Basic Calcium Phosphate Crystals Activate p44/42 MAPK Signal Transduction Pathway via Protein Kinase C\(\mu\) in Human Fibroblasts*

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Although basic calcium phosphate (BCP) crystals are common in osteoarthritis, the crystal-induced signal transduction pathways in human fibroblasts have not been fully comprehended. We have previously demonstrated that the induction of matrix metalloproteinases (MMP) 1 and 3 by BCP crystals follows both the calcium-dependent protein kinase C (PKC) pathway and the calcium-independent p44/42 mitogen-activated protein kinase (p44/42 MAPK) pathway. Although we showed that the calcium-dependent PKC pathway was characterized by calcium-dependent PKCa, here we show that the calcium-independent p44/42 MAPK pathway is mediated by calcium-independent PKC\(\mu\). Inhibition of PKC\(\mu\) synthesis and activity by antisense oligodeoxynucleotides and H-89, N-(2-[\(\beta\]-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide, respectively, results in the inhibition of p44/42 MAPK activation, thus demonstrating that p44/42 MAPK activity is dependent upon PKC\(\mu\). Reverse transcription-polymerase chain reaction and Western blotting also show that inhibition of PKC\(\mu\) results in the inhibition of MMP-1 and MMP-3 mRNA and protein expression as a result of p44/42 MAPK inhibition. These results now lead us to the conclusion that BCP crystal activation of human fibroblasts follows two pathways: 1) the calcium-dependent PKC pathway characterized by PKCa and 2) the calcium-independent p44/42 MAPK pathway mediated by PKC\(\mu\), which operate independently leading to an increase in mitogenesis and MMP synthesis and ultimately complementing each other for the efficient regulation of cellular responses to BCP crystal stimulation of human fibroblasts.

Basic calcium phosphate (BCP)\(^*\) crystals are among the most common forms of pathologic articular minerals. They frequently occur in the joints of osteoarthritis patients and can be phlogistic (1, 2). There is compelling evidence that these crystals engender multiple biological effects that promote joint degeneration. The presence of BCP crystals correlates strongly with radiographic evidence of cartilaginous degeneration and synovial thickening and is associated with larger joint effusions when compared with joint fluid from osteoarthritis knees where BCP crystals are absent (3, 4). Conversely, osteoarthritis is both more common and more severe in patients with calcium-containing crystals. We have demonstrated that BCP crystals stimulate the proliferation and synthesis of matrix metalloproteinases (MMPs) in cultured human foreskin and synovial fibroblasts (5–9). The addition of BCP crystals to the growth medium yielded an immediate 10-fold rise in the intracellular calcium level and a second rise starting at 60 min and lasting for 3 h. This second rise in the intracellular calcium level is probably because of the intracellular dissolution of phagocytosed crystals, which may activate a variety of calcium-dependent signals and induce sustained cell proliferation and MMP synthesis (10).

To date, BCP crystal-induced signal transduction has not been fully comprehended. We have previously demonstrated that the induction of human MMP-I and MMP-3 expression by BCP crystals, in part, follows the p44/42 mitogen-activated protein kinase (p44/42 MAPK) signal transduction pathway (11, 12). We have also shown that BCP crystal stimulation of MMP-1 and MMP-3 mRNA and protein expression in human fibroblasts are mediated, in part, through the calcium-dependent protein kinase C (PKC) signal transduction pathway and that the PKCa isoform is specifically involved in the pathway (13). We found that these two pathways operate independently since the p44/42 MAPK inhibitors, PD098058 and U0126, had no effect on the BCP crystal activation of p44/42 MAPK (13). However, the possibility that a PKC isoform that was not sensitive to the PKC inhibitors might be required for the BCP crystal activation of p44/42 MAPK signal transduction could not be ruled out.

Therefore, we undertook the current study to identify all of the PKC isoforms involved in the BCP crystal activation of human fibroblasts and to further examine the interrelationship between the PKC and the p44/42 MAPK signal transduction pathways involved in the process. Activation of p44/42 MAPK in response to various agonists can occur via mechanisms that may be PKC-dependent (14, 15) or PKC-independent (16–18).

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‡ The abbreviations used are: BCP, basic calcium phosphate; AP-1, activating protein-1; DAG, 1,2-dioctanoyl-sn-glycerol; DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular signal-related protein kinase; FBS, fetal bovine serum; H-89, N-(2-[\(\beta\]-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; HF, human fibroblasts; MAP, mitogen-activated protein kinase; MEK, MAPK-ERK kinase; MMP, matrix metalloproteinase; PC, phosphocholine; PDBa, phorbol 12,13-dibutyrate; PKC, protein kinase C; RT, reverse transcription; ODN, oligodeoxynucleotide; TPA, 12-O-tetradecanoylphorbol-13-acetate; PH, pleckstrin homology.

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BCP Crystal Induction of Matrix Metalloproteinases

Even in the same cell type, p44/42 MAPK activation can be PKC-dependent or PKC-independent depending upon the stimulus and the corresponding cellular response (19). On the other hand, the PKC family of isozymes consists of three subfamilies: the conventional, the novel, and the atypical isozymes. Whereas the conventional isozymes (α, βI, βII, and γ) require calcium, phospholipid, and 1,2-dioctanoyl-sn-glycerol (DAG) for their activities, the novel isozymes (δ, ε, η, and θ) require neither calcium nor DAG for their maximal activities (20, 21). Human PKCμ and its murine analog, protein kinase D (PKD), form a distinct class (22-24) and are activated by DAG in the absence of calcium (23, 25). Recently, this class has been named the PKD family, which comprises three isoforms: PKCμ/PKD1; PKD2; and PKD3 (26). In contrast to other PKC isozymes, PKCμ has a hydrophobic N terminus, a transmembrane domain, a regulatory C1 domain with an extended spacing of two cysteine clusters, and an additional 270 amino acid sequence that separates the protein kinase domain from the regulatory C1 domain, resulting in an unusually large isozyme with an apparent molecular mass of 115 kDa (22, 25). In addition, PKCμ contains a pleckstrin homology (PH) domain that may exert an inhibitory effect on its kinase activity and also lacks the C2 domain that is responsible for the calcium sensitivity of the conventional PKC subgroup, thereby making it calcium-unresponsive (22). Because of the lack of a pseudosubstrate sequence present in the other PKC family members, PKCμ also displays a distinct inhibitor and substrate specificity (27, 28).

In our previous work, only the PKC isozymes in the conventional subfamily were examined (13). In this work, we extended our study to include other PKC isozymes in the novel, atypical, and PKD subfamilies to determine their expression and potential involvement in the BCP crystal-induced activation of the p44/42 MAPK signal transduction pathway in the human fibroblasts (HF). We also further examined the relationship between the BCP crystal-induced PKC and p44/42 MAPK signal transduction pathways.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM), phospho–buffered saline, fetal bovine serum (FBS), penicillin, streptomycin, fungizone, protein A-agarose, ThermoScript RT-PCR system, and TRIZol reagent were obtained from Invitrogen. Phorbol 12,13-dibutyrate (PDBu), 1,4-phosphatidyl-t-serine, DAG, N-[2-(lg-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), leupeptin, and aprotinin were obtained from Sigma. U0126 and monoclonal antibodies against MMP-1 and MMP-3 were from Calbiochem. Concentrated serum-free medium containing MMP-1 and MMP-3 control proteins was from Chemicon International Inc. (Temecula, CA). Monoclonal phospho-p44/p42 MAPK antibody and polyclonal p44/p42 MAPK antibody were from Cell Signaling Technology, Inc. (Beverly, MA). Monoclonal antibodies against PKC α, βI, βII, γ, δ, ε, η, and ι were from Pan Vera (Madison, WI). Monoclonal antibody against PKCα, polyclonal antibodies against PKCβ, PKCs, and PKCμ, rat brain extract, rat thymus, NIH 3T3, and K-562 whole cell lysates were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-mouse IgG-horseradish peroxidase conjugate and Anti-rabbit IgG-horseradish peroxidase conjugate were from Promega (Madison, WI). SuperSignal WestPico chemiluminescent substrate was from Pierce.

Cell Culture—HF cells were established from explants and transfected as described previously (29). They were grown and maintained in DMEM supplemented with 10% heat-inactivated FBS containing 1% penicillin, streptomycin, and fungizone. All of the cultures were third or fourth passage cells. All of the experiments were performed on confluent monolayers that had been rendered quiescent by removing the medium, washing the cells with DMEM alone, and subsequently incubating the cells in the same medium containing 0.2% FBS for 24 h. This medium was then removed, the cells were washed with phosphate-buffered saline, and serum-free DMEM was added to the cells and allowed to equilibrate before being used for the experiments. For the inhibition experiments, the cells were pretreated with the appropriate concentration of the inhibitors for 30 min, unless otherwise stated, before being stimulated with the required concentrations of the agonists and for the indicated length of time.

Synthesis of BCP Crystals—BCP crystals were synthesized by a modification of previously published methods (30). These crystals have a calcium/phosphate ratio of 1.59 and contain partially carbonated hydroxyapatite mixed with octacalcium phosphate, as indicated by Fourier transform infrared spectroscopy. The crystals were crushed and sieved to yield 10-20-μm aggregates, which were sterilized and rendered pyrogen-free by heating at 200 °C for at least 90 min.

RT-PCR—Total RNA was isolated using the TRIzol reagent according to the manufacturer’s instructions. 1 μg of each sample was reverse-transcribed at 60 °C for 60 min followed by enzyme inactivation at 85 °C for 5 min using the ThermoScript RT-PCR system. PCR primers for MMP-1, MMP-3, and β-actin were designed and synthesized as described previously (13). PCR primers for PKCμ were as follows: sense, 5'-GAGATATTCCTCCTGAGTATTCA-3', corresponding to positions 1968-1981, and antisense, 5'-ACACATTCTC- GGTTGAGGTCAC-3', corresponding to positions 2349-2372 of the nucleotide sequence of the human PKCμ and giving a PCR product of 465 bp (22). Amplifications were carried out as previously described (13) with the exception that MMP-1 was done at 25 cycles while PKCμ and MMP-3 were done at 30 cycles. The PCR products were analyzed by electrophoresis on 2% agarose gel containing ethidium bromide.

Cell Lysis—After the experiment, the cells were washed with cold phosphate-buffered saline and the plates were drained. 1 ml of cold lysis buffer (50 mM Tris, pH 7.6, 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin, 10 μg/ml aprotinin, and 1% Triton X-100) then was added to each 100-mm plate and placed on ice with occasional shaking. The cells were then scraped into 1.5 ml Microfuge tubes and centrifuged at 10,000 × g for 10 min at 4 °C. The supernatants were transferred to fresh tubes, and the protein concentrations were determined with the Bio-Rad protein assay reagent (Hercules, CA) according to the manufacturer’s instructions. The lysates were either used immediately or stored at -70 °C.

Immunoprecipitation—Following an experiment, the cells were lysed and aliquots of the lysates were incubated with the appropriate antibodies on ice for 4 h to allow formation of the immune complexes. Protein A-agarose gel was added to each mixture and gently rotated overnight at 4 °C to allow the immune complexes to bind by adsorption of the antibody to protein A. The unbound proteins were removed by washing the solid phase several times with the lysate buffer by centrifugation, leaving the immune complexes bound to the agarose gel. The purified immune complexes were ready for further analysis by gel electrophoresis and immunoblotting after adding an equal volume of 2× SDS sample buffer and boiling for 5 min, or the immune complexes were used for enzyme activity studies.

Expression of PKC Isozymes in HF—HF cells were grown on 100-mm plates to confluency in DMEM containing 10% FBS and then washed with phenol red-free DMEM containing 0.2% FBS and starved in the same medium for 24 h. This medium was then replaced with serum-free and phenol red-free DMEM and allowed to equilibrate in that medium before the cells were treated with or without BCP crystals for 15 min. After removing the medium and washing with phosphate-buffered saline, the cells were lysed and total proteins were extracted using the mammalian protein extraction reagent or M-PER obtained from Pierce according to the manufacturer’s instructions. Protein concentrations in the extracts were determined as described before. Aliquots of the extracts with equal protein concentrations were electrophoresed on a 10% SDS-polyacrylamide gel and subjected to Western blotting.

Western Blotting—Aliquots of cell lysates were electrophoresed through a 10% (P) or 12% (p44/p42 MAPK) SDS-polyacrylamide gel and then transferred onto Immobilon-P polyvinylidene fluoride membranes (Millipore, Bedford, MA). After the transfer, the membranes were incubated for 4 h at room temperature in the blocking buffer TBS (20 mM Tris, 136 mM NaCl, 0.1% and Tween 20) containing 5% nonfat dry milk to eliminate nonspecific binding. The membranes were washed several times and then incubated in TBS containing 5% bovine serum albumin at 4 °C overnight with the following antibodies: a monoclonal antibody against MMP-1 (Chemicon, Temecula, CA); a polyclonal MAPK antibody recognizing p44/p42 MAPK phosphorylated at Tyr-204 and Thr-202; or a polyclonal p44/p42 MAPK antibody or a polyclonal antibody against each of the PKC isozymes (α, βI, βII, γ, δ, ε, η, ι, ε, and μ). The membranes were washed again several times with TBS.
and incubated with the appropriate anti-mouse or anti-rabbit heredidish peroxidase-conjugated secondary antibody in TBST with 5% bovine serum albumin for 30 min at room temperature. Finally, the membranes were washed in TBST and Tris-buffered saline, and the protein bands were detected with the Supersignal West Pico Chemiluminescent substrate according to the manufacturer’s instructions and exposed to Kodak X-OMAT AR film.

**Down-regulation of PKC with TPA**— Cultured HF cells in 100-mm plates were starved and pretreated with or without TPA (1 μM) for 24 h to down-regulate PKC. They were treated with or without BCP crystals (50 μg/ml) for 15 min. The cells were also treated with PDBu (200 nm) and DAG (1 mM), both of which were used as positive controls for the BCP crystal stimulation of PKC. After cell lysis, aliquots of the lysates with equal protein concentrations were electrophoresed through a 10% (for PKC) or 12% (for phospho-p44/42) SDS-polyacrylamide gel, subjected to Western blotting, and probed with antibodies against PKCα, PKCβ, and phospho-p44/42.

**Treatment of HF Cells with Sense and Antisense Oligodeoxynucleotides**—An 18-base-long phosphorothioated antisense oligodeoxynucleotide (ODN) against PKCβ mRNA and its corresponding sense ODN were synthesized by Sigma. The antisense sequence for PKCβ was 5'-GACGGAGGGCAGCTCAT-3', based on the start codon (ATG) plus the 15 additional downstream bases in the human PKCβ sequence (22, 31). The corresponding sense sequence, 5'-ATGGCTGACGTTTACCCG-3', was used as the control. As previously reported (31), semi-constitutive HF cells in 100-mm plates were treated with or without 20 μM ODN, 20 μM Lipofectin for 6 h at 37°C. The cells then were washed, and the medium was replaced with fresh medium containing 2 mM glutamine and 10% heat-inactivated FBS in the presence or absence of 20 μM ODN and without Lipofectin at 37°C for 72 h. After these steps, the cells were treated with or without BCP crystals (50 μg/ml) for 15 min (for PKCα and phospho-p44/42) and 24 h (for MMP-1 and MMP-3). The lysates then were assessed for immunoreactive PKCα, phospho-p44/42, MMP-1, and MMP-3 protein levels by Western blotting and for PKCα, MMP-1, and MMP-3 mRNA levels by RT-PCR.

**Inhibition of BCP Crystal-induced PKCβ and Phospho-p44/42 Activation**—HF cells were cultured, starved, and preincubated with or without PKCβ inhibitor H-89 (100 μM) and phospho-p44/42 inhibitor U0126 (10 μM) for 30 min before the addition of BCP crystals (50 μg/ml) and PDBu (200 nm), which was used as a positive control for PKCβ activation. Cell lysates were immunoprecipitated with anti-PKCβ, and the immune complex was used to determine PKCβ activity by autophosphorylation assay.

**Autophosphorylation of PKCα**—After cell lysis and immunoprecipitation with anti-PKCα, the immune complexes were washed several times with the lysis buffer and then with the phosphorylation buffer (20 mM Tris, pH 7.4, 5 mM magnesium acetate, 2 mM sodium orthovanadate, 10 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Autophosphorylation was started by adding 30 μl of the phosphorylation buffer containing 0.3 μl of [γ-32P]ATP (5000 Ci/mmol and 10 mCi/ml) and α-phosphatidyl-d-serine (100 μg/ml) to the immunocomplexes and incubating at 37°C for 20 min (23, 25, 32). The reactions were terminated by adding an equal volume of 2× SDS sample buffer to each one. The proteins were released by boiling for 5 min, resolved by electrophoresis on 10% polyacrylamide gels and transferred to polyvinylidene fluoride membranes. The 32P-labeled proteins were visualized after a 4-hour exposure to autoradiography.

**p44/42 MAPK Activation**—Following experimental treatments, cell lysis, and immunoprecipitations, aliquots of the cell lysates or immunoprecipitates were subjected to 12% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose blotting with a monoclonal antibody specific for the activated and phosphorlated p44/42 MAPK (phospho-p44/42) and a polyclonal antibody specific for the constitutive and nonactivated p44/42.

**Statistics**—Statistical analysis was performed with the Student’s t test in Sigma Plot scientific graphing software, and p < 0.05 was considered significant. Data were expressed as the means ± S.E.

**RESULTS**

**Expression of PKC Isozymes in HF**—Because the expression and interactions of the PKC isozymes are cell type-specific (33), we sought to determine the specific PKC isozymes expressed in HF upon BCP crystal (50 μg/ml) stimulation. By Western blotting, we show in Fig. 1 that, of all the conventional isozymes (A–D), only the α isozyme (A) is expressed as previously reported (13). None of the novel (E–H) or atypical (I–J) isozymes is expressed. However, of the PKD family, PKCδ is highly expressed (K). These results indicate that both PKCα and PKCβ may have specific and distinct functions in the HF. The concentration of BCP crystals (50 μg/ml) used in these studies is consistent with our previously established optimal range of 50–100 μg/ml in vitro and is consistent with the in vivo concentration in articular joint fluids isolated from osteoarthritic patients, which range from 10 to 20 μg/ml, depending on the severity of the disease (34).

**Role of PKC in the BCP Crystal Stimulation of Phospho-p44/42**— Down-regulation of PKC is often used to determine its role in MAPK activation. Pretreatment of α-T3-1 cells with TPA had been shown to down-regulate PKCα and PKCε with no effects on ERK1 (p44) (35). To determine the roles of PKCα and PKCβ in the BCP crystal activation of phospho-p44/42 in HF, the cells were pretreated with TPA (1 μM) for 24 h to down-regulate the PKCs before stimulation with BCP crystals (50 μg/ml). Fig. 2 shows that pretreatment with TPA (+) completely depleted PKCα (A) but affected neither the expression of PKCβ (B) nor the stimulation of phospho-p44/42 by BCP, PDBu, and DAG (C) in lanes 5–8 compared with their respective controls that were not pretreated with TPA (−) in lanes 1–4. These results were further proof of our previous work that the PKCα and the p44–42 MAPK signal transduction pathways in response to BCP crystal stimulation in HF are independent of each other (13). However, the results do not exclude the fact that PKCα and phospho-p44/42 may share a common pathway in which the stimulation of phospho-p44/42 may be dependent upon the expression and/or activation of PKCβ.

**BCP Crystal Stimulation of Phospho-p44/42 Is Dependent upon PKCβ**—Since it was suggested in Fig. 2 that PKCα and phospho-p44/42 might share a common signal transduction pathway, the ideas that the two pathways were indeed related and/ or one pathway was dependent upon the other were tested by treating the BCP crystal and PDBu-stimulated cells with the inhibitors of the two pathways in Fig. 3. The PKCβ inhibitor used, H-89, is a competitive ATP antagonist previously regarded as a highly specific inhibitor of protein kinase A (36). However, it had been reported that PKCβ had an ~60-fold...
higher sensitivity to H-89 than the other PKCs and that it required 60 μM H-89 to inhibit PKCμ autophosphorylation in A549 carcinoma cell line (25, 27). Other studies had also shown by in vitro kinase assay that the lack of kinase activity shown by the transfection of COS-7 cells with a kinase-defective PKD mutant was not the result of PKD degradation as seen by Western blotting analysis of the PKD levels of the wild type expression vector and the kinase-defective PKD mutant (37, 38). In our preliminary studies (results not shown), treatment of HF cells with 60 μM H-89 affected neither the PKCμ kinase activity as assayed by autophosphorylation assay nor the expression level by Western blotting. However, treatment of BCP crystal and PDBu-stimulated cells with 100 μM H-89 inhibited not only the autophosphorylation (A) and expression (B) of PKCμ but also the stimulation of phospho-p44/42 (C), suggesting that the H-89 concentration needed to inhibit PKCμ kinase activity may be cell type-dependent. On the other hand, treatment of the BCP crystal and PDBu-stimulated cells with the phospho-p44/42 inhibitor, U0126 (10 μM), inhibited only phospho-p44/42 (C) and had no effect on the autophosphorylation and expression of PKCμ (A and B, respectively). To the contrary, the constitutively expressed or nonactivated p44/42 was seen with no changes in all of the samples (D). These results demonstrate that BCP crystal stimulation of phospho-p44/42 is dependent upon the expression and activity of PKCμ.

A more specific evidence for the dependence of BCP crystal stimulation of phospho-p44/42 upon PKCμ is provided by inhibiting the synthesis and expression of PKCμ with PKCμ antisense ODN to test whether such inhibition would affect the stimulation of phospho-p44/42 by BCP crystals in HF. Oligodeoxynucleotides are designed to hybridize to specific mRNA sequences (39) and have been used to inhibit the expression of a number of viral and mammalian genes in vitro (40–43). To inhibit PKCμ synthesis and expression, we used PKCμ-specific antisense phosphorothioated oligodeoxynucleotides in which one of the nonbridging oxygens in the backbone of the DNA is replaced by sulfur (44). As shown in Fig. 4A, treatment of HF cells with PKCμ antisense ODN completely inhibited the synthesis and expression of the 115-kDa PKCμ both in the absence (−) and presence (+) of BCP crystals, whereas treatment with the sense ODN showed no difference in the synthesis and expression of PKCμ from the control group. Fig. 4B shows that treatment of the cells with PKCμ antisense ODN also inhibited the stimulation of phospho-p44/42, whereas treatment with the sense ODN did not inhibit stimulation in comparison with the control group. These results provide specific and conclusive evidence that the stimulation of phospho-p44/42 by BCP crystals in HF depends upon the synthesis and expression of PKCμ.

**Inhibition of MMP-1 and MMP-3 mRNA and Protein Expression**—We had previously shown that BCP crystal induction of MMP-1 and MMP-3 is dependent on p44/42 MAPK because the specific inhibitor of p44, PD98059, at 100 μM and the specific inhibitor of p44/42, U0126, at 10 μM also inhibited both MMP-1 and MMP-3 mRNA and protein expression (12). We have now shown here that the BCP crystal induction of p44/42 MAPK is dependent on PKCμ, because the specific inhibition of PKCμ also results in the inhibition of p44/42 MAPK activation (Figs. 3 and 4). Therefore, it is probable that the inhibition of PKCμ would ultimately result in the inhibition of both MMP-1 and MMP-3 expression because of the inactivation of p44/42 MAPK. To test this probability, we again used PKCμ antisense ODN to inhibit the BCP crystal-induced PKCμ to show whether MMP-1 and MMP-3 expression would also be inhibited. The densitometric scan of the relative intensities (mean ± S.E.) of the RT-PCR results in Fig. 5 shows significant inhibition (p < 0.05) of the mRNA expression of PKCμ (A), MMP-1 (B), and MMP-3 (C) by PKCμ antisense ODN over the control and PKCμ sense ODN-treated samples both in the absence (−) and presence (+) of BCP crystals. These results show that the inhibition of PKCμ results in the inhibition of p44/42 MAPK (Fig. 4), which in turn results in the inhibition of both MMP-1
and MMP-3 mRNA as previously reported (12, 13). The corresponding expression of the housekeeping gene using β-actin primers did not show any changes between the samples in Fig. 5D. These results were also confirmed by Western blotting for MMP-1 and MMP-3 protein expression in Fig. 6, A and B, respectively.

**DISCUSSION**

In their repertoire of signal transduction mechanisms, almost all of the cell surface receptors utilize one or more of the mitogen-activated kinase pathways to coordinate appropriate responses to extracellular signals (45). One pathway activated upon crystal stimulation of human fibroblasts is the p44/42 MAPK signal transduction pathway also known as ERK1 and ERK2, respectively. We have previously shown that BCP crystal induction of MMP-1 and MMP-3 is dependent upon the p44/42 MAPK pathway (12) and the calcium-dependent PKCα pathway (13). It was proposed that these two pathways initially function independently, ultimately leading to an increase in mitogenesis and MMP synthesis, and that they may converge downstream of PKC and p44/42 MAPK to mediate BCP crystal-induced cellular responses. At that time, only the calcium-dependent PKC isozymes were examined; therefore, only the calcium-dependent PKCα isozyme was identified as a participant in the PKC pathway, but the possibility that another PKC isozyme, which was not sensitive to the calcium-dependent PKC inhibitors, might be required for the BCP crystal-induced activation of the p44/42 signal transduction pathway could not be ruled out (13). We demonstrate here that, in addition to the calcium-dependent PKCα (Fig. 1A) of the conventional subfamily, a calcium-independent PKCμ (Fig. 1K) of the PKD subfamily is also expressed in human fibroblasts and that the PKCμ is required for the activation of the p44/42 MAPK pathway (Figs. 3 and 4).

One of the objectives of this study was to determine the PKC isozymes expressed in HF. Evaluation of the PKC isozymes from all of the PKC subfamilies shows that only PKCα and PKCμ are expressed in HF as shown in Fig. 1, A and K, respectively. The fact that PKCα is calcium-dependent and PKCμ is calcium-independent and that they belong to two different PKC subfamilies suggests differential roles for these isozymes in the BCP crystal activation of HF. We had previously shown that BCP crystal-induced activation of p44/42 MAPK was independent of PKC, because the PKC inhibitors, bisindoylmaleimide I and Go6976, had no effects on p44/42 MAPK and, conversely, the p44/42 MAPK inhibitors, PD098058 and U0126, had no effects on PKC (13). At that time, only the calcium-dependent PKCα was identified. We now have also identified PKCμ, which is calcium-independent (Fig. 1K), and have shown that the BCP crystal activation of p44/42MAPK is dependent upon PKCμ (Figs. 3 and 4) and that, when p44/42 MAPK is inactivated by PKCμ inhibition, MMP-1 and MMP-3 mRNA and protein expressions are also inhibited (Figs. 5 and 6). The identification of PKCμ and the dependence of p44/42MAPK on PKCμ help to explain why, in our previous work, the inhibitors of PKCα did not affect p44/42MAPK and vice versa (13). In our present model in Fig. 7, BCP crystals modulate PKCα and PKCμ through the sequential hydrolysis of phospholipase C and phosphatidylinositol 1,4,5-bisphosphate, producing DAG, which independently activates PKCα and PKCμ that have different sensitivities to the inhibitors and different substrate specificities (27, 28). The PKC inhibitors are specific for the calcium-dependent PKCα, which has a 10-fold higher sensitivity to bisindoyl- maleimide I and Go6976 than PKCμ. Whereas 2 μM Go6976 completely inhibited BCP crystal-induced PKCα (13), it required 20 μM Go6976 and 300 μM Go6983 to suppress the
phorbol ester-enhanced autophosphorylation of PKC\(\mu\) (27). Therefore, at the concentrations of inhibitors that inhibit PKC\(\alpha\), PKC\(\mu\) is not inhibited and is still able to activate p44/42 MAPK, which is translocated to the nucleus to trans-mit extracellular stimuli by phosphorylating several trans-cription factors (46). This is in agreement with a previously published work in which PKC\(\mu\) selectively activated the MAPK p42 pathway (47).

We have demonstrated that a calcium-dependent PKC\(\alpha\) and a calcium-independent PKC\(\mu\) are the only two PKC isozymes expressed in HF in response to BCP crystal activation. We have also provided evidence that the BCP crystal-induced activation of p44/42 MAPK is dependent upon PKC\(\mu\), because inhibition of expression and activity of PKC\(\mu\) greatly attenuates the BCP crystal induction of p44/42 MAPK. In addition to showing an association of PKC\(\mu\) with p44/42 MAPK, we have also shown that both the activation of p44/42 MAPK and its translocation to the nucleus are coordinated by PKC\(\mu\). These new facts have now led us to the modified model shown in Fig. 7 and to the hypothesis that BCP crystal activation of HF follows two independent pathways. One pathway is the calcium-dependent PKC pathway characterized by PKC\(\alpha\) and modulated by mobilized intracellular Ca\(^{2+}\) generated by the sequential hydrolysis of phospholipase C-phosphatidylinositol 1,4,5-bisphosphate-phosphatidylinositol 1,4,5-triphosphate (48–50) by transient opening of the Ca\(^{2+}\) channel and by crystal endocytosis and dissolution (10). The mobilized Ca\(^{2+}\) in the cytosol then modifies the activation of PKC\(\alpha\) by DAG and induces its translocation to the plasma membrane where it becomes physiologically active (51). Some of the mobilized Ca\(^{2+}\) diffuses through the nuclear pores into the nucleus (52, 53) where it enhances the BCP crystal induction of c-fos mRNA (10). PKC\(\mu\) can also be redistributed from the cytosol to the nucleus as recently noted in NIH 3T3 fibroblasts (54). It has also been shown recently that PKC\(\alpha\) isozymes can either act in the cytoplasm and cause nuclear effects indirectly by triggering signaling pathways directed toward the cell nucleus or PKC itself can act in the cell nucleus (55). The other pathway is the calcium-independent p44/42 MAPK pathway, which is mediated by the calcium-independent PKC\(\mu\). BCP crystals activate the sequential hydrolysis of phospholipase C-phosphatidylinositol 1,4,5-bisphosphate, producing DAG, which activates PKC\(\mu\) (which in turn undergoes autophosphorylation) as a potential measure of activation (56, 57) and activates p44/42 MAPK generated from the Ras-Raf-MEK-p44/42 MAPK signaling cascade (58). The activated and phosphorylated p44/42 MAPK then migrates to the nucleus to mediate BCP crystal-induced cellular responses.

Therefore, we conclude that, although these two pathways operate independently, they seem to complement each other for the efficient regulation of cellular responses to BCP crystal stimulation of human fibroblasts.

BCP crystal activation of both the PKC and the p44/42 MAPK signal transduction pathways ultimately leads to the synthesis and secretion of the MMPs, which are responsible for cartilage degeneration in osteoarthritis (1). Because these two pathways are mediated by PKC\(\alpha\) and PKC\(\mu\), respectively, it remains to be seen what happens to the MMPs when these activities of PKC isozymes are increased by trans-}
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