Phosphocitrate Inhibits a Basic Calcium Phosphate and Calcium Pyrophosphate Dihydrate Crystal-induced Mitogen-activated Protein Kinase Cascade Signal Transduction Pathway* (Received for publication, February 12, 1997, and in revised form, May 7, 1997)

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Calcium deposition diseases caused by calcium pyrophosphate dihydrate (CPPD) and basic calcium phosphate (BCP) crystals are a significant source of morbidity in the elderly. We have shown previously that both types of crystals can induce mitogenesis, as well as metalloproteinase synthesis and secretion by fibroblasts and chondrocytes. These responses may promote degradation of articular tissues. We have also shown previously that both CPPD and BCP crystals activate expression of the c-fos and c-jun proto-oncogenes. Phosphocitrate (PC) can specifically block mitogenesis and proto-oncogene expression induced by either BCP or CPPD crystals in 3T3 cells and human fibroblasts, suggesting that PC may be an effective therapy for calcium deposition diseases. To understand how PC inhibits BCP and CPPD-mediated cellular effects, we have investigated the mechanism by which BCP and CPPD transduce signals to the nucleus. Here we demonstrate that BCP and CPPD crystals activate a protein kinase signal transduction pathway involving p42 and p44 mitogen-activated protein (MAP) kinases (ERK 2 and ERK 1). BCP and CPPD also cause phosphorylation of a nuclear transcription factor, cyclic AMP response element-binding protein (CREB), on serine 133, a residue essential for CREB's ability to transactivate. Treatment of cells with PC at concentrations of 10⁻³ to 10⁻⁵ M blocked both the activation of p42/p44 MAP kinases, and CREB serine 133 phosphorylation, in a dose-dependent fashion. At 10⁻³ M, a PC analogue, n-sulfo-2-aminotricarballylate and citrate also modulate this signal transduction pathway. Inhibition by PC is specific for BCP- and CPPD-mediated signaling, since all three compounds had no effect on serum-induced p42/P44 or interleukin-1β induced p38 MAP kinase activities. Treatment of cells with an inhibitor of MEK1, an upstream activator of MAPKs, significantly inhibited crystal-induced cell proliferation, suggesting that the MAPK pathway is a significant mediator of crystal-induced signals.

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Crystalline calcium pyrophosphate dihydrate (CPPD) and basic calcium phosphate (BCP; a term including carbonate-substitute apatite, octacalcium phosphate, and tricalcium phosphate) are two of the most common sources of pathologic articular mineral. Each occurs frequently in degenerative joints, and may be phlogistic, causing acute attacks of pseudogout in the case of CPPD crystals and acute calcific periarthritis in the case of BCP crystals (1, 2).

Calcium-containing crystals such as BCP and CPPD in concentrations found in pathologic human joint fluids, stimulate fibroblast, synovial cell, and chondrocyte mitogenesis in vitro by a process similar to that of platelet-derived growth factor (PDGF) (3). Moreover, BCP crystals and PDGF exert similar biologic effects on cultured cells, such as stimulation of PGE₂ production via the phospholipase A₂/cyclo-oxygenase pathway (4), activation of phospholipase C and inositol phospholipid hydrolysis (5), induction of collagenase and neutral protease synthesis (6–8), and induction of proto-oncogenes (c-fos and c-myc) (9, 10).

Based on clinical and synovial fluid findings and in vitro data (11), a hypothesis has been formulated concerning the pathogenesis of calcium-containing crystal deposition diseases. Synovial lining cells phagocytose crystals in the joint fluid. In the process and/or as a result of endocytosis, synovial cells respond with: 1) protease synthesis and secretion, which in turn releases additional crystals and collagen from the surrounding tissue, 2) PGE₂ production, 3) DNA synthesis as a result of protein kinase C activation and crystal dissolution. Proteases and PGE₂ cause the “degeneration” of the periarticular tissue. DNA synthesis leads to an increase in synovial cells that generate more proteases and PGE₂ (12).

Members of the mitogen-activated protein kinase (MAPK) family are key regulators of a variety of signal transduction cascades that play a central role of mediating cellular responses elicited by many different environmental agents. Three distinct MAPK-dependent signaling cascades have been identified in mammalian cells. These can be distinguished based on the particular MAPK members activated: p42/p44 MAPKs, p38 MAPK, or p46/p54 stress-activated protein kinase/c-Jun N-terminal kinases. p38 MAPK and the stress-activated protein kinase/c-Jun N-terminal kinases mediate signals in response to cytokines and environmental stress. The

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1. The abbreviations used are: CPPD, calcium pyrophosphate dihydrate; MAPK, mitogen-activated protein kinase; P-MAPK, phosphorylated MAPK; SAT, n-sulfo-2-aminotricarballylate; BCP, basic calcium phosphate; PC, phosphocitrate; PDGF, platelet-derived growth factor; SRF, serum response factor; EGF, epidermal growth factor; DMEM, Dulbecco’s modified Eagle’s medium; IL, interleukin; FBS, fetal bovine serum; CREB, cyclic AMP response element-binding protein.
PC Inhibits Crystal-induced MAPK Signal Transduction Pathway

p42/p44 MAPK pathway was the first identified and is the best understood ERK-based signaling pathway (reviewed in Ref. 13). This pathway is required for cell proliferation elicited by growth factors and Ras transformation of cells. The p42/p44 MAPKs are believed to regulate proliferation by a mechanism that involves activation of genes associated with cell proliferation, including primary response genes such as c-fos and c-jun. These kinases can also phosphorylate other kinases, such as the RSK family, thereby regulating their action. In the case of c-fos gene, p42/p44 MAPKs have been shown to activate expression by phosphorylating members of the Elk-1 family of transcription factors (reviewed in Ref. 14). The Elk-1 factors bind to the c-fos serum response element by virtue of their association with serum response factor (SRF), which directly interacts with the serum response element (14).

PC is a naturally occurring compound which has been identified in mammalian mitochondria (15) and crab hepatopancreas (16). It has been speculated that PC may have an important role in preventing calcium phosphate precipitation in cells or cellular compartments maintaining high concentration of calcium and phosphate (15). PC prevents soft tissue calcification in vivo and cellular compartments maintaining high concentration of calcium/phosphate ratio of 1.59 and contained partially carbonate-substituted hydroxyapatite mixed with octacalcium phosphate by FTIR spectroscopy. Crystals were crushed in an agate mortar and sieved to yield 10–20-μm aggregates, which were sterilized and rendered pyrogen-free by heating at 200 °C for 90 min. CPPD crystals were made by methods published earlier (22) and sieved, sterilized, and treated in a similar fashion as BCP crystals.

Human foreskin fibroblast cultures were established from explants and transferred. They were grown and maintained in DMEM supplemented with 10% FBS containing penicillin and streptomycin. All cultures used were third- or fourth-passage cells. Experiments were performed on confluent cell monolayers that had been rendered quiescent by removing the medium, washing with DMEM containing 0.5% FBS, and subsequently incubating in this medium for 24 h. PC, SAT, or citrate was added to the medium 30 min before stimulation at the desired concentration. Cells were stimulated with BCP (50 μM), IL-1β (10 ng/ml), or 20% serum for 15 min except in the time course studies. Cells were lysed with SDS sample buffer, heated to 100 °C for 5 min, and briefly centrifuged. Samples were then stored at −20 °C. To detect activated forms of p44/42 MAPK, antibodies that specifically recognize p44/42 MAPK phosphorylated on Tyr204, or antibodies that specifically recognize Tyr182 on p38 MAPK, were purchased from New England Biolabs and used in immunoblot analyses. Phosphorylation on Tyr204 is diagnostic for activated p44/42 MAPK, and phosphorylation on Tyr182 is diagnostic for activated p38 MAPK. Detection of antibody binding was carried out as described by the manufacturer using the Phototope chemiluminescent detection system (New England Biolabs).

Cells were grown to confluence in culture dishes containing 24 wells, each 16-mm in diameter (Multiwell, Life Technologies, Inc.) and rendered quiescent by incubating in 0.5% FBS for 24 h. Quiescent cells were then stimulated with 10% FBS, BCP crystals, or CPPD crystals in the presence and absence of inhibitors (PC, SAT, and citrate) in DMEM, 0.5% FBS. [3H]Thymidine (1 μCi/ml) was added to the wells 23 h after stimulation and pulsed for 1 h. The cells were washed three times with phosphate-buffered saline, and macromolecules were precipitated with 5% trichloroacetic acid solution. The precipitate was washed again with phosphate-buffered saline and dissolved in 1 ml of 0.1N NaOH, 0.1% sodium dodecyl sulfate. Trichloroacetic acid-precipitable radioactivity was determined in a liquid scintillation counter (Packard Instrument, Downer's Grove, IL). In previous experiments, the optimal concentrations to induce thymidine incorporation in human foreskin fibroblasts were BCP crystals (100 μM) and CPPD crystal (200 μg/ml) (12). Unless otherwise specified, these were the concentrations of BCP and CPPD crystals used in all experiments.

RESULTS

BCP Activates the p42/p44 MAPK Pathway and Can be Specifically Blocked by PC—To elucidate a pathway by which BCP elicits its many biological effects, the effect of these crystals on p42/p44 MAPK activity was examined. By measuring the levels of phosphorylated p42/p44 MAPK, the degree of activation was determined. After 15-min stimulation, serum, IL-1, and BCP induce phosphorylation of p42/p44 MAPK significantly over basal levels (Fig. 2a, lanes 2, 4, and 6). These results show that BCP stimulates p42/p44 MAPK pathway as strongly as serum-L-1 also induced phosphorylation of p42/p44 MAPK (Fig. 2a, lanes 2, 4) which suggests that this pathway is not always specific to mitogenic signals. The presence of 10−3 M PC has no effect on the serum- or IL-1-induced response, but completely blocks the BCP-induced response (Fig. 2a, lanes 3, 5, and 7) implying relative specificity.

Time Course Induction of p42/p44 MAPK by BCP—To clarify the time dependence of p42/p44 MAPK phosphorylation by...
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BCP, a time course study was carried out (Fig. 3). After an initial 5 min delay, the levels of P-p42/p44 MAPK induced by BCP increase quickly and by 15 min reach a maximum level. Surprisingly, IL-1β causes a transient increase in P-p42/p44 MAPK levels that ends 30 min after stimulation. Serum induces a rapid increase in P-p42/p44 MAPK levels that remains sustained throughout the time course.

p38 MAPK Pathway Is Not Activated by BCP—The p38 MAPK pathway, associated with stress and cytokine responses (23), was examined to further characterize cellular responses to BCP. The time course of p38 MAPK phosphorylation was studied. BCP did not significantly induce phosphorylation of p38 MAPK (Fig. 4). IL-1β, on the other hand, causes a transient increase in P-p38 MAPK levels that begins within 5 min of stimulation and ends by 30 min (Fig. 4). Serum does not cause any increase in P-p38 MAPK levels. The IL-1β-induced response is much stronger than the responses elicited by either serum or BCP.

CPPD Preferentially Activates the p42/p44 MAPK Pathway and Can Be Blocked by PC—CPPD was studied to see whether it also acted through the p42/p44 MAPK pathway. A time course study of P-p42/p44 MAPK levels shows that, like BCP, CPPD also induces phosphorylation of p42/p44 MAPK (Fig. 5a, lanes 2–4). Unlike BCP, there is no initial 5-min delay before phosphorylation of p42/p44 MAPK. By 5 min after stimulation, P-p42/p44 MAPK levels reach a maximal level, which remains sustained throughout the time course. As with BCP, incubation with 10−3 M PC completely blocks CPPD induction of p42/p44 MAPK phosphorylation. The PC analogues SAT and citrate have a less appreciable effect on this activation.

The p38 MAPK pathway is not activated by CPPD crystals (Fig. 5b). Even at 30 min after stimulation, P-p38 MAPK levels are not elevated over basal levels. The upper band in Fig. 3b is a cross-reactive band which is not relevant to p38 MAPK activation. Overall, CPPD has a very similar effect to BCP on the p42/p44 and p38 MAPK pathways.

PC, SAT, and Citrate Inhibit BCP-induced p42/p44 MAPK Phosphorylation in a Dose-dependent Manner—To ascertain what concentration of PC is needed to block BCP-induced p42/p44 MAPK phosphorylation, a dose-response study was carried out (Fig. 6). Using PC concentrations ranging from 10−3 to 10−6 M, P-p42/p44 MAPK levels induced by BCP were assayed. The study showed that PC inhibited BCP activation of the p42/p44 MAPK pathway in a dose-dependent manner. While 10−6 M PC did have an appreciable effect on activation, 10−3 M PC was necessary to completely block activation (Fig. 4, lanes 4–7).

A dose-response study of SAT and citrate was also carried out (Fig. 7). While 10−3 M SAT and 10−6 M citrate reduced BCP induction of p42/p44 MAPK, neither was as effective as PC. At lower concentrations, SAT seems to block the response more effectively than citrate. PC, SAT, and citrate, which only differ in the side group attached to their tricarboxylic acid chain, clearly have very different biological effects. PC is much more effective than either of its analogues, SAT and citrate, at blocking BCP-induced p42/p44 MAPK phosphorylation.

BCP and CPPD Crystal Treatment of Cells Results in Phosphorylation of CREB Serine 133—Both BCP and CPPD have been shown to exert their effects at least in part by a mecha-
nism that involves an influx of extracellular calcium.\(^2\) Since the CREB transcription factor has been shown to be an important regulator of gene expression in response to calcium signals, we investigated the activation of CREB by treatment of cells with BCP or CPPD crystals using a phosphoserine 133-specific antibody. Serum, CPPD, and BCP all induced CREB serine 133 phosphorylation (Fig. 8). However, serum and CPPD crystal induced maximal phosphorylation of CREB serine 133 within 15 min, while there appeared to be a delay of 15 min in the BCP crystal induction.

Effects of PC, SAT, and citrate on BCP crystal-induced and IL-1 induced thymidine incorporation are summarized in Fig. 9, a and b, respectively. As reported before, PC (10\(^{-3}\) to 10\(^{-5}\) M) blocked crystal-induced mitogenesis while SAT or citrate had little effect (Fig. 9a). Interleukin-1\(\beta\) did not induce \([\text{H}]\)thymidine incorporation in human foreskin fibroblasts above unstimulated control (Fig. 9b).

Crystal-induced Cell Proliferation Is Mediated by a MAPK-dependent Pathway—The observation that BCP and CPPD can activate p42/p44 MAPK suggested that crystal-induced biological effects may be mediated by a MAPK pathway. To begin to address this issue the effect of a selective inhibitor of the MAP kinase cascade, PD98059, on crystal-induced p42/p44 MAPK activities and \([\text{H}]\)thymidine incorporation was studied. As can be seen in Fig. 10, a and b, PD98059 blocked BCP and CPPD crystal-induced p42/p44 MAPK activities and thymidine incorporation in a dose-dependent fashion. These results indicate that the BCP and CPPD crystal-induced cell activation is mediated at least in part by a MAPK-dependent pathway.

DISCUSSION

In the present study, we have demonstrated that BCP and CPPD crystals differentially activate members of the MAPK signal transduction cascade. Calcium-containing crystals activate the MAPK p42/p44 and not the p38 protein kinase cascade pathway, and induction of p42/p44 MAPKs by BCP crystals occurs with a 5-min delay relative to serum and IL-1\(\beta\) induction. We also have shown that treatment of cells with BCP crystals leads to phosphorylation of CREB Ser\(^{133}\). CREB is a key transcriptional regulator of the c-fos gene that has been shown to be important for mediating c-fos activation in response to elevated levels of intracellular calcium (25) and cAMP (26). Serine 133 is a critical residue whose phosphorylation is necessary for CREB-mediated transactivation. Recently, CREB has also been shown to be important for mediating activation of the c-fos gene by purified growth factors, through a MAPK-dependent pathway (27). Since the p42/p44 MAPK pathway is necessary for cell proliferation our results suggest that calcium containing crystals may mediate their mitogenic effects by activating this pathway. Consistent with this, inhibition of MEK1, an upstream activator of MAPKs, with the inhibitor PD98059, significantly inhibits crystal-mediated cell proliferation. Since p42/p44 MAPKs are differentially activated by BCP and CPPD, relative to p38 MAPK, this observation suggests that p42/p44 MAPKs are important mediators of the crystal response. Our results also suggest that c-fos activation may occur by a mechanism that involves activation of the CREB factor.

The induction of c-fos and c-jun transcription are among the earliest responses to serum and growth factors in culture cells (28, 29). The Fos protein, in combination with the protein products of c-jun, can serve as a trans-acting activator of the expression of other genes such as collagenase and stromelysin (28–31). McDonnell et al. (29) reported that EGF stimulation of stromelysin required induction of c-fos, c-jun, and activation of PKC. Since BCP crystal-induced collagenase and stromelysin synthesis was preceded by a transient increase in the level of intracellular Ca\(^{2+}\), PKC activation, and c-fos, c-myc, c-jun induction (12), it is likely that these same factors are required for crystal-induced metalloproteinase synthesis and that BCP may be mediating its tissue destructive effects as a result of induction of the c-fos gene.

The RAS/p42/p44 MAPK pathway mediates activation of the c-fos gene in response to a variety of mitogenic agents, including serum and purified growth factors such as EGF. Transcriptional activation of the gene is a synergistic process in which multiple c-fos promoter elements are targeted to effect maximal activation. In one case, activated p42/p44 MAPKs directly phosphorylate members of the Elk-1 family of SRF-associated transcription factors, thereby stimulating transcription in an SRF-dependent manner (reviewed in Ref. 14). In addition, p42/p44 MAPK can also activate RSK family members, which can

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\(^2\) P. B. Halverson, A. Greene, and H. S. Cheung, submitted for publication.
than citrate (possessing carboxylates only) with calcium ions distributed on the (−1 0 1) and (0 1 0) surfaces of calcium oxalate monohydrate crystal, thus blocking growth. We speculate that the inhibitory action of PC on the CPPD unit cell lattice, although different from calcium oxalate monohydrate or BCP, would be achieved by a similar inhibitory mechanism. SAT is not as efficient an inhibitor as PC for calcium salts examined so far, which probably reflects its stereochemistry and the fact that both a sulfate moiety (one less charge than PO₄) and the presence of a nitrogen atom reduce its capacity to position itself correctly for crystal face interaction.

Our working hypothesis of the mechanism of action of PC on crystal is that the binding of PC to calcium-containing crystals change the ζ potential of the crystal surface, thus interfering with the crystal-membrane interactions that lead to cellular responses. This would explain why PC does not inhibit the monosodium urate crystal and peptide growth factor induction of mitogenesis and metalloproteinase synthesis (19).

Our earlier studies had shown that PC specifically inhibits CPPD and BCP crystal-induced metalloproteinase synthesis and mitogenesis in human fibroblasts in vitro, while PC has no effect on similar biologic responses induced by EGF, PDGF, and serum (19). PC (10−10 μM) blocked both the BCP and CPPD crystal formation in a dose-dependent fashion in both articular cartilage vesicle and articular cartilage models, while SAT and citrate (1 mM) blocked only BCP crystal formation in similar models (23, 36). PC prevents disease progression in murine progressive ankylosis (an animal model of crystal deposition diseases), a condition marked by extensive BCP deposition (24). Taken together with the present data, PC may be considered a potential therapeutic agent for both CPPD and BCP crystal deposition diseases. Our working hypothesis is that PC will have dual beneficial effects of blocking the degeneration-promoting effects of crystals, e.g. metalloproteinase synthesis and mitogenesis (12), and of inhibiting further BCP and CPPD crystal formation in articular tissue (23).

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