Extracellular Calcium and Platelet-derived Growth Factor Promote Receptor-mediated Chemotaxis in Osteoblasts through Different Signaling Pathways

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The discovery of a calcium receptor has stimulated interest in the signaling events underlying extracellular calcium ([Ca\(^{2+}\)])-induced cell-specific responses. In osteoblasts, elevated levels of extracellular calcium mediate both mitogenesis and chemotaxis. Here we provide evidence that [Ca\(^{2+}\)]\(_i\)-stimulated chemotaxis of MC3T3-E1 osteoblast-like cells involves a G-protein-linked calcium-sensing receptor. [Ca\(^{2+}\)]\(_i\) promotes chemotaxis in a concentration-dependent manner. Pertussis toxin blocked almost all of [Ca\(^{2+}\)]\(_i\)-stimulated chemotaxis but had only a small effect on platelet-derived growth factor (PDGF)-stimulated chemotaxis. Consistent with the signaling model for PDGF-mediated chemotaxis, activation of phospholipase C played a critical role in [Ca\(^{2+}\)]\(_i\)-initiated chemotaxis: U-73122, an inhibitor of the activation of phospholipase C, blocked approximately 50% of PDGF-stimulated chemotaxis but blocked nearly all of the [Ca\(^{2+}\)]\(_i\)-stimulated chemotaxis. Down-regulation of protein kinase C also blocked about 50% of PDGF-stimulated chemotaxis but did not block [Ca\(^{2+}\)]\(_i\)-stimulated chemotaxis. Thus, unlike PDGF-mediated chemotaxis, chemotaxis stimulated by [Ca\(^{2+}\)]\(_i\) does not appear to require protein kinase C activation. This finding suggests events downstream of inositol 1,4,5-trisphosphate production rather than diacylglycerol production are critical to [Ca\(^{2+}\)]\(_i\)-promoted chemotaxis of MC3T3-E1 cells. The signal transduction mechanism underlying PDGF-induced chemotaxis involves the activation of phosphoinositide 3-kinase, as judged by the in vivo production of phosphatidylinositol 3,4-diphosphate and 3,4,5-trisphosphate and the partial sensitivity of chemotaxis to wortmannin, an inhibitor of phosphoinositide 3-kinase. In contrast, [Ca\(^{2+}\)]\(_i\)-stimulated chemotaxis was not blocked by wortmannin and elevations in [Ca\(^{2+}\)]\(_i\) did not increase the production of lipid products of phosphoinositide 3-kinase. Overall, [Ca\(^{2+}\)]\(_i\)-promoted chemotaxis of osteoblasts appears to utilize a unique signaling mechanism via a calcium-sensing receptor.

Multiple cell types sense extracellular calcium (1). Cellular responses to alterations in external calcium are diverse and cell-specific. Elevations in extracellular calcium signal parathyroid cells to reduce parathyroid hormone secretion (2). Removal of calcium ions is inhibited by high [Ca\(^{2+}\)]\(_i\). (3). During bone remodeling, osteoclasts (bone-resorbing cells) and osteoblasts (bone-forming cells) function in local environments characterized by dramatic fluctuations in [Ca\(^{2+}\)]\(_i\) concentrations (4). Elevated external calcium in the resorption lucunae acts as a negative feedback on osteoclasts, inhibiting their resorptive capacity (5). In contrast, high [Ca\(^{2+}\)]\(_i\) concentrations enhances DNA synthesis and promotes chemotaxis of osteoblasts (6, 7). The signal transduction mechanisms underlying [Ca\(^{2+}\)]\(_i\)-stimulated chemotaxis have yet to be defined; however, [Ca\(^{2+}\)]\(_i\) is capable of producing a transient elevation of intracellular calcium in osteoblasts at concentrations that initiate these two physiological processes (8). Since the transient rise in intracellular calcium was not a result of a [Ca\(^{2+}\)]\(_i\)-influx, the existence of a cation-sensing receptor on the surface of osteoblasts was suggested, similar to that reported earlier in parathyroid cells (9).

Recently, calcium receptor cDNA has been cloned and transcripts of this gene were recovered from bovine and human parathyroid, kidney, thyroid and brain cells (10). This 120-kDa polypeptide receptor, BoPCaR1, consists of a seven-transmembrane region and belongs to the G-protein-linked receptor superfamily (10). Recent reports indicate that MC3T3-E1 osteoblast-like cells may have a [Ca\(^{2+}\)]\(_i\)-sensing receptor that is homologous, yet different, to the BoPCaR1 (11).

The aim of this report is to define the initial post-receptor signal transduction events in [Ca\(^{2+}\)]\(_i\)-stimulated chemotaxis of the murine osteoblast clonal cell line MC3T3-E1. Platelet-derived growth factor (PDGF) also promotes chemotaxis in these cells, and we examined the effects of perturbations of various signaling pathways on both PDGF- and [Ca\(^{2+}\)]\(_i\)-promoted chemotaxis. In previous studies of other cells, the signaling mechanisms underlying PDGF-induced chemotaxis were examined by mutating specific PDGF receptor tyrosine residues, and it was observed that PDGF-stimulated chemotaxis relies on both phospholipase C and the phosphoinositide 3-kinase (12). Hepatocyte growth factor also activates both PI 3-kinase and PLC, and both signaling events are necessary for hepatocyte growth factor-promoted chemotaxis (13, 14). However, not all chemot-
trastacts use this dual pathway paradigm. For example, basic fibroblast growth factor mediates chemotaxis without activating PLC (15). Therefore, the signal transduction mechanism utilized among chemotastacts is not universal.

We report that PDGF-mediated chemotaxis of MC3T3-E1 osteoblast-like cells is dependent on both PLC and PI 3-kinase. In contrast, extracellular calcium-stimulated chemotaxis requires PLC activation but not PI 3-kinase activation. Our results suggest that a G-protein-linked [Ca\(^{2+}\)]-sensing receptor is present on osteoblasts. The presence of such a receptor would allow extracellular calcium to act as a coupling agent, linking the bone resorptive phase of bone remodeling to bone formation.

MATERIALS AND METHODS

Reagents—All chemicals were reagent grade or better. a-modified Eagle’s medium (α-MEM), fetal calf serum, penicillin/streptomycin, and wortmannin were purchased from Sigma. Dulbecco’s modified Eagle’s medium, amphotericin B (Fungizone), and phorbol 12-myristate 13-acetate (PMA) were obtained from Life Technologies, Inc. Human PDGF-BB was acquired from Upstate Biochemicals Inc. (Lake Placid, NY). U-73122 and U-73343 were purchased from Biomol (Plymouth Meeting, PA). List Biologicals (Campbell, CA) supplied the pertussis toxin (Ptx). [γ-32P]ATP (specific activity, 3,000 Ci/mmol) and ortho-[32P]P phosphate (specific activity, 8,500–9,120 Ci/mmol) was obtained from DuPont NEN. The anti-phosphotyrosine antibody (anti-Tyr(P)), a murine monoclonal antibody, was generously contributed by Dr. Thomas Roberts (Dana-Farber Cancer Institute, Boston, MA).

Cell Culture—MC3T3-E1 cells (16), a clonal line of normal murine osteoblasts, were kindly provided by Peter Hansbeck (The Children’s Hospital, Boston, MA). The cells were grown in a 95% air, 5% CO2 atmosphere in 100-mm diameter dishes containing α-MEM plus 10% fetal calf serum, 100 µg/ml streptomycin, 100 IU/ml penicillin, and 0.025 µg/ml amphotericin B at 37°C. All experiments were conducted with cells at or near confluence. Where noted, serum-starvation of the MC3T3-E1 cells was accomplished through exposure to low serum medium (α-MEM plus 0.1% fetal calf serum) 12 h prior to stimulation. Protein kinase C (PKC) down-regulated cells were generated by 12-h incubation of cells in low serum α-MEM containing 1 µg PMA. G-protein inhibition was accomplished by 12-h exposure of cells to low serum α-MEM containing 100 ng/ml pertussis toxin. Strict cell culture parameters were maintained, and cells were used early in their passage to avoid phenotypic maturation that might accompany long term cell culture. For example, we observed that U-73122 had an enhanced inhibitory effect on chemotaxis of late passage osteoblasts (data not shown).

Alterations in the Extracellular Calcium Concentration—α-MEM has a basal calcium concentration of 1.8 mM. In all experiments, alterations in the extracellular calcium concentration from basal (1.8 mM) levels were produced by the addition of CaCl\(_2\) (Sigma) to the final concentration specified.

Chemotaxis—A 48-well modified Boyden chemotaxis chamber (NeuroProbe Inc., Baltimore, MD) and polyvinylpyrrolidone-free polycarbonate filters (8-µm pore size) (Poretics Corp., Livermore, CA) were utilized as described previously (17) with the following alterations. The filters were coated with 50 µg/ml rat type I collagen (Collaborative Biomedical Products, Bedford, MA). Chematotic agent was diluted in low serum α-MEM, which was then added to the lower wells of the chamber. When dimethyl sulfoxide was used as a vehicle for an inhibitor, the final concentration of dimethyl sulfoxide was less than 0.01%. A coated filter was fixed atop the bottom wells. Cells were diluted in low serum α-MEM to a final concentration of 4 × 10\(^4\) cells/ml. Inhibitors were added to the cell suspension where noted, and 50 µl of this suspension were loaded into each of the upper wells. In each experiment, at least 6 of the chamber’s 48 wells were used for each condition examined. The chamber was incubated for 4 h at 37°C in a 95% air, 5% CO\(_2\) atmosphere.

Following incubation, the chamber was disassembled, and the cells on the upper surface of the filter were removed. The cells on the lower surface were fixed and stained with Diff-Quik (Baxter Healthcare Corp., Orange, CA). Chemotaxis was quantified by overlapping fields (× 200 magnification). For the purpose of comparison between multiple assays, the data were normalized by expressing it as the fold increase in cell chemotaxis relative to that of the control.

Assay of In Vitro Phosphoinositide 3-Kinase Activity—Growth factors and inhibitors were exposed to serum-starved cells for the indicated time at 37°C. The cells were washed twice with 4 ml of cold phosphate buffered saline (137 mM NaCl, 15.7 mM Na\(_2\)HPO\(_4\), 1.47 mM KH\(_2\)PO\(_4\), 2.68 mM KCl (pH 7.4)) and were lysed in 1 ml lysis buffer (137 mM NaCl, 20 mM Tris, 1 mM MgCl\(_2\), 0.2 mM vanadate, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 4.5 mM sodium pyrophosphate, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM ZnCl\(_2\), 48 mM NaF, 9 mM β-glycerophosphate). The lysates were vortexed and centrifuged at 16,000 × g (Eppendorf 5414 microcentrifuge). The cleared supernatants were transferred to new Eppendorf tubes and incubated with anti-phosphotyrosine (6.6 µg/ml) for 1 h at 4°C, followed by the addition of protein A-Sepharose (4 mg/ml lysis) for an additional 2 h at 4°C.

The immunoprecipitates were pelleted, washed three times in phosphate-buffered saline, 1% Nonidet P-40, twice in 0.1 M Tris (pH 7.5), 0.5 M LiCl, and twice in TNE (10 mM Tris, 100 mM NaCl, 1 mM EDTA (pH 7.5)). All three wash solutions contained 200 µM vanadate. To assay the phosphoinositide 3-kinase activity of the immunoprecipitates, sonicated phosphatidylinositol (PI) (0.2 mg/ml final concentration, in 10 mM HEPES, 1 mM EGTA (pH 7.5)) and [γ-32P]ATP (10–20 µCi/sample) were added to the immunoprecipitates for 10 min at room temperature. The reaction was stopped by the addition of 80 µl of HCl and 160 µl of methanol/chloroform (1:1 (v/v)) mixture. The lipid-containing organic phase was separated on oxalate-coated thin-layer chromatography (Silica Gel 60; MCB Reagents, Merck) and developed in chloroform: methanol:water and filtered using a 0.2-µm filter. The organic phase was separated on oxalate-coated thin-layer chromatography (Silica Gel 60; MCB Reagents, Merck) and developed in chloroform: methanol:water (60:47:11.3:2, v/v). Radiolabeled spots corresponding to phosphatidylinositol 3-phosphate were quantified using a phosphomager (Bio-Rad).

Measurements of in Vivo Phosphoinositide 3-Kinase Activity—MC3T3-E1 cells were serum-starved for 24 h in α-MEM containing 0.1% (v/v) fetal calf serum. The cells were incubated in 5 ml of phosphate-free Dulbecco’s modified Eagle’s medium for 45 min, followed by exposure to 2 ml of the same medium plus carrier-free ortho-[32P]P phosphate (0.5–2 M Ci/mmol). The cells were allowed to incorporate the radioisotope at 37°C for 3 h in a humified atmosphere containing 5% CO₂, PDGF (10 ng/ml) or calcium (5 mM final concentration) was subsequently added to the dishes for 1–5 min. Cells were washed twice with 20 mM Tris (pH 7.5), followed by addition of 750 µl of 1 M HCl/methanol (1:1, v/v). The cells were scraped with a cell scraper, and the mixture was vortexed in a 2-ml microcentrifuge tube. Chloroform (380 µl) was added to the lysates and vortexed vigorously. The samples were centrifuged briefly at 16,000 × g. The lower chloroform phase was extracted twice with 400 µl of methanol/EDTA (0.1 M, pH 7.5) in a 1.09 (v/v) mixture. The organic (lower) was transferred to glass scintillation vials and dried under N₂. Deacetylation of the lipids was performed using methylene reagent as described previously (18).

The glycerophosphoinositides were resuspended in 120 µl of distilled water and filtered using a 0.2-µm filter. The glycerophosphoinositides were then separated by HPLC using a 12.5-cm Partisphere 5-μm column (Beckman Instruments). Data—Data given as the means ± the S.E. of the means, with the number of determinations (n) representing separate experiments.

RESULTS

Extracellular Calcium and PDGF Induce Chemotaxis of MC3T3-E1 Cells—There was a dose-dependent relationship between the extracellular calcium concentration and the rate of chemotaxis of the MC3T3-E1 osteoblast-like cells. The maximum chemotactic effect was attained at a calcium concentration of 5 mM (Fig. 1). A substantial enhancement of chemotaxis was also seen at 3 mM calcium, a concentration less than twice the basal concentration (1.8 mM). At 10 mM calcium, the rate of chemotaxis was greater than the basal rate, but was less than the peak rate. Chemotaxis of the MC3T3-E1 cells was not increased when sodium chloride was used to produce osmolar increases equivalent to the CaCl\(_2\) additions, suggesting that slight increases in medium osmolality were not responsible for the induction of chemotaxis by increases in [Ca\(^{2+}\)] (data not shown). Using the Zigmund-Hirsch (19) checkerboard analysis, the [Ca\(^{2+}\)]-induced cell movement was found to be directed chemotaxis rather than random chemokinesis (data not shown).

PDGF-BB also promoted an increase in chemotaxis of the
Ca\(^{2+}\) Receptor- and PDGF Receptor-mediated Chemotaxis

**Ca\(^{2+}\) Receptor- and PDGF Receptor-mediated Chemotaxis**

**FIG. 1.** Concentration dependence of extracellular calcium on chemotaxis. Low serum α-MEM solutions with various concentrations of CaCl\(_2\) were placed in the bottom wells of a 48-well Boyden chemotaxis chamber. MC3T3-E1 cells in low serum α-MEM (1.8 mM calcium) were placed in the upper wells and allowed to migrate through a collagen-coated filter for 4 h at 37 °C in a 95% O\(_2\), 5% CO\(_2\) humidified environment. Cells were removed from the top of the filter, and the cells that chemotaxed through the 8 μm pores were stained using Diff-Quik. Quantification of chemotaxis was performed at 200 × magnification, counting 100 grids in each of the wells. The number of cells that chemotaxed to a specific calcium concentration was normalized to chemotaxis toward basal conditions (1.8 mM). Values with error bars represent the mean ± S.E. from three experiments. *p < 0.05 versus basal (1.8 mM calcium); **p < 0.01 versus basal (1.8 mM calcium).

MC3T3-E1 osteoblasts. In 12 experiments in which the effects of PDGF and [Ca\(^{2+}\)]\(_{i}\) were compared directly, PDGF (10 ng/ml) increased chemotaxis to 20.3 ± 1.2 times basal (no stimulus) levels, and [Ca\(^{2+}\)]\(_{i}\) (5 mM) increased chemotaxis to 7.9 ± 0.4 times basal levels. Previous studies in this cell line have described a dose-dependent relationship between PDGF concentration and chemotaxis, and PDGF-promoted chemotaxis has been observed in other cell lines (20–22). In contrast, the chemotactic effect of extracellular calcium appears to be cell type specific. [Ca\(^{2+}\)]\(_{i}\)-induced increases in chemotaxis were not seen using NIH-3T3 fibroblasts exposed to 5 or 10 mM calcium, although PDGF (10 ng/ml) stimulated chemotaxis of these cells to 9 times the basal level (data not shown).

**Pertussis Toxin Inhibits Extracellular Calcium-stimulated Chemotaxis of MC3T3-E1 Cells—**Previous studies have reported that several cell types have a G-protein-coupled calcium receptor (1). Since the stimulatory effect of extracellular calcium on chemotaxis suggests that MC3T3-E1 cells have calcium receptors, we investigated whether the chemotaxis response in MC3T3-E1 cells was sensitive to pertussis toxin. Pertussis toxin prevents receptor activation of some G-proteins by catalyzing ADP-ribosylation of the G-protein α-subunits (23). Following 12-h exposure to 100 ng/ml pertussis toxin, [Ca\(^{2+}\)]\(_{i}\)-directed chemotaxis of the MC3T3-E1 cells was almost completely inhibited (Table I). In contrast, pertussis toxin had hardly any effect on PDGF-induced chemotaxis of these cells (Table I). These data suggest that a G-protein-linked calcium receptor plays a critical role in [Ca\(^{2+}\)]\(_{i}\)-stimulated chemotaxis, whereas a pertussis toxin-sensitive G-protein activation is not involved in PDGF-directed chemotaxis.

**Extracellular Calcium- and PDGF-stimulated Chemotaxis of MC3T3-E1 Cells Require Phospholipase C Activation—**U-73122 inhibits the activation of phosphatidylinositol-specific phospholipase C (PI-PLC) (24). Although the precise mechanism of inhibition has yet to be defined, U-73122 probably disrupts the link between G-proteins and PLC (25). [Ca\(^{2+}\)]\(_{i}\)-directed chemotaxis was almost completely inhibited by this compound, whereas PDGF-stimulated chemotaxis was reduced by only about 50% (Table I). U-73433, an inactive analog of U-73122, had no effect on either [Ca\(^{2+}\)]\(_{i}\) or PDGF-stimulated chemotaxis (data not shown). Previous studies have shown that PLC activation is involved in PDGF-directed chemotaxis (12).

**PI 3-Kinase Is Involved in PDGF-directed Chemotaxis, but Not Extracellular Calcium-stimulated Chemotaxis of MC3T3-E1 Cells—**Wortmannin, a fungal metabolite, acts as a specific irreversible inhibitor of the catalytic p110 subunit of PI 3-kinase (29). Previous studies have shown that wortmannin will inhibit both PDGF- and hepatocyte growth factor-stimulated chemotaxis, linking PI 3-kinase activation to chemotaxis (13, 14, 30). Consistent with these findings, PDGF-stimulated chemotaxis of MC3T3-E1 cells was inhibited by at least 50% when 100 nM wortmannin was present in both the upper and lower chambers when chemotaxis studies were initiated. PKC was down-regulated by pretreating cells with 1 μM PMA for 12 h.

Our results indicate that PLC activation may be responsible for about half of the PDGF-directed chemotaxis response in MC3T3-E1 cells. However, the role of PI-PLC is even more critical in [Ca\(^{2+}\)]\(_{i}\)-directed chemotaxis of MC3T3-E1 cells, and accounts for nearly the entire response. Consistent with the data obtained using pertussis toxin, these findings suggest the chemotactic effect of [Ca\(^{2+}\)]\(_{i}\) is mediated by a calcium receptor linked to PLC.

**TABLE I**

| Chemoattractant | Pertussis toxin | PKC down-regulation | Wortmannin |
|-----------------|-----------------|---------------------|------------|
| PDGF (10 ng/ml) | 11 ± 1 \(^{a}\) | 48 ± 7 \(^{a}\) | 54 ± 5 \(^{a}\) |
| [Ca\(^{2+}\)]\(_{i}\) (5 mM) | 90 ± 5 \(^{a}\) | 88 ± 4 \(^{a}\) | 5 ± 4 |
| (n = 3) | (n = 3) | (n = 4) | (n = 3) |

* \(^{a}\) p < 0.001 versus no inhibitory effect.

PI 3-Kinase is involved in PDGF-directed chemotaxis, but not extracellular calcium-stimulated chemotaxis of MC3T3-E1 cells.
Results suggest that the promotion of chemotaxis by extracellular calcium differs from that promoted by PDGF, since PI 3-kinase does not appear to be involved in the signal transduction pathway for [Ca$^{2+}$]o-stimulated chemotaxis of MC3T3-E1 cells.

**PDGF, but Not Extracellular Calcium, Activates PI 3-Kinase in MC3T3-E1 Cells**—In anti-phosphotyrosine immunoprecipitates from MC3T3-E1 cells exposed to 10 ng/ml PDGF, there was an elevation of PI 3-kinase activity to 12.1 ± 2.2 (n = 8) times that found under basal conditions (Fig. 2). In contrast, there was no increase (1.1 ± 0.1, n = 3) in PI 3-kinase activity above basal levels in anti-phosphotyrosine immunoprecipitates from calcium (5 mM)-treated cells. This suggests that elevation in external calcium does not affect PI 3-kinase activity. To examine the in vivo production of phosphoinositides phosphorylated on the D-3 position of the inositol ring, the lipid products of PI 3-kinase, the osteoblasts were loaded with inorganic ortho-[32P]phosphate and then exposed to PDGF (10 ng/ml) or increases in extracellular calcium (5 mM final concentration). PDGF produced substantial increases in D-3 lipids. There was a 3-fold elevation in PI-3,4-P$_2$ and a 13-fold increase in PI-3,4,5-P$_3$ levels after a 1-min exposure to PDGF (Fig. 3). A 5-min exposure to PDGF produced a 5- and 13.5-fold elevation in PI-3,4-P$_2$ and PI-3,4,5-P$_3$, respectively. In contrast, extracellular calcium (1 or 5 min) did not enhance production of either PI-3,4-P$_2$ or PI-3,4,5-P$_3$ in MC3T3-E1 cells (Fig. 3). These results are consistent with the in vitro immunoprecipitation data that in extracellular calcium that activated PI 3-kinase in MC3T3-E1 cells, whereas PDGF-DB significantly increased PI 3-kinase activity.

 Pretreatment of 32P-labeled cells with 100 mM wortmannin (20 min) produced a complete inhibition of 5-min PDGF-stimulated PI-3,4-P$_2$ production and a 60% reduction in PDGF-induced PI-3,4,5-P$_3$ production (Fig. 3). Wortmannin had a negligible effect on the D-3 lipids produced in cells exposed to 5 mM extracellular calcium, consistent with the lack of effect of calcium on PI 3-kinase activity in vivo (Fig. 3). These results support the wortmannin-sensitivity of the chemotaxis assay which indicated a requirement for PI 3-kinase activation in PDGF-stimulated, but not [Ca$^{2+}$]o-directed, chemotaxis of MC3T3-E1 cells (Table I).

**DISCUSSION**

The results of the present study suggest that different chemoattractants can stimulate chemotaxis of the same cell type through chemoattractant-specific signal transduction mechanisms. PDGF produced large increases in PI 3-kinase activity, and the activation of PI 3-kinase and PLC appeared to contribute equally to PDGF-promoted chemotaxis (Table I). In contrast, while elevation in extracellular calcium promoted the chemotaxis of MC3T3-E1 cells, this response appeared to be dependent on the activation of PLC. Extracellular calcium did not activate PI 3-kinase in MC3T3-E1 cells, and [Ca$^{2+}$]o-stimulated chemotaxis was not sensitive to wortmannin, an inhibitor of PI 3-kinase activity. Our results strongly suggest that MC3T3-E1 cells have a pertussis toxin-sensitive G-protein-linked calcium receptor, and that activation of this receptor stimulates chemotaxis utilizing a PLC-dependent mechanism.

Brown et al. (10) recently cloned a calcium-sensing receptor from bovine parathyroid cells. In addition to parathyroid cells, BoPcaR1 mRNA transcripts were found in kidney, brain, and thyroid cells (10). The BoPcaR1 belongs to the seven-transmembrane receptor family and is linked via a G-protein to PLC (10). Activation of this receptor by extracellular calcium leads to elevation in IP$_3$ with subsequent increases in the intracellular calcium concentration (1). A recent report suggests that MC3T3-E1 osteoblast-like cells do not express the BoPcaR1, at least not in great abundance, but may produce a calcium-sensing receptor that is similar, yet distinct, to BoPcaR1 (11).

Evidence for receptor-mediated signaling by extracellular calcium in MC3T3-E1 cells was indicated by the results obtained using two inhibitors, pertussis toxin and U-73122. Pertussis toxin, an inhibitor of G-protein activation, blocked almost all of the [Ca$^{2+}$]o-stimulated chemotaxis (Table I). This result suggests that a G-protein-linked receptor must be involved in [Ca$^{2+}$]o-mediated chemotaxis. In some cells, including osteoblasts (31), pertussis toxin has been shown to inhibit agonist-stimulated transient elevations in intracellular calcium. Pertussis toxin also affects the activation of adenylate cyclase through inhibition of $G_0$. The role that adenylate cyclase and cAMP play in [Ca$^{2+}$]o-stimulated chemotaxis of MC3T3-E1 cells has yet to be examined. Several other chemoattractants activate G-protein-linked receptors. For example, neutrophil chemotaxis in response to leukotriene B$_4$ and formyl-methionyl-lysyl-phenylalanine is inhibited by pertussis toxin (32, 33). The limited effect of pertussis toxin on PDGF-mediated chemotaxis (Table I) was expected, since PDGF acts through a tyrosine kinase receptor rather than a G-protein-linked receptor (34).

Signaling events downstream of both G-protein-linked receptors and receptor tyrosine kinases activation include the stimulation of PLC activity. In the presence of U-73122, which blocks the activation of PI-PLC, [Ca$^{2+}$]o-directed chemotaxis was almost completely inhibited (Table I). This result indicates that [Ca$^{2+}$]o-mediated chemotaxis of MC3T3-E1 cells requires PLC. U-73122 had a substantial, but less complete, inhibitory effect on PDGF-directed chemotaxis of these cells, confirming previous evidence suggesting that PLC is one of two signal transduction pathways that are critical for PDGF-directed chemotaxis (12). The fact that PDGF promotes a substantial chemotaxis response in U-73122-treated cells suggests that the near complete inhibition of [Ca$^{2+}$]o-directed chemotaxis by U-73122 was not due to a nonspecific toxic effect of this inhibitor on MC3T3-E1 cells.

Activation of PLC leads to hydrolysis of PI-4,5-P$_2$ to form DAG and IP$_3$ (26). DAG activates PKC, a serine/threonine...
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**Fig. 3.** PDGF, but not extracellular calcium, stimulated the *in vivo* production of D-3 phosphorylated phosphoinositides in MC3T3-E1 cells. MC3T3-E1 cells were loaded with organic ortho-[32P]phosphate for 3 h, then exposed to either PDGF (10 ng/ml) or calcium (5 mM) for 1–5 min. The D-3 phosphorylated lipids were extracted and deacylated as described under "Materials and Methods." HPLC analysis was performed to separate the lipids and quantify their production based on disintegrations/min. The PI-4-phosphate and PI-4,5-P_2 levels were summed and used to represent total phospholipids. The PI-3,4-P_2 and PI-3,4,5-P_3 levels were then expressed as a percentage of total phospholipids. There were significant increases in PI-3,4-P_2 and PI-3,4,5-P_3 levels in cells exposed to PDGF for 1 and 5 min. In contrast, neither a 1- or 5-min exposure to 5 mM calcium produced a significant elevation in PI-3,4-P_2 or PI-3,4,5-P_3. Pretreatment of the organic ortho-[32P]phosphate-loaded cells with 100 nM wortmannin (20 min) resulted in a complete inhibition of the PDGF-stimulated (5 min) elevation in the PI-3,4-P_2 level, and the PDGF-mediated increase in PI-3,4,5-P_3 was reduced by 60% after wortmannin pretreatment. Wortmannin had no substantial effect on [Ca^{2+}]_o-initiated PI 3-kinase activity. Values with error bars represent the mean ± S.E. of four experiments performed in duplicate determinations for each experiment. Values without error bars represent the average of duplicate determinations from one experiment. *p < 0.05 versus basal; **p < 0.01 versus basal.

...kinase that is involved in multiple signaling events (26). In studies in which cells were exposed to phorbol ester to down-regulate PKC, we found that PKC is critical to PDGF-stimulated chemotaxis of MC3T3-E1 cells (Table I). In contrast, [Ca^{2+}]_o-stimulated chemotaxis did not appear to require PKC activation, even though PLC activation was necessary. Alternatively, it is possible that a PKC isoform(s) which is relatively slowly down-regulated or is insensitive to PMA (e.g., PKC ζ) contributes to [Ca^{2+}]_o-promoted chemotaxis. When PMA (100 nM) was added to the bottom wells of the Boyden chamber, direct migration of MC3T3-E1 cells was not observed, suggesting that PMA alone is not a chemoattractant for these cells. Hemolytically inactive complement component complex C5b67 induces chemotaxis of neutrophils through a G-protein-linked receptor which activates PLC, but not PKC (34). Our data suggests that the IP_3 pathway rather than the DAG pathway is important in [Ca^{2+}]_o-initiated chemotaxis of MC3T3-E1 cells. Since extracellular calcium and PDGF both have been reported to elevate the intracellular calcium concentration, it is possible that a rise in intracellular calcium contributes to chemotaxis in MC3T3-E1 cells. Consistent with this, both PDGF- and extracellular calcium-stimulated chemotaxis were reduced in cells loaded with the calcium-chelator BAPTA (data not shown). However, this may have been due to an effect on regulatory components in cell migration/chemotaxis that are far downstream from receptor activation.

PDGF increased PI 3-kinase activity and the production of PI-3,4-P_2 and PI-3,4,5-P_3 in MC3T3-E1 cells (Figs. 2 and 3). Wortmannin, a PI 3-kinase inhibitor, significantly reduced both the *in vivo* and *in vitro* PDGF-mediated elevations of the D-3 phosphorylated lipids. Furthermore, PDGF-directed chemotaxis of the MC3T3-E1 cells was significantly inhibited by wortmannin (Table I). Thus, PDGF-stimulated chemotaxis requires PI 3-kinase activity in addition to PLC activation, as others have reported in other cells (12). In contrast, cells exposed to increases in [Ca^{2+}]_o did not display an increase in PI 3-kinase activity in the *in vitro* assays of anti-phosphotyrosine immunoprecipitates (Fig. 2). To rule out activation of PI 3-kinase via a G-protein-linked receptor (35), which may not recruit PI 3-kinase to a tyrosine phosphorylated protein, we measured the D-3 lipid production *in vivo*. PI-3,4-P_2 and PI-3,4,5-P_3 levels were not elevated by an increase in extracellular calcium (Fig. 3). In addition, wortmannin had an insignificant effect on [Ca^{2+}]_o-stimulated chemotaxis of MC3T3-E1 cells (Table I). These data suggest that PI 3-kinase is not involved in extracellular calcium-stimulated chemotaxis. In neutrophils, similar findings demonstrated that PI 3-kinase was essential for PDGF-mediated chemotaxis but was not necessary for chemotaxis directed by chemoattractants that act through G-protein-linked receptors (36).

...Previous studies have described a dose-dependent relationship between extracellular calcium concentration and chemotaxis in MC3T3-E1 osteoblast-like cells (6). Our results are consistent with these studies in that concentration-dependent increases in chemotaxis were observed between 1.8 and 5 mM calcium. In contrast to our results shown in Fig. 1, Sugimoto et al. (6) reported that the chemotactic effect of [Ca^{2+}]_o was further enhanced at a 10 mM concentration. We found that the stimulation of chemotaxis of MC3T3-E1 cells at this calcium concentration was less than that found at 5 mM. Other investigators have described a reduction of chemotaxis in the presence of high concentrations of chemoattractant and have attributed this effect to a more rapid dissolution of the chemoattractant gradient responsible for directed cell movement (36).

Increases in extracellular calcium may act as a physiological...
stimulus for bone cells. Bone remodeling consists of two components, a resorptive phase followed by a reparative phase (37). Osteoclasts resorb bone by creating a local environment capable of demineralizing bone and proteolyzing bone matrix proteins (38). The initiation of the reparative phase, known as reversal, requires the recruitment and activation of osteoclasts (37). Several cytokines and growth factors, including PDGF, have been implicated as coupling agents, capable of inducing reversal (39, 40). Osteoblasts incorporate the growth factors into the bone matrix during the reparative phase of bone remodeling (39). These coupling agents are then released from the bone matrix throughout the resorptive phase (39–41). In addition to liberating growth factors, osteoclasts dramatically alter extracellular calcium concentration at local sites of resorption. The extracellular calcium concentration can reach 40 mM within the resorption lucunae (4). By inhibiting osteoclastic activity while stimulating chemotaxis and mitogenesis of osteoblasts, extracellular calcium may serve as a key coupling agent between bone formation and resorption (5, 6, 42). Under-activity while stimulating chemotaxis and mitogenesis of osteblasts may also reverse (39, 40). Osteoblasts incorporate the growth factors into the bone matrix during the reparative phase of bone remodeling (39). These coupling agents are then released from the bone matrix throughout the resorptive phase (39–41). In addition to liberating growth factors, osteoclasts dramatically alter extracellular calcium concentration at local sites of resorption. The extracellular calcium concentration can reach 40 mM within the resorption lucunae (4). By inhibiting osteoclastic activity while stimulating chemotaxis and mitogenesis of osteoblasts, extracellular calcium may serve as a key coupling agent between bone formation and resorption (5, 6, 42). Understanding the signaling events responsible for the induction of extracellular calcium events in this system will lead to further developments in the treatment of bone metabolism disorders.

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