected after centrifugation was chromatographed over a wheat germ agglutinin column (EY Laboratories, San Mateo, CA). The eluent containing 100

μ g of protein was incubated with 2 μ g polyclonal PLC- γ 1 antibodies (BD Biosciences, San Jose, CA) at 4°C overnight and then 20 μ l of UltraLink immobilized protein G (Pierce Chemical) at 4°C for 1 h. After centrifugation, the pellet was washed with reaction buffer containing 10 mM HEPES, pH 7.0, 10 mM NaCl, 120 mM KCl, 2 mM EGTA, 0.05% deoxycholate, 5 μ g/ml BSA, and 10 μ M CaCl₂ and resuspended in 800 μ l of reaction buffer. In triplicates, 50 μ l of suspension was incubated with 10 μ M [³H]PIP₂ (6.5 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA), phosphatidylcholine (Sigma-Aldrich), and phosphatidylserine (Sigma-Aldrich) in a molar ratio of 1:3:3 in the reaction buffer. The reaction was ended by adding 200 μ l of 10% trichloroacetic acid (TCA) at 1, 2, 3, 4, and 5 min and then 200 μ l of 10% BSA. The radioactivity of 50 μ l of supernatant after centrifugation was determined by a scintillation counter. The concentration of [³H]IP₃ produced was calculated based on the specific activity of the substrate.

src, *fyn*, and *lyn* Kinase Activity Assays

src kinase assays were carried out using the *src* assay kit according to the manufacturer's instructions (Upstate Biotechnology, Charlottesville, VA). *fyn* and *lyn* kinase assays were carried out according to a protocol modified based on the instructions for the *src* kinase assay. Briefly, 0.5 mg of total cellular protein isolated from human keratinocytes in 1 ml of buffer A [50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM activated sodium orthovanadate, 10 mM sodium β -glycerol phosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 0.1% (wt/vol) Triton X-100, 0.1% 2-mercaptoethanol, 0.27 M sucrose, and 1 μ M microcystin LR] containing Complete protease inhibitors (Roche Diagnostics) was incubated with 2 μ g of *src* polyclonal antibody, 2 μ g of *fyn* polyclonal antibody, or 2 μ g of *lyn* polyclonal antibody at 4°C for 1 h. Immunocomplexes were collected by incubation with 20 μ l of UltraLink immobilized protein G (Pierce Chemical) overnight. For the *src* activity assay, the immunoprecipitate was washed three times with ice-cold buffer A and then incubated with 10 μ l (150 μ M final concentration) of the substrate peptide, 10 μ l of *src* reaction buffer, and 10 μ l (0.01 μ Ci) of [³²P]ATP (Amersham Biosciences) in a total volume of 40 μ l for 30 min at 30°C with agitation. To precipitate the peptide, 20 μ l of 40% TCA was added and incubated for 5 min at room temperature. In triplicate, a 25- μ l aliquot was transferred onto the center of a numbered P81 paper square. The assay squares were washed five times for 5 min each with 0.75% phosphoric acid and once with acetone. The assay squares were transferred to a scintillation vial, 10 ml of scintillation cocktail added, and the level of radioactivity was determined by a scintillation counter. For the *fyn* or *lyn* activity assay, the immunoprecipitate was washed three times with *fyn/lyn* assay buffer and then incubated with 2.5 μ l (250 μ M final concentration) of the appropriate substrate peptide, 6.25 μ l of assay buffer, and 10 μ l (0.01 μ Ci) of [³²P]ATP (Amersham Biosciences) in a total volume of 25 μ l for 30 min at 30°C with agitation. In triplicate, a 6- μ l aliquot was transferred onto the center of a numbered P81 paper square. The assay squares were washed three times for 5 min each with 0.75% phosphoric acid and once with acetone for 1 min. The assay squares were transferred to a scintillation vial, 10 ml of scintillation cocktail added, and the level of radioactivity was determined by a scintillation counter.

PI3K Activity Assay

The PI3K activity assay was performed according to the experimental procedure described recently (Bourguignon *et al.*, 2003). Briefly, cells were washed with PBS and solubilized in buffer A (1% NP-40, 1% sodium orthovanadate, 1% phenylmethylsulfonyl fluoride [PMSF], and 25 ng/ml okadaic acid) containing Complete protease inhibitors (Roche Diagnostics) at 4°C for 1 h followed by immunoprecipitation with antibody-conjugated beads against p110 α , p110 β , p110 γ , or p110 δ . The antibody conjugated beads were then incubated in 50 μ l of buffer A containing 20 μ g of sonicated PIP₂ (Sigma-Aldrich) and 10 μ Ci of [³²P]ATP for 30 min at 32°C. The total ATP concentration in the reaction was 1 mM. Subsequently, the reaction was terminated by the addition of acidified chloroform:methanol [1:1 (vol/vol)]. Extracted lipids were then spotted and developed using a solvent system containing chloroform:acetone:methanol:acetic acid/water (80:30:26:24:14). The thin layer chromatography plates were dried, and radioactively labeled PIP₃ spots were visualized by autoradiography.

PIP₃ Assay

One hundred micrograms of total protein extracted from human keratinocytes with 1% NP-40 was incubated with PLC- γ 1 antibody at 4°C overnight and then with UltraLink immobilized protein G (Pierce Chemical) for 1 h. PIP₃ bound to PLC- γ 1 was extracted by chloroform and then spotted onto a PVDF membrane. After incubation in blocking buffer (100 mM Tris base, 150 mM NaCl, 5% nonfat milk or 2% BSA, and 0.5% Tween 20), the membrane was incubated overnight at 4°C with a monoclonal antibody against PIP₃ (Echelon Research Laboratories, Salt Lake City, UT) at a concentration of 1 μ g/ml. After washes in the blocking buffer, the membranes were incubated for 1 h with rabbit anti-IgG secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences) in the blocking buffer. After a second series of washes, bound antibody complexes were visualized using the Super-

Signal ULTRA chemiluminescent kit (Pierce Chemical) and subsequent exposure to x-ray film.

RESULTS

Several members of the *src* family of tyrosine kinases are expressed in keratinocytes, including *src* and *fyn*, and each has been shown to be increased in protein levels and activities in human or mouse keratinocytes induced to differentiate by calcium (Zhao *et al.*, 1992; Calautti *et al.*, 1995). To confirm these results, human keratinocytes were treated with 1.2 mM calcium, and *src* and *fyn* activities in cells were determined and normalized to the protein level in the immunoprecipitates. We found that calcium increased the intrinsic activity and protein level of *src* above control in a time-dependent manner (Figure 1A). The induction of *src* activity by calcium occurred as early as 5 min, with a peak more than threefold increase by 30 min. The induction of *src* protein levels in the immunoprecipitates by calcium occurred as early as 1 h, with a plateau starting at 6 h (Figure 1A). The induction of the intrinsic activity of *fyn* by calcium also occurred as early as 5 min (Figure 1B). However, *fyn* protein levels in the immunoprecipitates were not induced by calcium (Figure 1B). Similar data were obtained when total cellular extracts of *src* and *fyn* were analyzed for protein levels (our unpublished data). These results demonstrate that calcium induces both activity and protein levels of *src* but only activity of *fyn*. To investigate the role of *src* family tyrosine kinases in human keratinocyte differentiation, human keratinocytes were pretreated with a specific *src* family inhibitor PP2 for 1 h, and the responses of keratinocyte differentiation markers involucrin and transglutaminase to 1.2 mM calcium were examined (Figure 2A). The results showed that the levels of involucrin and transglutaminase were induced >3- and 12-fold, respectively, with 24 h of calcium administration. The induction of involucrin by calcium was reduced over threefold with PP2 treatment. The level of transglutaminase in PP2-treated keratinocytes was reduced to a level undetectable by Western analysis. In contrast, calcium-induced involucrin and transglutaminase were not affected by the vehicle control (dimethyl sulfoxide, DMSO) and an inactive analogue PP3 (Figure 2A). These results indicate that the *src* family is critical in calcium-induced human keratinocyte differentiation. To identify the isoforms of the *src* family involved in human keratinocyte differentiation, we transfected *src* and *fyn* dominant negative constructs into human keratinocytes. To enrich the fraction of transfected cells, cells were then treated with neomycin (G418) for 2 d. To determine the transfection efficiency, in pilot experiments, we first transfected an enhanced green fluorescent protein (EGFP)-expression vector into human keratinocytes at a density of 40% confluence. Two days later, cell density reached 85 to 90% confluence, and the EGFP was expressed in ~40% of the cell population. To determine the efficiency of G418, killing curves of nontransfected keratinocytes by G418 were established in pilot experiments by treating 100% confluent keratinocyte cultures with different doses of G418 (50–400 μ g/ml) for various lengths of time (1–5 d). We found that treatment with 200 μ g/ml G418 for 2 d consistently killed >85% of the cells without affecting cell differentiation. Using this transfection method, equal amounts of dominant negative constructs for *src* and *fyn* or the combination of both were transfected into human keratinocytes, and the cells were then treated with 1.2 mM calcium for 24 h after a 2-d G418 selection. The results showed that neither dominant negative *src* nor *fyn* alone affected the calcium induction of involucrin and transglu-

Figure 1. Calcium stimulates *src* and *fyn* activities. Cultured human keratinocytes were treated with 1.2 mM calcium. The cells were harvested at the time points indicated. The activities of *src* (A) and *fyn* (B) were assayed as described in *Materials and Methods* and normalized to the relative protein levels in the immunoprecipitates. The relative activities are expressed as the percentage of that at zero time point. Protein levels of *c-src* (A) and *fyn* (B) in the immunoprecipitates were measured by Western analysis.

taminase. However, the combination of dominant negative *src* and *fyn* completely blocked this induction (Figure 2B). The expression levels of dominant negative *src* and *fyn* in human keratinocytes were determined by Western analysis by using *src* and *fyn* antibodies. Although calcium increased endogenous *src* levels, it did not alter the expression of the dominant negative constructs. Because the total amount of DNA transfected into these cells was the same for the combination of dominant negative *src* and *fyn* as when transfected individually, the expression of either *src* or *fyn* in the

Figure 2. The *src* family kinase inhibitor PP2 and the combination of dominant negative *src* and *fyn* block calcium-induced involucrin and transglutaminase expression. (A) Cultured human keratinocytes were treated with a specific *src* family kinase inhibitor (PP2) or its inactive analogue (PP3) at a concentration of 10 μ M for 1 h. Cells were then stimulated with calcium for 24 h, and cells were harvested. The protein levels of involucrin and transglutaminase were determined by Western analysis. (B and C) Human keratinocytes cultured in KGM containing 0.03 mM calcium were transfected with dominant negative *src* (*src*-DN) and/or *fyn* (*fyn*-DN). Cells in each plate contained equal amounts of DNA during the transfection. Cells were then stimulated with 1.2 mM calcium for 24 h, and cells were harvested. The protein levels for involucrin, transglutaminase, *src*, and *fyn* were measured by Western analysis (B). The activities of *src*, *fyn*, and *lyn* (a control for determining the specificity of *src*-DN and *fyn*-DN) were assayed as described in *Materials and Methods* and are expressed as the percentage of the vector control at 0.03 mM calcium (C).

Figure 3. Calcium stimulates PLC- γ 1 activity. Cultured human keratinocytes were treated with 1.2 mM calcium. The cells were harvested at the time points indicated after the addition of calcium. The activity of PLC- γ 1 was assayed as described in *Materials and Methods* by using a time course from zero to 15 min for each reaction. The activity of PLC- γ 1 was expressed as picomoles of IP₃ formed and normalized to the total protein content used for the immunoprecipitation.

combined transfection was ~50% of that for either construct transfected by itself (Figure 2B). To determine the specificity of *src* and *fyn* dominant negative constructs, the activities for *src*, *fyn*, and *lyn* were evaluated. The activity of *lyn* was used as a negative control for the inhibitory effect of dominant negative *src* and *fyn* constructs. The results showed that the *src* or *fyn* dominant negative construct specifically inhibited *src* or *fyn* activity. Neither the *src* nor *fyn* dominant negative construct affected *lyn* activity. The data indicate that the effects of both *src* and *fyn* dominant negative constructs are specific (Figure 2C).

To determine whether the calcium-induced *src* and *fyn* activities are associated with activation of PLC- γ 1, human keratinocytes were treated with 1.2 mM calcium, cells were then harvested after 5, 15, and 30 min and 1 h, and PLC- γ 1 activity was measured (Figure 3). Activity was assessed by the level of IP₃ production. The results showed that stimulation of PLC- γ 1 activity occurred as early as 15 min after

Figure 4. The *src* family kinase inhibitor PP2 and the combination of dominant negative *src* and *fyn* reduce calcium-stimulated PLC- γ 1 activity. (A) Cultured human keratinocytes were treated with a specific *src* family kinase inhibitor (PP2) or its inactive analogue (PP3) for 1 h. Cells were then stimulated with calcium for 1 h, and cells were harvested. The PLC- γ 1 activity was assayed as described in *Materials and Methods* and expressed as the percentage of the vehicle control at 0.03 mM calcium. (B) Human keratinocytes cultured in KGM containing 0.03 mM calcium were transfected with dominant negative *src* (*src*-DN) and/or *fyn* (*fyn*-DN). Equal amounts of DNA were added to cells in each plate during the transfection. Cells were then stimulated with calcium for 1 h, and cells were harvested. The PLC- γ 1 activity was assayed as described in *Materials and Methods* and expressed as the percentage of the vector control at 0.03 mM calcium.

Figure 5. Calcium does not stimulate the phosphorylation of PLC- γ 1. Cultured human keratinocytes were treated with 1.2 mM calcium or 50 ng/ml EGF. The cells were harvested at the time points indicated. The protein levels of PLC- γ 1 and its phosphorylated forms (p-PLC- γ 1-pY783 and p-PLC- γ 1-pY1254) were analyzed by Western analysis. In addition, the total phospho-PLC- γ 1 was immunoprecipitated by antibodies against phosphotyrosine (p-Tyr) or PLC- γ 1 followed by Western analysis with antibodies against PLC- γ 1 or p-Tyr.

calcium administration. One-hour calcium treatment stimulated PLC- γ 1 activity by up to 14-fold (Figure 3). To determine whether the activation of PLC- γ 1 by calcium requires *src* kinases, human keratinocytes were pretreated with the *src* family inhibitor PP2 before the administration of calcium for 1 h. PLC- γ 1 activity was determined 1 h later (Figure 4A). The results showed that calcium-stimulated PLC- γ 1 activity was reduced by fivefold by PP2 (Figure 4A). To determine whether calcium-activated *src* and *fyn* contribute to the activation of PLC- γ 1, human keratinocytes were transfected with dominant negative *src* and *fyn* or the combination of both (Figure 4B). PLC- γ 1 activity in the cells was measured. The results showed that calcium stimulated PLC- γ 1 activity by eightfold in cells transfected with the vector pcDNA3.1. Transfection either with dominant negative *src* or dominant negative *fyn* had little or no effect on calcium-stimulated PLC- γ 1 activity, but the combination of dominant negative *src* and *fyn* reduced calcium-induced PLC- γ 1 activity more than threefold (Figure 4B). These data indicate that both *src* and *fyn* are required for the activation of PLC- γ 1 by calcium. To test whether the induced PLC- γ 1 activity by calcium is due to tyrosine phosphorylation of PLC- γ 1 by *src* and *fyn*, the effect of calcium on the levels of PLC- γ 1 phosphorylated at Tyr 783 and Tyr 1254 were determined (Figure 5). These sites are known to be required for the activation of PLC- γ 1. The results showed that neither of these two phosphorylation sites of PLC- γ 1 was regulated by calcium (Figure 5). Immunoprecipitation with either anti-PLC- γ 1 or anti-p-Tyr followed by Western with either anti-p-Tyr or anti-PLC- γ 1 showed no stimulation of any tyrosine phosphorylation in PLC- γ 1 by calcium (Figure 5). As a positive control, EGF treatment stimulated tyrosine phosphorylation of PLC- γ 1 within 5 min (Figure 5). These data indicate that calcium induces PLC- γ 1 activity through *src* and *fyn* by a mechanism that does not involve tyrosine phosphorylation of PLC- γ 1. To explore other mechanisms, we tested whether PIP₃ could activate PLC- γ 1 as has been reported in other systems. Immunoprecipitated PLC- γ 1 in human keratinocytes was incubated with PIP₃, and then PLC- γ 1 activity was assayed. The results showed that PIP₃ induced PLC- γ 1 activity (Figure 6A). To determine whether endogenous PIP₃ remains bound to PLC- γ 1 after immunoprecipitation and whether this binding is increased by calcium, human keratinocytes

were treated with 1.2 mM calcium and lysed in 1% NP-40. PLC- γ 1 was then immunoprecipitated by PLC- γ 1 antibody. PIP₃ in the immunoprecipitate was then measured by PIP₃

Figure 6. PIP₃ stimulates PLC- γ 1 activity, and the binding of PIP₃ to PLC- γ 1 is induced by calcium. (A). Human keratinocytes were cultured in KGM with 0.03 mM calcium. The total cell lysate was isolated with the cell lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 20 mM MgCl₂, 1.0% NP-40, 0.4 mM Na₃VO₄, 40 mM NaF, 50 μ M okadaic acid, 0.2 mM PMSF, and Complete protease inhibitors (Roche Diagnostics). PLC- γ 1 in the lysate was immunoprecipitated with PLC- γ 1 antibody. The immunoprecipitate was incubated with PIP₃ (Sigma-Aldrich) dissolved in the cell lysis buffer at a final concentration of 1 μ M for 1 h. PLC- γ 1 activity was assayed as described in *Materials and Methods* and expressed as the percentage of the activity in the absence of PIP₃ with 1 min [³H]PIP₂ incubation. (B). The total cell lysate was isolated from human keratinocytes treated with or without calcium for 1 h, and PLC- γ 1 in the lysate was immunoprecipitated with PLC- γ 1 antibody. The immunoprecipitate was extracted with chloroform and then spotted onto a PVDF membrane. The amount of PIP₃ on the membrane was determined by PIP₃ antibody.

antibody. The results showed that calcium increases the amount of PIP₃ bound to PLC- γ 1 even after lysis in 1% NP-40 and immunoprecipitation, supporting the conclusion that the increased PLC- γ 1 activity after calcium administration is due to its stimulation by PIP₃ (Figure 6B).

Because PI3K is a major means by which PIP₃ is produced in cells, we next evaluated whether calcium could activate PI3K, and whether *src* kinases participated in that activation. We first wanted to know whether calcium regulates the phosphorylation of PI3K-p85 α , a regulatory subunit of PI3K-p110 α , β , and δ . The results showed that calcium increased the level of phospho-p85 α in a time-dependent manner (Figure 7A). The increase was seen as early as 5 min and reached a peak threefold increase at 8 h. The total protein level of p85 α was not altered by calcium. As a control, the protein level of involucrin was induced by calcium in a time-dependent manner (Figure 7A). The 1-h treatment with calcium also increased p110 α , β , γ , and δ activity, although p110 β activity was less stimulated than the others (Figure 7B). Phosphorylation of Akt is another downstream effect after PI3K activation. Therefore, we determined the effect of calcium on serine phosphorylation of Akt and found that calcium increases phosphorylated Akt for at least an hour (Figure 7C), correlating with the activation of PLC- γ 1 by calcium, and confirming the activation of PI3K as assessed by PIP₃ production.

These data indicate that calcium activates p110 α , β , and δ by inducing phosphorylation of p85 α . Activation of p110 γ is presumably by another mechanism in that it is controlled by the regulatory subunit p101 (Vanhaesebroeck and Waterfield, 1999). To determine whether *src* and *fyn* are responsible for the calcium-stimulated p85 α phosphorylation, human keratinocytes were pretreated with PP2 for 1 h, and the response to calcium was examined (Figure 8A). The results showed that PP2 prevented the increase in phosphorylated p85 α (p-p85 α) by calcium, whereas treatment with vehicle (DMSO) or the inactive analogue PP3 had no effect (Figure 8A). To confirm these results, dominant negative constructs for *src* and *fyn* or the combination of both were then transfected into human keratinocytes, the cells were treated with 1.2 mM calcium, and the effect on p85 α phosphorylation was measured (Figure 8B). The results showed that neither dominant negative *src* nor *fyn* alone affected calcium-induced phosphorylation of p85 α , whereas the combination of dominant negative *src* and *fyn* blocked calcium-induced phosphorylation of p85 α (Figure 8B). Furthermore, the combination of dominant negative *src* and *fyn* blocked the activation of p110 α and δ and reduced the activation of γ (Figure 8C). In contrast, inhibition of PI3K activity by LY294002 affected neither *src* nor *fyn* activity. The inhibitory effect of LY294002 on the Akt phosphorylation induced by calcium was used as a positive control for the experiment (Figure 8D). These data suggest that both *src* and *fyn* are required for the activation of PI3K by calcium, whereas PI3K is not required for the activation of either *src* or *fyn* by calcium. Having shown that PIP₃, a product of PI3K, stimulated PLC- γ 1 activity, we then wanted to know whether the activation of PLC- γ 1 requires PI3K activity. To address this issue, human keratinocytes were pretreated with LY294002 for 1 h followed by calcium for 1 h at which point PLC- γ 1 activity was determined (Figure 9). The results showed that LY294002 blocked the induction of PLC- γ 1 activity by calcium (Figure 9). These data indicate that PI3K is required for calcium-induced PLC- γ 1 activation. To determine whether PI3K is involved in calcium-induced human keratinocyte differen-

Figure 7. Calcium stimulates PI3K-p85 α phosphorylation, PI3K activity, and Akt phosphorylation. (A). Cultured human keratinocytes were treated with 1.2 mM calcium for the indicated times. The protein levels of PI3K-p85 α and its phosphorylated form (p-PI3K-p85 α) were determined by Western analysis. Involucrin was used as a positive control for calcium induction (B). Human keratinocytes were treated with 1.2 mM calcium for 1 h. Cells were then harvested and PI3K-p110 α , β , γ , and δ activities were measured as described in *Materials and Methods* (C). Cultured human keratinocytes were treated with 1.2 mM calcium for the indicated times. The protein levels of Akt and its phosphorylated form (p-Akt) were determined by Western analysis.

tiation, human keratinocytes were pretreated with LY294002 for 1 h before calcium administration, and the differentiation markers for keratinocyte differentiation, involucrin, and transglutaminase were examined 24 h later (Figure 10). The results showed that LY294002 prevented the induction of involucrin and transglutaminase by calcium (Figure 10). The data indicate that PI3K is required for calcium-induced human keratinocyte differentiation.

Figure 8. The *src* kinase inhibitor and dominant negative *src* and *fyn* block calcium-stimulated PI3K-p85 α phosphorylation and PI3K activity, whereas PI3K inhibition does not affect calcium stimulation of either *src* or *fyn* activity. Cultured human keratinocytes were preincubated for 1 h with PP2 or its inactive analog PP3 at a concentration of 10 μ M (A), transfected with dominant negative *src* (*src*-DN), and/or *fyn* (*fyn*-DN), or vector alone (B and C). For the p85 α phosphorylation (A and B) and p110 α , β , γ , and δ activity (C) assay, transfected cells were then treated with 1.2 mM calcium for 1 h. The protein levels of the phosphorylated PI3K-p85 α were determined by Western analysis. The activities of PI3K-p110 α , β , γ , and δ were measured as described in *Materials and Methods*. (D) Cultured human keratinocytes were preincubated with 10 μ M LY294002 or vehicle DMSO for 1 h. Cells were then stimulated with 1.2 mM calcium for 15 min, and cells were harvested. The *src* and *fyn* activities was assayed as described in *Materials and Methods* and expressed as the percentage of the vehicle control of the *src* activity. The levels of Akt phosphorylation and total Akt were assayed by Western analysis as described in *Materials and Methods*.

Figure 9. PI3K inhibition blocks the stimulation of PLC-γ1 activity by calcium. Cultured human keratinocytes were preincubated with 10 μM LY294002 (+) or vehicle DMSO (−) for 1 h. Cells were then stimulated with 1.2 mM calcium for 1 h, and cells were harvested. The PLC-γ1 activity was assayed as described in *Materials and Methods* and expressed as the percentage of the activity at 0.03 mM calcium in the absence of LY294002.

DISCUSSION

In mouse keratinocyte cultures, IP₃ and DAG levels increase within seconds to minutes after adding high calcium to the cells, suggesting an activation of the PLC pathway (Jaken and Yuspa, 1988; Tang *et al.*, 1988; Moscat *et al.*, 1989; Reiss *et al.*, 1991). The increased IP₃ production is thought to account for the initial rise in intracellular calcium by release of calcium from intracellular stores. However, the levels of inositol phosphates (IPs) remain elevated for hours after adding high calcium to mouse keratinocytes, indicating ongoing activation of the PLC pathway. This prolonged increase in IPs seems to be due to calcium activation of PLC-γ1 (Punnonen *et al.*, 1993). Our data demonstrate that the activation of PLC-γ1 by calcium remains for at least 1 h after the calcium switch, consistent with previous findings. We have previously reported that PLC-γ1 is required for the calcium-induced increase in intracellular calcium and subsequent differentiation of human keratinocytes (Xie and Bickle, 1999). The present study shows for the first time that activation of PLC-γ1 by both *src* and *fyn*, but not *src* or *fyn* alone, is required for calcium-stimulated human keratinocyte differentiation. It was reported previously that *fyn* but not *src* is induced by calcium in mouse keratinocytes (Calautti *et al.*, 1995). Mice lacking *fyn* display skin abnormalities, suggesting impaired epidermal differentiation (Ilic *et al.*, 1997). However, in human keratinocytes, calcium induces *c-src* but not *fyn* (Zhao *et al.*, 1992; current study). However, inhibition of both *src* and *fyn* activity and expression was required to block calcium-induced human keratinocyte differentiation,

suggesting that both *src* and *fyn* are involved. PLC-γ1 is known to be tyrosine-phosphorylated by receptor kinases at a number of positions. The tyrosine phosphorylation sites on PLC-γ1 resulting from EGF stimulation have been mapped to Tyr 771 and Tyr 783, which sit between the C-SH2 and SH3 domains, and to Tyr 1254 in the C terminus (Kim *et al.*, 1990; Nishibe *et al.*, 1990). Mutagenesis indicates that the phosphorylation at Tyr 783 is essential for IP₃ formation, phosphorylation of Tyr 771 is dispensable, and phosphorylation of Tyr 1254 is necessary to achieve maximal IP₃ formation (Kim *et al.*, 1991). In addition to the receptor kinases, *src*, *fyn*, *lck*, *lyn*, and *hck* kinases are all capable of phosphorylating PLC-γ1 in vitro (Liao *et al.*, 1993; Lee and Rhee, 1995; Noh *et al.*, 1995). However, it is not clear that any of the *src* family kinases are responsible for tyrosine phosphorylation of PLC-γ1 in vivo. Although tyrosine phosphorylation has a limited effect on the activity of PLC-γ1 in vitro, it has been reported to be essential for activation of the phospholipase in vivo (Kim *et al.*, 1991). Data presented in this article indicate that both *src* and *fyn* are required for the calcium-induced PLC-γ1 activation, but this activation does not involve tyrosine phosphorylation of PLC-γ1. PLC-γ1 also is phosphorylated on serine and threonine in other cell types. However, the functional significance of such phosphorylation is unknown, and there is no evidence to suggest it alters enzyme activity (Kim *et al.*, 1991). Furthermore, in studies not reported here, we have found that calcium does not affect serine phosphorylation of PLC-γ1 (our unpublished data).

Several studies indicate that PLC-γ1 can be activated by phosphatidic acid (Jones and Carpenter, 1993b), arachidonic acid (Hwang *et al.*, 1996; Sekiya *et al.*, 1999), and PIP₃ (Bae *et al.*, 1998; Falasca *et al.*, 1998; Rameh *et al.*, 1998) in the absence of tyrosine phosphorylation. These lipid-derived messengers are the immediate products of phospholipase D, phospholipase A₂, and PI3K, respectively, and they provide alternative mechanisms to tyrosine phosphorylation by which PLC-γ1 can be activated. Recent observations indicate that *src* activates PI3K by an unknown mechanism in other cell types (Encinas *et al.*, 2001; Kubota *et al.*, 2001). Several classes of PI3K have been distinguished on the basis of sequence similarity and substrate selectivity (Toker and Cantley, 1997). The best-studied class Ia PI3Ks consist of a p110 catalytic subunit (p110α, β, or δ) and a tightly associated adapter (or regulatory) subunit encoded by at least three distinct genes (p85α, p85β, or p55γ) (Escobedo *et al.*, 1991;

Figure 10. PI3K inhibition blocks the induction of involucrin and transglutaminase expression by calcium. Cultured human keratinocytes were preincubated with 10 mM LY294002 (+) or vehicle DMSO (−) for 1 h and then treated with 1.2 mM calcium for 24 h. The protein levels of involucrin and transglutaminase were determined by Western analysis.

Figure 11. Proposed model for the signaling pathway of PLC- γ 1 activation mediating calcium-induced human keratinocyte differentiation. Calcium (Ca^{2+}) binding to the calcium receptor (CaR) (Tu *et al.*, 2001) stimulates *src* and *fyn*, which activate PI3K via phosphorylation of p85 α leading to PIP_3 accumulation. PIP_3 then stimulates PLC- γ 1 activity, which increases IP_3 production. Intracellular calcium ($[\text{Ca}^{2+}]_i$) release from endoplasmic reticulum (ER) and Golgi stores mediated by IP_3 binding to the IP_3 receptor (IP_3R) triggers human keratinocyte differentiation.

Domin and Waterfield, 1997). This subunit has no known enzymatic activity but serves a regulatory function. Alternative splicing of the p85 α gene results in the generation of two small splice variants, p55 α and p50 α , which also associate with p110 catalytic subunit isoforms. Class Ib PI3Ks contain a p110 γ catalytic subunit, and they do not interact with p85 adaptor molecules but with a p101 regulatory subunit. Furthermore, they are directly activated by $\beta\gamma$ subunits of G proteins rather than by kinases. The p85 α is the most abundantly expressed regulatory subunit of PI3Ks. Our data indicate that in human keratinocytes the phosphorylation of p85 α and the activation of PI3K α , β , and δ by calcium requires *src* and *fyn*. Mouse keratinocytes seem to have a different mechanism in the regulation of PI3K by calcium. Filvaroff *et al.* (1992) reported that in these cells the phosphorylation of p85 α was not induced by calcium. Although 110 γ does not associate with p85 α , calcium activation of p110 γ also requires *src* and *fyn*, suggesting other mechanisms are involved. Activated PI3K converts PIP_2 into PIP_3 at the plasma membrane. PIP_3 binds to the N-terminal pleckstrin homology domain (Falasca *et al.*, 1998) and the C-terminal SH2 domain (Bae *et al.*, 1998; Rameh *et al.*, 1998) of PLC- γ 1 to target PLC- γ 1 to the membrane. Our data demonstrate that PIP_3 binds to and activates PLC- γ 1, supporting our conclusion that PI3K is upstream of PLC- γ 1 activation. Together, we conclude that in human keratinocytes PIP_3 produced by *src* and *fyn* activated PI3K provides an "anchor" for PLC- γ 1 at the membrane that is crucial for the positioning and activation of PLC- γ 1 after calcium administration (Figure 11). Our data further indicate that this mechanism is required for calcium-induced human keratinocyte differentiation.

REFERENCES

- Bae, Y. S., Cantley, L. G., Chen, C. S., Kim, S. R., Kwon, K. S., and Rhee, S. G. (1998). Activation of phospholipase C- γ by phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* 273, 4465–4469.
- Berridge, M. J., and Irvine, R. F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* 312, 315–321.
- Boudot, C., Dasse, E., Lambert, E., Kadri, Z., Mayeux, P., Chretien, S., Haye, B., Billat, C., and Petitfrere, E. (2003). Involvement of the Src kinase Lyn in phospholipase C- γ 2 phosphorylation and phosphatidylinositol 3-kinase activation in Epo signaling. *Biochem. Biophys. Res. Commun.* 300, 437–442.
- Bourguignon, L. Y., Singleton, P. A., and Diedrich, F. (2004). Hyaluronan/CD44 interaction with Rac1-dependent PKN γ kinase promotes PLC γ 1 activation, Ca^{2+} signaling and cortactin-cytoskeleton function leading to keratinocyte adhesion and differentiation. *J. Biol. Chem.* 279, 29654–29659.
- Bourguignon, L. Y., Singleton, P. A., Zhu, H., and Diedrich, F. (2003). Hyaluronan-mediated CD44 interaction with RhoGEF and Rho kinase promotes Grb2-associated binder-1 phosphorylation and phosphatidylinositol 3-kinase signaling leading to cytokine (macrophage-colony stimulating factor) production and breast tumor progression. *J. Biol. Chem.* 278, 29420–29434.
- Calautti, E., Cabodi, S., Stein, P. L., Hatzfeld, M., Kedersha, N., and Paolo Dotto, G. (1998). Tyrosine phosphorylation and src family kinases control keratinocyte cell-cell adhesion. *J. Cell Biol.* 141, 1449–1465.
- Calautti, E., Grossi, M., Mammucari, C., Aoyama, Y., Pirro, M., Ono, Y., Li, J., and Paolo Dotto, G. (2002). Fyn tyrosine kinase is a downstream mediator of Rho/PRK2 function in keratinocyte cell-cell adhesion. *J. Cell Biol.* 156, 137–148.
- Calautti, E., Missero, C., Stein, P. L., Ezzell, R. M., and Paolo Dotto, G. (1995). *fyn* tyrosine kinase is involved in keratinocyte differentiation control. *Gene Dev.* 9, 2279–2291.
- Carpenter, G., and Ji, Q. (1999). Phospholipase C- γ as a signal-transducing element. *Exp. Cell Res.* 253, 15–24.
- Domin, J., and Waterfield, M. D. (1997). Using structure to define the function of phosphoinositide 3-kinase family members. *FEBS Lett.* 410, 91–95.
- Encinas, M., Tansey, M. G., Tsui-Pierchala, B. A., Comella, J. X., Milbrandt, J., and Johnson, Jr., E. M. (2001). c-Src is required for glial cell line-derived neurotrophic factor (GDNF) family ligand-mediated neuronal survival via a phosphatidylinositol-3 kinase (PI-3K)-dependent pathway. *J. Neurosci.* 21, 1464–1472.
- Escobedo, J. A., Navankasattusas, S., Kavanaugh, W. M., Milfay, D., Fried, V. A., and Williams, L. T. (1991). cDNA cloning of a novel 85 kD protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF β -receptor. *Cell* 65, 75–82.
- Falasca, M., Logan, S. K., Lehto, V. P., Baccante, G., Lemmon, M. A., and Schlessinger, J. (1998). Activation of phospholipase C γ by PI 3-kinase-induced PH domain-mediated membrane targeting. *EMBO J.* 17, 414–422.

- Filvaroff, E., Calautti, E., McCormick, F., and Paolo Dotto, G. (1992). Specific changes of Ras GTPase-activating protein (GAP) and a GAP-associated p62 protein during calcium-induced keratinocyte differentiation. *Mol. Cell. Biol.* 12, 5319–5328.
- Hwang, S. C., Jhon, D. Y., Bae, Y. S., Kim, J. H., and Rhee, S. G. (1996). Activation of phospholipase C- γ 1 by the concerted action of tau proteins and arachidonic acid. *J. Biol. Chem.* 271, 18342–18349.
- Ilic, D., Kanazawa, S., Nishizumi, H., Aizawa, S., Kuroki, T., Mori, S., and Yamamoto, T. (1997). Skin abnormality in aged *fyn*^{-/-} *fak*^{+/-} mice. *Carcinogenesis* 18, 1473–1476.
- Jaken, S., and Yuspa, S. H. (1988). Early signals for keratinocyte differentiation: role of Ca²⁺-mediated inositol lipid metabolism in normal and neoplastic epidermal cells. *Carcinogenesis* 9, 1033–1038.
- Jones, G. A., and Carpenter, G. (1993a). The regulation of phospholipase C- γ 1 by phosphatidic acid. *J. Biol. Chem.* 268, 20845–20850.
- Jones, G. A., and Carpenter, G. (1993b). The regulation of phospholipase C- γ 1 by phosphatidic acid. Assessment of kinetic parameters. *J. Biol. Chem.* 268, 20845–20850.
- Kim, H. K., Kim, J. W., Zilberstein, A., Margolis, B., Kim, J. G., Schlessinger, J., and Rhee, S. G. (1991). PDGF stimulation of inositol phospholipid hydrolysis requires Plc-gamma-1 phosphorylation on tyrosine residues 783 and 1254. *Cell* 65, 435–441.
- Kim, J. W., Sim, S. S., Kim, V. H., Nishibe, S., Wahl, M. I., Carpenter, G., and Rhee, S. G. (1990). Tyrosine residues in bovine phospholipase C- γ phosphorylated by the EGF receptor in vitro. *J. Biol. Chem.* 265, 3940–3943.
- Kubota, Y., Tanaka, T., Kitanaka, A., Ohnishi, H., Okutani, Y., Waki, M., Ishida, T., and Kamano, H. (2001). Src transduces erythropoietin-induced differentiation signals through phosphatidylinositol 3-kinase. *EMBO J.* 20, 5666–5677.
- Lee, S. B., and Rhee, S. G. (1995). Significance of Pip2 hydrolysis and regulation of phospholipase C isozymes. *Curr. Opin. Cell Biol.* 7, 183–189.
- Liao, F., Shin, H. S., and Rhee, S. G. (1993). In vitro tyrosine phosphorylation of PLC- γ 1 and PLC- γ 2 by src family protein tyrosine kinases. *Biochem. Biophys. Res. Commun.* 3, 1028–1033.
- Majerus, P. W., Connolly, T. M., Deckmyn, H., Ross, T. S., Bross, T. E., Ishii, H., Bansal, V. S., and Wilson, D. B. (1986). The metabolism of phosphoinositide-derived messenger molecules. *Science* 234, 1519–1526.
- Moscat, J., Fleming, T. P., Molloy, C. J., Lopez-Barahona, M., and Aaronson, S. A. (1989). The calcium signal for Balb/MK keratinocyte terminal differentiation induces sustained alterations in phosphoinositide metabolism without detectable PKC activation. *J. Biol. Chem.* 264, 11228–11235.
- Nishibe, S., Wahl, M. I., Hernandez-Sotomayor, S. M., Tonks, N. K., Rhee, S. G., and Carpenter, G. (1990). Increase of the catalytic activity of phospholipase C- γ 1 by tyrosine phosphorylation. *Science* 250, 1253–1256.
- Noh, D. Y., Shin, S. H., and Rhee, S. G. (1995). Phosphoinositide-specific phospholipase C and mitogenic signaling. *Biochim. Biophys. Acta* 1242, 99–113.
- Ozdener, F., Dangelmaier, C., Ashby, B., Kunapuli, S. P., and Daniel, J. L. (2002). Activation of phospholipase C γ 2 by tyrosine phosphorylation. *Mol. Pharmacol.* 62, 672–679.
- Pillai, S., Bikle, D. D., Hincenbergs, M., and Elias, P. M. (1988). Biochemical and morphological characterization of growth and differentiation of normal human neonatal keratinocytes in a serum-free medium. *J. Cell. Physiol.* 134, 229–237.
- Punnonen, K., Denning, M., Lee, E., Li, L., Rhee, S. G., and Yuspa, S. H. (1993). Keratinocyte differentiation is associated with changes in the expression and regulation of phospholipase C isoenzymes. *J. Invest. Dermatol.* 101, 719–726.
- Rameh, L. E., Rhee, S. G., Spokes, K., Kazlauskas, A., Cantley, L. C., and Cantley, L. G. (1998). Phosphoinositide 3-kinase regulates phospholipase C γ -mediated calcium signaling. *J. Biol. Chem.* 273, 23750–23757.
- Reiss, M., Lipsey, L. R., and Zhou, Z. L. (1991). Extracellular calcium-dependent regulation of transmembrane calcium fluxes in murine keratinocytes. *J. Cell Physiol.* 147, 281–291.
- Rhee, S. G., and Bae, Y. S. (1997). Regulation of phosphoinositide-specific phospholipase C isozymes. *J. Biol. Chem.* 272, 15045–15048.
- Rhee, S. G., Suh, P. G., Ryu, S. H., and Lee, S. Y. (1989). Studies of inositol phospholipid-specific phospholipase C. 1989 244, 546–550.
- Sekiya, F., Bae, Y. S., Jhon, D. Y., Hwang, S. C., and Rhee, S. G. (1999). AHNK, a protein that binds and activates phospholipase C- γ 1 in the presence of arachidonic acid. *J. Biol. Chem.* 274, 13900–13907.
- Tang, W., Ziboh, V. A., Isseroff, R., and Martinez, D. (1988). Turnover of inositol phospholipids in cultured murine keratinocytes: possible involvement of inositol triphosphate in cellular differentiation. *J. Invest. Dermatol.* 90, 37–43.
- Toker, A., and Cantley, L. C. (1997). Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature* 387, 673–676.
- Tu, C. L., Chang, W., and Bikle, D. D. (2001). The extracellular calcium-sensing receptor is required for calcium-induced differentiation in human keratinocytes. *J. Biol. Chem.* 276, 41079–41085.
- Twamley-Stein, G. M., Pepperkok, R., Ansorge, W., and Courtneidge, S. A. (1993). The Src family tyrosine kinases are required for PDGF-mediated signal transduction in NIH 3T3 cells. *Proc. Natl. Acad. Sci. USA* 90, 7696–7700.
- Vanhaesebroeck, B., and Waterfield, M. D. (1999). Signaling by distinct classes of phosphoinositide 3-kinases. *Exp. Cell Res.* 253, 239–254.
- Xie, Z., and Bikle, D. D. (1999). Phospholipase C- γ 1 is required for calcium-induced keratinocyte differentiation. *J. Biol. Chem.* 274, 20421–20424.
- Zhao, Y., Sudol, M., Hanafusa, H., and Krueger, J. (1992). Increased tyrosine kinase activity of c-src during calcium-induced keratinocyte differentiation. *Proc. Natl. Acad. Sci. USA* 89, 8298–8302.