Extracellular Calcium Mimics the Actions of Platelet-derived Growth Factor on Mouse Fibroblasts

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Abstract

Microprecipitates of calcium phosphate (CaPO₄) can substitute for platelet-derived growth factor (PDGF) to stimulate the growth of cultured 3T3 cells. In two-part complementation assays, CaPO₄ behaves as a PDGF-like “competence factor”—that is, the mitogenic response to CaPO₄ is enhanced synergistically by “progression factors” contained in platelet-poof plasma. In studies described here, we show that early cytoplasmic and intranuclear events in the mitogenic response to CaPO₄ are equivalent to those induced by PDGF. However, no net increase in tyrosine kinase activity of either the PDGF-α or PDGF-β receptor is seen following exposure to CaPO₄. Our data suggest that calcium acts within the cell, regulating events which normally proceed from activation of PDGF receptors. Alternatively, microprecipitates of CaPO₄ could act externally by activating a growth factor receptor which escapes detection with available reagents.

Introduction

Calcium has long been recognized to be an important cell signaling molecule. In 1975, Dulbecco and Elkington (1) observed that addition of calcium chloride to the culture medium of 3T3 cells stimulates DNA synthesis and cell division. Their findings were subsequently confirmed and extended by two independent groups who showed that neither free calcium nor free chloride media

d the mitogenic response (2–4). Rather, it is the microprecipitate of CaPO₄ (which forms in conventional culture medium when the concentration of free calcium exceeds 3 mM) that stimulates cell growth. Other small particulate materials do not necessarily stimulate 3T3 cell growth: strontium phosphate (a microprecipitate formed from a related divalent cation) is mitogenic, but finely ground glass or aluminum particles are without effect (1). Our own laboratory made the observation that microprecipitates of CaPO₄ can substitute for PDGF to stimulate the growth of cultured 3T3 cells. In two-part complementation assays, CaPO₄ microprecipitates behave as a PDGF-like “competence factor”; that is, the mitogenic response of 3T3 cells to CaPO₄ microprecipitates is enhanced synergistically by “progression factors” contained in platelet-poof plasma (5).

In recent years, there have been few additional insights into the molecular action of CaPO₄ microprecipitates. Much, however, has been learned about the molecular action of PDGF. Early events in the PDGF signal transduction cascade include autophosphorylation of PDGF-α and -β receptor subunits (6), transphosphorylation of both EGF receptor (7) and c-erbB-2 receptor (8), activation of phosphatidylinositol kinase (9) and ral (10), and induction of “immediate response genes” (11). Since CaPO₄ mimics PDGF in growth factor complementation assays, it is reasonable to hypothesize that particles of CaPO₄ somehow activate PDGF receptors on the 3T3 cell surface. In this study, we examine early events in the response of 3T3 cells to CaPO₄ and to PDGF. We show that the recognized mitogenic (2), morphological (1), and gene-inducing properties (11) of PDGF—as well as the ability of PDGF to transmodulate the c-erbB-2 cell surface receptor (8)—are closely mimicked by addition of calcium chloride to 3T3 tissue culture medium. Tyrosine kinase activity of PDGF receptors in quiescent 3T3 cells is not, however, stimulated by calcium addition.

Results

Calcium Mimics the Mitogenic Activity of PDGF in 3T3 Cells. Our first experiments analyzed the effect of calcium, serum, and PDGF on the growth of G8/DHFR cells, a strain of 3T3 cells which expresses a transfected c-erbB-2 gene. The c-erbB-2 protein, a close structural homologue of the EGF receptor, is expressed at high levels in this cell line, allowing sensitive detection of transphosphorylation events in response to PDGF (8). As shown in Fig. 1, serum and PDGF stimulate G8/DHFR cell growth in a concentration-dependent fashion, with peak stimulation occurring at concentrations of 10% and 10 ng/ml, respectively (Fig. 1, A and B); the magnitude of the serum effect varies with different serum batches (data not shown). The mitogenic response to incremental calcium is sharply concentration dependent, with an optimal stimulatory concentration of 5–10 mM CaCl₂ and a steep decline in cell growth at higher concentrations (Fig. 1C). Since the physiological concentration of calcium in DME is 1.85 mM (1), we assessed the effect of longer-term continuous cultivation of G8/DHFR cells in

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3 The abbreviations used are: PDGF, platelet-derived growth factor; DME, Dulbecco's minimal essential medium; PKC, protein kinase C; PdBU, phorbol dibutyrate; PBS, phosphate-buffered saline; kDa, kilodalton(s); PPP, platelet-poof plasma.
DME containing an additional 10 mM CaCl₂ to exclude the possibility that such concentrations might prove rapidly cytotoxic. Continued growth stimulation is seen during a 72-h period of high-calcium incubation in which several cell cycle traverses would be expected (Fig. 1D), indicating that the calcium-dependent mitogenic signaling pathway remains active throughout this time and that toxicity due to ambient high calcium is not a short-term problem.

**Calcium Induces PDGF-like Morphological Transformation in Nontransformed 3T3 Cells.** In a separate series of experiments, calcium-induced growth stimulation of BALB/c and NIH 3T3 cells was found to be of similar magnitude to that seen in G8/DHFR cells, although this effect was less marked or absent in other cell lines, including ras- and src-transformed 3T3 cells and human breast cancer cell lines (data not shown). Induction of morphological changes by calcium is particularly evident in G8/DHFR (Fig. 2D) and NIH 3T3 cell lines (data not shown) when compared with control cells (Fig. 2A) and closely resembles the transforming changes induced by PDGF (Fig. 2C). The elongated, crisscrossing, highly refractile cell morphology seen in these calcium-treated cells is not seen with serum stimulation (Fig. 2B). Hence, like mitogenic stimulation, morphological transformation of 3T3 cells appears to be induced by calcium exposure with similar efficiency to that induced by PDGF.

**The Time-Course and PKC Dependence of PDGF-induced c-fos and JE Gene Expression Is Mimicked by Calcium.** Expression of JE, an early response PDGF-inducible gene (11), is induced within 2 h by calcium exposure, but this expression [like that induced by PDGF

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Fig. 1. Comparative mitogenic effects of extracellular calcium, PDGF, and bovine calf serum in G8/DHFR 3T3 cells. All cultures were sparsely plated in 6-cm tissue culture dishes, medium changed with DME containing 5% PPP and 1.85 mM CaCl₂ for 24 h, and then treated for a further 24 h prior to trypsinization and Coulter counting of triplicate plates. The percentage cell growth stimulation in these experiments was calculated by expressing the difference between treated and control cell growth as a proportion of control cell growth; bars, SEM. A, mitogenic effects of increasing concentrations of bovine calf serum for 24 h. B, 24-h growth stimulation by different concentrations of PDGF. C, 24-h growth stimulation by different concentrations of CaCl₂; labeled values represent the amount of calcium added to cells growing in ordinary DME containing 1.85 mM CaCl₂ (i.e., total calcium = 1.85 + designated concentration). D, 72-h growth curve of cells grown either in DME containing 5% platelet-poor plasma alone (open symbols) or with added 10 mM CaCl₂ (closed symbols); the percentage cell growth stimulation in this experiment was calculated as a ratio of initial (control) cell counts.

Fig. 2. Morphological effects of calcium, serum, and PDGF on G8/DHFR cells. Stock cultures were detached using 0.1% trypsin-0.29 g/liter EDTA, resuspended in DME containing 10% bovine calf serum, plated sparsely in 10-cm tissue culture dishes, allowed to adhere, and then washed and overlaid with DME containing 5% PPP, antibiotics, and glutamine for 24 h. Cultures then remained in PPP (A), or else received 10% bovine calf serum (B), 50 ng/ml PDGF-BB (C), or 10 mM CaCl₂ (D) for a further 24 h and then were photographed.

Fig. 3. Comparative effects of calcium and PDGF on gene expression in BALB/c 3T3 cells as assessed by Northern analysis. A, JF gene induction in the presence and absence of 24-h phorbol ester pretreatment. Significant induction is seen with PdBU alone, but mild synergism is seen both with 2-h calcium (Lanes 4 and 5) and with 2-h PDGF cotreatment. B, c-fos gene induction in the presence and absence of 24-h PdBU treatment. PDGF induction at 30' (Lane 6) is markedly attenuated by phorbol ester pretreatment (Lane 8), as is calcium-induced fos expression (Lanes 3 and 5, respectively). For quantitation of phorbol ester effects, see Table 1.

(12)] is resistant to phorbol ester-mediated PKC down-regulation (Fig. 3A). Quantitation of this induction confirms that 24-h phorbol ester-induced PKC down-regulation is associated with a 2-2.5-fold enhancement of both calcium-induced and PDGF-induced JF expression (Table 1). Addition of calcium to BALB/c 3T3 cells results in c-fos expression within 30 min of exposure, as does addition of PDGF (11), and in both cases, this induction is abrogated by prolonged exposure to the phorbol ester PdBU (Fig. 3B); reduction of PDGF-induced c-fos expression in this experiment provides an internal control for the adequacy of PKC down-regulation (12). Band quantitation confirms a 14-fold reduction of both calcium- and PDGF-induced c-fos expression following PKC down-regulation (Table 1).

Calcium Induces c-erbB-2 Receptor Tyrosine Dephosphorylation in a Concentration-dependent and Calcium-specific Manner. PDGF transmodulates the tyrosine kinase activity of both EGF receptors (7) and c-erbB-2 receptors (8). In the case of EGF receptor, this involves conversion of high-affinity receptor-binding sites to low-affinity sites, thus reducing ligand binding. Like PDGF, incremental calcium exposure progressively reduces c-erbB-2 receptor tyrosine phosphorylation in GB/DHFR cells with virtual abolition of immunoreactive phosphotyrosine following 15 min exposure to 100 mmoL CaCl2 (Fig. 4A). This tyrosine dephosphorylation is not attenuated by pretreatment with suramin, a putative inhibitor of PDGF binding to its receptor (13), suggesting that calcium does not transmodulate c-erbB-2 by stabilizing PDGF binding (data not shown). In contrast, extracellular magnesium fails to mimic the effect of calcium even at high concentrations (Fig. 4A). These data indicate that calcium is at least 10-fold more potent than MgCl2 on a molar basis in transmodulating the c-erbB-2 receptor. Interestingly, high concentrations of calcium do not affect c-erbB-2 receptor phosphotyrosine content in several human breast cancer cell lines (data not shown); this observation may be related to the finding of Ochieng et al. (14) that such cell lines do not alter intracellular calcium levels significantly in response to extracellular calcium flux.

Curiously, the time course of c-erbB-2 receptor transmodulation in response to PDGF is not identical to that induced by calcium. PDGF-induced tyrosine dephosphorylation of the c-erbB-2 receptor becomes maximal within 15 min of initial exposure, remains maximal after 2 h continuous exposure (Fig. 4B), and persists for more than 4 h (8). In contrast, calcium-induced receptor transmodulation is also maximal within 15 min (Fig. 4B) but is largely reversed within 1 h (data not shown) and completely disappears within 2 h (Fig. 4B). This time course is in striking distinction to the well-sustained mitogenic effect of calcium in this cell line (Fig. 1D). Phosphorylation of c-rac-1 Is Differentially Regulated by Calcium and PDGF. PDGF activates the downstream serine-threonine kinase, Raf-1, by tyrosine phosphorylation (10). To determine whether the similarities between the signal-transducing properties of calcium and PDGF include this pathway, the effect of calcium on Raf-1 phosphorylation in BALB/c 3T3 cells was assessed using immunoblotting. Both PDGF and serum exposure are associated with retardation of immunoreactive Raf-1 electrophoretic mobility (Fig. 5, lanes 2 and 3), indicating phosphorylation of this protein following (although not

| Table 1 Quantitative effect of PKC down-regulation on PDGF- and calcium-induced c-fos and JF gene expression |
|-------------------------------------------------|-----------------|-----------------|-----------------|
| PDGF                                           | Calcium         |
|                                                | c-fos           | JF              | c-fos           |
|                                                | 13.8-fold decrease | 14.1-fold decrease | 2.4-fold increase | 2.2-fold increase |

Confluent BALB/c 3T3 cell monolayers were preincubated for 24 h in DME containing 5% platelet-poor plasma and 100 ng/ml PdBU. Fold reductions (FRs) of PDGF- and calcium-induced c-fos expression (when compared with non-PdBU-treated controls) were calculated using ImageQuant band quantifications (see "Materials and Methods") and the formula, FR = c-fosgene/c-foscontrol, where the numerator and denominator represent the quantitation of c-fos mRNA expression following 30 min exposure to calcium (or PDGF) plus calcium (or PDGF) plus 24 h phorbol ester pretreatment, respectively. Because of the substantial induction of JF by 24 h PdBU (Fig. 5A), fold enhancements (FEs) of JF gene expression due to PDGF and calcium were calculated from the formula, FE = (JFgene/IRFcontrol) / (JFcontrol/IRFcontrol), where the numerator represents the net enhancement of JF mRNA expression by 24 h phorbol ester pretreatment of samples subsequently exposed to PDGF (or calcium) for 2 h, and the denominator represents the absolute level of JF mRNA expression following 2 h PDGF (or calcium) exposure.
Calcium-induced transmodulation of the c-erbB-2 receptor in G8/DHFR mouse fibroblasts. A, concentration dependence and calcium specificity of c-erbB-2 tyrosine dephosphorylation. G8/DHFR cell samples were treated for 15 min with varying concentrations of CaCl2 or MgCl2. One hundred-μg aliquots of each protein lysate were then immunoprecipitated with phosphotyrosine antibody, electrophoresed, and blotted with anti-c-erbB-2. B, comparative time course of calcium- and PDGF-induced c-erbB-2 receptor tyrosine dephosphorylation. G8/DHFR cell samples were treated and lysed, and then 100-μg aliquots were immunoprecipitated using antiphosphotyrosine and subsequently blotted using c-erbB-2 antibody. Upper 175-185kDa band, position of the c-erbB-2 receptor; lower band, immunoprecipitated antibody heavy chain which is cross-reactive with the alkaline phosphatase-conjugated second antibody.

Fig. 4. }

Effect of calcium on Raf-1 phosphorylation. Density-arrested serum-starved G8/DHFR cells in 10-cm tissue culture dishes were treated for 30 min with either PDGF (50 ng/ml), bovine calf serum (10%), calcium chloride (CaCl2) (10 mM), the calcium ionophore A23187 (5 μM), thapsigargin (5 μM), or dimethyl sulfoxide (DMSO) (0.1%). Cell samples were lysed, following which 100-μg aliquots were electrophoresed, transferred to nitrocellulose, and probed using polyclonal rabbit anti-Raf antiserum. Bands were developed using alkaline phosphatase colorimetry (see "Materials and Methods").

PDGF Receptor Tyrosine Kinase Activity in Quiescent 3T3 Cells Is Not Enhanced by Calcium. Antiphosphotyrosine immunoblotting of immunoprecipitated PDGF-β receptor indicates that no increase in receptor phosphotyrosine is detectable when PDGF-deprived G8/DHFR cells are exposed to calcium (data not shown). Antiphosphotyrosine immunoprecipitates of calcium-treated quiescent G8/DHFR cells (Lane 3) contain negligible in vitro tyrosine kinase activity in the 160–180kDa range, indicating calcium antagonism of c-erbB-2 kinase activity as well as failure of calcium to activate either the PDGF-α (160kDa) or PDGF-β (180kDa) receptor (8) (Fig. 6A). Kinase activity associated with the 180kDa PDGF receptor seen in PDGF-stimulated cells (Lane 2) is also associated with an 85kDa band, whereas the 175kDa kinase c-erbB-2-associated kinase activity seen in PDGF-deprived cells (Lane 1) is not. Similarly, antiphosphotyrosine immunoprecipitates of quiescent BALB/c cells exposed to calcium for various durations exhibit negligible in vitro tyrosine kinase activity (Fig. 6B), indicating that neither PDGF-α nor PDGF-β receptors are activated by calcium during the period of c-fos and Jκ gene induction or c-erbB-2 transmodulation. Finally, antiphosphotyrosine immunoblotting of BALB/c cell lysates reveals no calcium-inducible tyrosine phosphorylation of any proteins larger
Fig. 6. Effect of calcium on PDGF receptor activation. A, in vitro kinase activity of G8/DHFR antiphosphotyrosine immunoprecipitates. PDGF (30 ng/ml) or calcium chloride (10 mM) was added for 10 min prior to lysis, and 100 µg of each protein lysate were immunoprecipitated at 4°C prior to in vitro autophosphorylation (see "Materials and Methods"). B, in vitro kinase activity of BALB/c 3T3 cell antiphosphotyrosine immunoprecipitates. Cell samples were exposed either to PDGF for 10 min or to calcium for 15 min, 40 min, or 2 h, followed by lysis, antiphosphotyrosine immunoprecipitation of 100-µg aliquots, in vitro kinase reaction, and electrophoresis. C, antiphosphotyrosine immunoblot of BALB/c 3T3 cell lysates. Quiescent cells were exposed to PDGF (30 ng/ml), calcium chloride (10 mM), A23187 (5 µM), or thapsigargin (thaps) (5 µM) for 10 min prior to lysis, and 100-µg aliquots of the lysates were examined by gel electrophoresis and immunoblotting.

than about 40 kDa (Fig. 6C), making it unlikely that calcium exerts its effects by activating other tyrosine kinase receptors. These findings indicate that the PDGF-like effects of calcium are not mediated by PDGF receptor activation.

Transfection Using Calcium Phosphate Permeabilization of BALB/c 3T3 Cells Induces JE in the Absence of Growth Factors. As a parenthetical observation, we note that our data have practical implications for other investigators studying the promoter regions of mitogen-inducible immediate response genes. As shown in Fig. 7, routine calcium phosphate transfection induces expression of JE in the absence of serum or PDGF. As with PDGF stimulation, induction of JE is readily apparent within 2–4 h of calcium phosphate transfection using salmon sperm DNA, and this induction disappears within
Fig. 7. \( \beta \)E gene induction by calcium phosphate transfection. A, Northern blot of \( \beta \)E gene expression in BALB/c 3T3 cells. Density-stained quiescent cells (Lane 1) were incubated in DME containing 10% bovine calf serum for 24 h prior to calcium phosphate transfection using salmon sperm DNA. Cells were lysed 4 h (Lane 2) following transfection and were maintained in transfection medium throughout this time. Cells were also transfected, media changed to DME containing 5% platelet-poor plasma after 4 h, and lysed after an additional 14-h incubation (Lane 3). B, ribonuclease protection assay of \( \beta \)E gene expression (represented here by the 272-base pair protected fragment) in BALB/c 3T3 cells before and after mock (no DNA) calcium phosphate transfection. Lane 1, nontransfected cultures stimulated with 10% bovine calf serum for 15 min; Lane 2, nontransfected quiescent cells; Lane 3, mock-transfected cells exposed to serum; and Lane 4, mock-transfected quiescent cells. Top band (344-base pair fragment), undigested probe.

18 h (Fig. 7A). Transcript abundance is relatively low when compared with that induced by optimal concentrations of PDGF or serum, but it is still clearly evident even when transfection is carried out in the absence of DNA (Fig. 7B). Other methods of cell permeabilization carried out in calcium-containing media also induce the \( \beta \)E gene (data not shown).

Discussion

Our findings concerning the mitogenic and transforming effects of calcium in 3T3 cells confirm and extend those of Dulbecco and Ellkington (1). Rubin and Sanui (3), Barnes and Colowick (2), Bowen-Pope and Rubin (4), and Mitchell et al. (15). In particular, our data reveal several striking similarities between the effects of 3T3 cell exposure to calcium and PDGF: (a) the intensity and duration of the peak mitogenic response is similar; (b) the efficiency of morphological transformation is comparable for equivalent mitogenic exposures; (c) transduction of the c-erbB-2 receptor occurs with similar efficiency; and (d) both the induction and the PKC dependence of early response genes are similar. Despite these similarities, our results indicate that the effects of calcium are not mediated via activation of the PDGF receptor itself.

The finding that calcium-induced c-fos expression is reduced by PKC down-regulation (Fig. 3) suggests that, like the PDGF receptor, calcium may act not only by influencing the intracellular inositol triphosphate-Ca\(^{2+} \) axis but also by interacting with the PKC-sensitive diacylglycerol pathway. It is also conceivable that calcium could be activating the intracellular signal transduction pathway of the receptor via an indirect mechanism, perhaps by negatively interacting with a cytoplasmic repressor domain (16). A previous report of c-fos induction by basic calcium phosphate crystal (17) raises the possibility of a cell surface mechanism, since calcium does not begin to be solubilized in the latter experimental system for 3 h (18).

Definitive interpretation of our findings is limited by several problems. Foremost among these is the lack of a simple and reproducible technique for measuring intracellular free calcium. Assays developed for this purpose are often restricted to measuring total intracellular calcium content (19) even though locally quantized free calcium release is now recognized to be critical in regulating signal transduction (20). Moreover, calcium influx blockers (such as those blocking dihydropyridine-sensitive channels) may not abolish intracellular nonspecific ingress of calcium (21); intracellular calcium chelators such as Quin-2 may exert nonspecific effects on calcium-independent processes (22); and the use of calcium- and/or phosphate-free media to dissect the relative roles of extracellular free versus microphysiologically calcium is complicated by the probable heterogeneous (and toxic) effects of these two methods in cellular microenvironment. Another interpretation limitation relates to the possibility that calcium-induced cell membrane permeabilization per se may contribute to the cellular behaviors described, thus reducing the specificity of the calcium effect. Definitive resolution of this issue will require analyzing the effects induced by several methods of cell permeabilization in a variety of chemically defined (e.g., calcium-depleted) tissue culture media.

Despite these experimental obstacles, it seems clear that manipulation of extracellular calcium may prove to be a valuable technique for studying cell growth and behavior. Currently, we are engaged in clarifying the physiological relevance of the effects described in this report. Circumstantial evidence suggests that pathological extracellular calcium deposits may play a central role in the genesis of diseases as diverse as arthritis (23) and breast cancer (24). The remarkable similarities demonstrated in this report between the actions of calcium and those of PDGF suggest that this experimental approach may provide key insights into mechanisms of intracellular signal transduction and cell surface tyrosine kinase regulation.

Materials and Methods

Cell Culture and Manipulation. G8/DHFR cells (NIH 3T3-derived murine fibroblasts) expressing a transfected and methotrexate-amplified dicistronic rat c-neu/dihydrofolate reductase construct (25) were a gift of Dr. Robert Weinberg (Whitehead Institute, Cambridge, MA). BALB/c mouse 3T3 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in a humidified 10% CO\(_2\) incubator at 37°C in DME supplemented with 10% bovine calf serum (HyClone), glutamine, and antibiotics (hereinafter referred to as growth medium), whereas stock cultures of G8/DHFR cells were also maintained in 0.3 μM methotrexate. Experimental cell samples were seeded into 10-cm tissue culture dishes and grown to confluence over 3 days without medium changing; cultures were then medium changed overnight with DME containing 5% platelet-poor plasma. Cell samples were treated at this point by adding the designated concentrations of v-sis PDGF (Amgen), bovine calf serum, CaCl\(_2\)-2H\(_2\)O (Sigma), MgCl\(_2\)
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Northern Blotting and Ribonuclease Protection Assays. Cell samples were treated, lysed in guanidinium isothiocyanate, and centrifuged for 18 h on cesium chloride gradients, and total cellular RNA was recovered by ethanol precipitation as previously described (27). RNA samples (10 μg) were then denatured by heating to 65°C for 15 min in formamide buffer, electrophoresed on formaldehyde gels, blotted onto nitrocellulose, and then UV-irradiated (120 joules) for 2 min. Following overnight prehybridization at 42°C, RNA blots were probed with nick-translated inserts from the relevant complementary DNAs for a further 16 h. After washing and drying, blots were exposed using PhosphorImager model 400S, and band quantification was carried out using ImageQuant v. 2.0 software (Molecular Dynamics, Sunnyvale, CA).

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