Molecular Mechanism of the Induction of Metalloproteinases 1 and 3 in Human Fibroblasts by Basic Calcium Phosphate Crystals

ROLE OF CALCIUM-DEPENDENT PROTEIN KINASE Ca*

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Synovial fluid basic calcium phosphate (BCP) crystals are common in osteoarthritis and are often associated with destructive arthropathies involving cartilage degeneration. These crystals are mitogenic and induce oncogene expression and matrix metalloproteinase (MMP) synthesis and secretion in human fibroblasts. To date, BCP crystal-elicited signal transduction pathways have not been completely studied. Because protein kinase C (PKC) is known to play an important role in signal transduction, we investigated the participation of this pathway in the BCP crystal induction of MMP-1 and MMP-3 mRNA and protein expressions in human fibroblasts. Using reverse transcription/polymerase chain reaction (RT-PCR) and Northern and Western blotting techniques, we show here that BCP crystal stimulation of MMP-1 and MMP-3 mRNA and protein expressions in human fibroblasts is dependent upon the calcium-dependent PKC signal transduction pathway and that the PKCa isozyme is specifically involved in the pathway. We have previously shown that BCP crystal induction of MMP-1 and MMP-3 is also dependent on the p44/42 mitogen-activated protein kinase (p44/42 MAPK) signal transduction pathway. We now show that these two pathways operate independently and seem to complement each other. This leads to our hypothesis that the two pathways initially function independently, ultimately leading to an increase in mitogenesis and MMP synthesis, and may converge downstream of PKC and p44/42 MAPK to mediate BCP crystal-induced cellular responses.

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† The abbreviations used are: BCP, basic calcium phosphate; AP-1, activating protein-1; BAPTA-AM, 1,2-bis(O-aminophenoxy)ethane-N,N',N″,N‴-tetraacetate acid-acetoxymethyl ester; Bis, bisindolylmaleimide; CPPD, calcium pyrophosphate dihydrate; DAG, diacylglycerol; DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular signal-related protein kinase, FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HF, human fibroblasts; HBSS, Hanks’ balanced salt solution; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; PC, phosphocriptate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PVDF, polyvinylidene fluoride; RT, reverse transcription; TBS, Tris-buffered saline; TMB-8, 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxy benzoate; HCl; PI-PLC, phosphatidylinositol-specific phospholipase C.

Another messenger with an apparent role in crystal-activated signal transduction is calcium. We have previously shown that treatment of HF with BCP crystals induces a rapid transient rise of intracellular calcium levels in seconds due to calcium influx from outside the cell, followed by a slow and sustained increase of intracellular calcium within 60 min after stimulation, due to crystal dissolution (18). Removal of calcium from the cell culture medium attenuates the BCP crystal-induced responses.
duction of c-fos mRNA (18), suggesting that an influx of extracellular calcium is required for maximal induction of c-fos expression. Perhaps related to the rise of intracellular calcium is the crystal activation of adenosine 3',5'-cyclic monophosphate (cAMP) response element (CRE)-binding protein (CREB) (15), 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 (19).

Treatment of cells with BCP crystals also results in the activation of phospholipase C, leading to the hydrolysis of phosphatidylinositol 4,5-biphosphate and production of the intracellular messengers, inositol triphosphate and diacylglycerol (DAG) (5, 20). Inositol triphosphate modulates the activities of calcium-dependent enzymes such as protein kinases by releasing calcium from the endoplasmic reticulum (21) whereas diacylglycerol is a potent activator of protein kinase C (PKC) (22). In humans, the PKC family consists of at least 11 structurally related serine/threonine protein kinases. These isozymes are further divided into three subfamilies: the conventional, the atypical, and the novel isozymes. The conventional isozymes include alpha (α), beta I (βI), beta II (βII), and gamma (γ), and their activities are calcium- and phospholipid-dependent. The novel isozymes comprise delta (δ), epsilon (ε), eta (η), and theta (θ), whose activities are calcium-independent but phospholipid-dependent. The atypical isozymes are made up of zeta (ζ), iota (ι), and mu (μ), and their activities are neither calcium- nor phospholipid-dependent (23, 24).

We have previously shown that crystal treatment of HF results in the translocation of the PKC enzyme from the cytosolic to the membrane fraction of the cell, an indicator of PKC activation. The BCP crystal-induced PKC activity is blocked by co-treatment of crystal-stimulated cells with the PKC inhibitor, staurosporine and bisindolylmaleimide I (25). Furthermore, an increase in PKC activity associated with the membrane fraction is seen following BCP crystal stimulation of chondrocytes (26). Down-regulation of PKC activity by chronic treatment with the phorbol ester, 12-O-tetradecanoyl-phorbol 13-acetate, an analog of DAG, blocks crystal-induced c-fos and c-myc expressions and mitogenesis in Balb/c 3T3 cells (7) whereas co-treatment with the PKC inhibitor, staurosporine, blocks BCP-induced c-fos expression and mitogenesis in HF (25), indicating that PKC activity is essential for these crystal-induced effects to occur.

In this study, we investigated the participation of the PKC signal transduction pathway in the BCP crystal induction of MMP-1 and MMP-3 mRNA and proteins in HF. Because the PKC family comprises several isozymes, we further sought to identify the specific isozyme of the PKC family that is translocated to the putative membrane fraction as an indication of PKC activity in the human fibroblasts. We also examined the requirement of calcium signaling in the crystal activation of PKC in HF as well as 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), Hanks’ balanced salt solution (HBSS), phosphate-buffered saline (PBS), fetal bovine serum (FBS), penicillin, streptomycin, fungizone, ThermoScript RT-PCR System, PCR primers, and TRIzol reagent were obtained from Invitrogen, Gaithersburg, MD. MMP-1-specific probe (a 2.02-kb HindIII/SmaI insert from the pCllase 1 clone), MMP-3-specific probe (a 1.7-kb EcoRI insert from the pTRI plasmid), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific probe (a mouse 0.8-kb HindIII insert from the pBS-GAPDH plasmid) were obtained from American Type Culture Collection, Rockville, MD. Concentrated medium containing MMP-1- and MMP-3-positive control proteins was from Chemicon International Inc, Temecula, CA. Bisindolylmaleimide, 8-(N,N-diethy lamino)-octyl-3,4,5-trimethoxy benzoate, HCl (TMB-8), staurosporine, 1,2-bis(O-aminophenox yethane-N,N,N’,N’‘,tetraacetic acid-acetoxyethyl ester (BAPTA-AM), PD98059, U0126, Go6976, monoclonal MMP-1 antibody, monoclonal MMP-3 antibody, and monoclonal PKC antibody were from Calbiochem, La Jolla, CA. Monoclonal Phospho p44/42 MAPK antibody and polyclonal p44/42 MAPK antibody were from New England Biolabs Inc. Rabbit polyclonal antibodies against the PKCα, βI-βII, and γ isozymes were from Panvera, Madison, WI. Anti-mouse IgG horseradish peroxidase conjugate was from Promega, Madison, WI. EDTA, EGTA, 3,3'-diaminobenzidine, levpeptin, and aprotinin were from Sigma Chemical Co., St. Louis, MO.

**Cell Culture**—HF were established from explants and transferred as previously described (27). The cells were grown and maintained in DMEM supplemented with 10% heat-inactivated FBS containing 1% penicillin, streptomycin, and fungizone. All cultures were third or fourth passage cells. All 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 0.5% FBS for 24 h (MMPs and PKC) or for 48 h (p44/42 MAPK). Then this medium was removed, the cells were washed with PBS, and serum-free DMEM was added to the cells. For the inhibition experiments, the cells were pretreated with the appropriate concentrations of the inhibitors for 30 min before being stimulated with BCP crystals or PMA for the indicated length of time.

**BCP Crystals and PC Preparations**—BCP crystals were synthesized by modification of previously published methods (28). These crystals were isolated from New England Biolabs Inc. and rendered pyrogen-free by heating at 200°C for at least 90 min. FC was prepared as previously described (29).

**RT and PCR**—Total RNA was isolated using the reagent TRIzol according to the manufacturer’s instructions. Then 1 µg of each sample was reverse-transcribed at 50°C for 60 min, followed by enzyme inactivation at 85°C for 5 min using the ThermoScript RT-PCR system. The resulting cDNA samples were amplified by the PCR method. PCR primers for MMP-1 were: sense, 5'-GATCATCGGGAGCAACTCTC'T-3', corresponding to positions 567–587, and antisense, 5'-TCCGGTTGA- GAAAGGATTGTG-3', corresponding to positions 980–1000 of the published nucleotide sequence of the human skin collagenase cDNA and giving a PCR product of 434 bp (30). The primers for MMP-3 were: sense, 5' -GAAAGGTTCCGGAAGAGGTGACCTCCAC'-3', corresponding to positions 414–440, and antisense, 5'-CATGTTCGGTCAAGAGAAAGAGACCC-3', corresponding to positions 671–697 of the nucleotide and amino acid sequence for human MMP-3 and giving a PCR product of 284 bp (31). As an internal control, 353 bp of the constitutively expressed housekeeping gene, β-actin, was also synthesized and used to normalize the amount of mRNA in each RT-PCR reaction. All primers were synthesized by Invitrogen (Gaithersburg, MD). Amplifications were carried out for 30 cycles by denaturing at 95°C for 30 s, annealing at 55°C for 30 s, and extending at 72°C for 45 s, with a final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis on 2% agarose gel containing ethidium bromide.

**Northern Blotting**—Total RNA samples (10 µg each) were denatured and electrophoresed through a 1.2% agarose gel containing 2.2% formaldehyde followed by transfer and cross-linking with a UV Stratalinker 1800 (Stratagene, La Jolla, CA) to Nytran Supercharge nylon membrane (Schleicher & Schuell, Inc., Keene, NH). The membranes were prehybridized at 42°C for 4 h and then hybridized at 42°C overnight to 100000 cpm each of the following: mouse MMP-1 and MMP-3-3-specific cDNA probes that were radiolabeled with [α-32P]dATP (6000 Ci/mmol, Amersham Biosciences, Inc., Piscataway, NJ). The blots were subsequently stripped and reprobed with GAPDH cDNA as a control. After washing, the hybridized membranes were exposed to Kodak X-OMAT-AR films with intensifying screen at –80°C.

**Western Blotting**—Aliquots of conditioned media (MMPs), cell lysates (p44/42 MAPK), and membrane fractions (PKC) were electrophoresed through a 10% (MMPs) or 7.5% (PKC) or 12% (p44/42 MAPK) SDS-polyacrylamide gel and then transferred onto Immobilon-P PVDF membranes (Millipore, Billerica, MA). After transfer, the membranes were incubated for 4 h at room temperature in the blocking buffer, TBST (20 mM Tris, 136 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk to eliminate nonspecific binding. The membranes were washed several times and then incubated in TBST containing 5% bovine serum albumin at 4°C overnight with the following antibodies: a monoclonal antibody against MMP-1 or MMP-3, a monoclonal antibody against PKC, a phospho-specific monoclonal PKC antibody recognizing p44/42 MAPK phosphorylated at Tyr-204 and Thr-202 or a polyclonal
p44/42 MAPK antibody or a polyclonal antibody against each of the PKC isozymes, α, βII, βI, and γ. The membranes were again washed several times with TBST and incubated with the appropriate antimouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody in TBST with 5% bovine serum albumin for 1 h at room temperature. Finally, the membranes were washed in TBST and TBS, and the protein bands were visualized colorimetrically with a solution containing 3,3-diaminobenzidine (25 mg/100 ml) and hydrogen peroxide in 0.05 m Tris-HCl, pH 7.5.

PKC Translocation—After treatment, the cells were washed twice with cold PBS. The cells were then harvested on ice in 1.5 ml of translocation buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.33 mM sucrose). The cells were sonicated on ice for 15 s and then centrifuged at 100,000 × g for 45 min. The supernatant was collected as the cytosolic fraction. The pellet was then dissolved in 0.5 ml of translocation buffer containing 0.1% Triton X-100, shaken at 4 °C overnight, and then centrifuged again at 100,000 × g for 45 min. The supernatant was used as the membrane fraction. Samples (25 μl) each of the fractions were subjected to 7.5% SDS-polyacrylamide gel electrophoresis and Western blotting with a p44/42 MAPK monoclonal antibody or p44/42 polyclonal antibody.

Statistics—Statistical analysis was performed by the Student t test in SigmaPlot Scientific Graphing software, and p < 0.05 was considered significant. Data were expressed as the means ± S.E.

RESULTS

Participation of a Protein Kinase Pathway in the BCP Crystal-induced MMP-1 and MMP-3 Expression—Treatment of cultured human fibroblasts with calcium-containing gels increases the expression levels of MMP-1 and MMP-3. To determine whether a protein kinase signaling is necessary for the BCP crystal-induced expression of these MMPs, we examined the effects of staurosporine, a potent, cell-permeable and broad spectrum inhibitor of protein kinases on the BCP crystal-induced MMP mRNA and protein levels by reverse transcription (RT)-PCR and Northern and Western blots. The RT-PCR with MMP-1- and MMP-3-specific primers show that, after 24 h of stimulation of HF with BCP crystals, levels of MMP-1 and MMP-3 mRNA increased approximately 4-fold over the control levels as shown in Fig. 1, A and B, respectively. The inhibition of the MMP-1 and MMP-3 mRNA by staurosporine is concentration-dependent, with the greatest inhibition at 100 μM, which is similar to the inhibition by 1 mM of PC, a well known inhibitor of the biological effects of BCP crystals (17) and suggests the participation of a protein kinase signaling pathway. The corresponding expression of the housekeeping gene, using β-actin primers, did not show any change in Fig. 1C. The densitometric scan of the relative intensities (means ± S.E.) of three such independent experiments showed a significant inhibition of the BCP crystal-induced MMP-1 and MMP-3 by staurosporine at 100 μM (p < 0.05) (data not shown).

Northern blotting of the RNA samples in Fig. 2 shows no degradation of the RNA at all concentrations of staurosporine in Fig. 2A. Fig. 2, B and C, shows that, at 100 μM staurosporine, the complete inhibition of MMP-1 and MMP-3 mRNA, respectively, is similar to the inhibition by 1 μM of PC whereas panel D shows no change in the housekeeping gene, GAPDH.

These results were confirmed with Western blotting of the culture medium in Fig. 3. Here, there is also a concentration-dependent inhibition of the BCP crystal-induced MMP-1- and MMP-3-secreted proteins in Fig. 3, A and B, with the greatest inhibition again at 100 μM staurosporine and with the molecular mass of the proteins corresponding to MMP-1 and MMP-3 control standard at 53–55 kDa and MMP-3 control standard at 57–59 kDa. All the results suggest the participation of a protein kinase pathway.

Identification of Protein Kinase C as the Signaling Pathway—Our aim was to determine whether PKC was involved in the BCP crystal activation of MMP-1 and MMP-3 expression in human fibroblasts. Using bisindolylmaleimide I (Bis I), a highly selective, cell-permeable PKC inhibitor that is structurally similar to staurosporine (33), we have shown that, by Northern blotting, that there is a concentration-dependent inhibition of MMP-1 and MMP-3 mRNA by Bis I in Fig. 4, A and B, respectively, and that, at 10 μM, the inhibition is similar to the inhibition by 1 mM PC. To determine the specificity of the inhibition, we also used bisindolylmaleimide V (Bis V), which is a structural analog of Bis I and a negative control inhibitor for PKC (34) and which shows no inhibition even at the same 10 μM concentration as Bis I. We also used PMA as a positive control for PKC stimulation. The samples were normalized with GAPDH as the housekeeping gene (Fig. 4C). These results were confirmed by Western blotting for the MMP-1 and MMP-3 protein expressions in Fig. 5, A and B, respectively. The results identify the PKC signaling pathway as a participant in the BCP crystal induction of MMP-1 and MMP-3 in HF.

Requirement for Calcium in the PKC Signaling Pathway—To identify the particular subfamily of PKC that participates in the signaling pathway upon the BCP crystal induction of MMP-1 and MMP-3 mRNA, we used the indolocarbazole
MMP-1 and MMP-3 mRNA expression in the Northern blotting

Simultaneous treatment of the cells with BCP crystals and G6976 led to a concentration-dependent inhibition of MMP-1 and MMP-3 mRNA expression in the Northern blotting results in Fig. 6, A and B, respectively and with the maximum inhibition at 25 mM G6976 similar to the inhibition by PC at 1 mM. PMA was used as a positive control for the PKC activity, and the samples were again normalized with the housekeeping gene, GAPDH (Fig. 6C). These results were also confirmed by Western blotting for the MMP-1 and MMP-3 protein expressions in Fig. 7, A and B. The results show convincingly that the calcium-dependent PKC subfamily is required for the BCP crystal induction of MMP-1 and MMP-3 mRNA and protein expressions in human fibroblasts.

Further evidence for the involvement of a calcium-dependent PKC signaling pathway is provided by determining PKC activity in the absence and presence of calcium. Because PKC is known to be physiologically active only in the membrane-associated state and translocation of the PKC enzyme from the cytosol to the membrane of the cell is used to monitor its intracellular activation (25,26), we determined PKC activity in the membrane fractions of the cells in calcium- and magnesium-free HBSS and compared it with the activity in HBSS containing calcium and magnesium. Using G6976 as the specific inhibitor of the calcium-dependent PKC and PMA as the positive control for the PKC activity, we have shown that there was no PKC activity in the absence of calcium and magnesium as seen in Fig. 8A. On the other hand, BCP crystal induction resulted in increased PKC activity in the presence of calcium and magnesium, similar to that of PMA as the positive control,
and is totally inhibited by 2 μM Gö6976 (Fig. 8B). However, this concentration is different from the concentration of 25 nM that completely inhibited the MMPs in Figs. 6 and 7. Our dose-dependent study (data not shown) found 2 μM to be the concentration of Gö6976 that would inhibit PKC in HF. This is in agreement with a previous study, which found that 2 μM Gö6976 was not toxic to NALM-6 cells (36). Taken together, all these results confirm the necessity for the calcium-dependent PKC in the signaling pathway for the BCP crystal induction of MMP-1 and MMP-3.

Identification of the Specific PKC Isozyme—The only calcium-dependent subfamily of PKC is the conventional subfamily, which is a pool of isozymes consisting of alpha (α), beta I (βI), beta II (βII), and gamma (γ) (23, 24). To evaluate the extent and specificity of the PKC activation induced by BCP crystals, we also sought to identify the specific isozyme/isozymes involved in the induction. As seen in Fig. 9, blotting of the membrane fractions with the total pool PKC antibody and with the antibodies to the individual isozymes showed an induction of PKC in the total pool by BCP crystals (panel A) and a more specific induction of PKCα isozyme in panel B, whereas there was no induction at all of the βI, βII, and γ isozymes in panels C, D, and E, respectively. The specificity of the PKCα isozyme was confirmed with the use of the Gö6976, which is a specific inhibitor of the calcium-dependent PKC α and β isozymes (35). Complete inhibition was seen in the PKC in the total pool as well as in the PKCα, thus unequivocally identifying the α isozyme of the calcium-dependent PKC as being activated by BCP crystals.

Cooperativity of PKC with PKC-independent MAPK—We have previously shown that treatment of human fibroblasts with calcium-containing crystals activate the p44/42 MAPK signal transduction pathway (15) and recently reported that this pathway is required for maximal induction of MMP-1 and MMP-3 mRNA and proteins by BCP crystals (16). Here, we were interested in determining whether p44/42 MARK induction by BCP crystals is PKC-dependent and whether the two pathways are coupled in HF. Treatment of the cells with BCP crystals resulted in an increased level of Phospho p44/42 MAPK activation shown in Fig. 10. When the same concentrations of the protein kinase inhibitor, staurosporine, which inhibited BCP crystal-induced MMP-1 and MMP-3 mRNA and proteins shown in Figs. 2 and 3, respectively, were used with the BCP crystal-treated cells, there were no changes in all the samples in Fig. 10. These results demonstrate that BCP crystal-induced Phospho p44/42 could be inhibited, 1 μM PC was used as a control inhibitor, which resulted in a marked inhibition of the BCP crystal-induced Phospho p44/42. To the contrary, the constitutively expressed or nonactivated p44/42 was seen with no changes in all the samples in Fig. 10B. These results demonstrate that the BCP crystal activation of the p44/42 signal transduction pathway is independent of the PKC pathway.

Further evidence for the two independent pathways is provided by treatment of the BCP crystal-stimulated cells with inhibitors of the two different pathways in Fig. 11. Treatment of the BCP crystal-stimulated cells with the PKC inhibitors, Bis I and Gö6976, inhibited only PKC whereas treatment with the...
three independent experiments. Membrane (fractions were used to determine the levels of PKC translocation to the membrane (A) whereas the cell lysates were used to determine the levels of activated Phospho p44/42 (B) and the constitutively expressed p44/42 (C) by Western blotting. Blots shown are representatives of three independent experiments.

Phospho p44/42 inhibitors, PD98059 and U0126, did not inhibit PKC at all as shown in Fig. 11A. Conversely, the PKC inhibitors did not inhibit phospho p44/42, which was only inhibited by its own inhibitors, PD98059 and U0126, as shown in Fig. 11B, thus indicating that the two pathways are independent of each other. On the other hand, the constitutively expressed or nonactivated p44/42 was not affected by any of the inhibitors in Fig. 11C.

**BCP Crystal-activated p44/42 MAPK Pathway Is Calcium-independent**—We have shown in Fig. 8 that BCP crystal induction of MMP-1 and MMP-3 requires the calcium-dependent PKC signaling pathway. Similarly, we wanted to know if the p44/42 MAPK pathway was also calcium-dependent. Cellular calcium requirement can be met by either an influx of extracellular calcium from the culture medium into the cells (18) or by the stimulation of a phosphatidylinositol-specific phospholipase C (PI-PLC), leading to the generation of inositol triphosphate and diacylglycerol (DAG) (20), which are involved in intracellular calcium mobilization (21) and PKC activation (22), respectively. Although extracellular calcium chelation by EGTA and intracellular calcium chelation by BAPTA-AM and TMB blocked the BCP crystal-induced PKC in Fig. 12A, they had no effect on Phospho p44/42 (Fig. 12B). These results show that neither calcium influx nor calcium release is necessary for the BCP crystal-mediated activation of the p44/42 MAPK pathway, thus establishing that this pathway is distinct from the calcium-dependent PKC pathway through which BCP crystals activate MMP-1 and MMP-3 in the human fibroblasts.

**DISCUSSION**

The ultimate biological effects of calcium-containing crystals on cells in vitro are an increase in MMP synthesis and secretion and increased mitogenesis. These effects are hypothesized to be correlated with calcium deposition disease in vivo. The increased production of matrix-degrading MMPs by synoviocytes results in articular damage and degeneration and the release of additional crystals from the surrounding tissue, whereas mitogenesis leads to an increase in synoviocytes that generate more MMPs (37). Of interest are the signal transduction mechanisms by which crystal-induced up-regulation of MMP synthesis and secretion and increased mitogenesis are mediated. Here, we demonstrate for the first time that the calcium-de-
ulation of HF results in a rapid transient increase in intracellular calcium levels due to an influx of extracellular calcium from the culture medium into the cells (18). To determine whether BCP crystal activation of PKC in HF also requires an influx of extracellular calcium, we determined PKC activity in a culture medium with and without calcium. Our results show conclusively that, in the absence of any calcium influx from the culture medium into the cells, there is no PKC activity, contrary to the PKC activity in the medium containing calcium as seen in Fig. 8. Further proof of this phenomenon is provided in Fig. 12A in which the chelation of both extracellular calcium with EGTA and intracellular calcium with BAPTA-AM and TMB results in no BCP crystal activation of PKC.

Because there are several PKC subfamilies, each with a number of different isoforms that can be calcium-dependent or calcium-independent (23, 24), we then sought to identify the specific PKC isoform that is activated upon BCP crystal stimulation. To this end, we used Go6976, a methyl- and cyanoalkyl-substituted nonglycosidic indolocarbazole, which selectively inhibits the calcium-dependent PKC α and β isoforms (35). In our results in Fig. 9, we have identified PKCβ as the only isoform that is activated upon BCP crystal stimulation and inhibited by Go6976 in HF. Such selective inhibition of an overactivated PKC isoform may provide a potential target for the design of pharmacological drugs and thereby offer a unique therapeutic prospect. As seen in Fig. 12B, chelation of extracellular calcium influx with EGTA and intracellular calcium release with BAPTA-AM and TMB, had no effect on BCP crystal activation of p44/42 MAPK by BCP crystals occurs via a PKC-independent pathway. However, these results cannot rule out the possibility that a PKC isoform that is not sensitive to staurosporine may be required for the BCP crystal activation of the p44/42 MAPK signal transduction pathway.

BCP crystal activation of HF likely involves an interplay or “cross-talking” among several second messengers and signal transduction pathways. Our present results and previous work (18) indicate that calcium plays an important role in BCP crystal activation of HF. Because PKC does not appear to be required for the BCP crystal activation of p44/42 MAPK, the prospect arises that calcium may be a necessary factor in the activation. Results of our investigation into the role of calcium in BCP crystal activation of p44/42 MAPK argue against this prospect. As seen in Fig. 12B, chelation of extracellular calcium influx with EGTA and intracellular calcium release with BAPTA-AM and TMB, had no effect on BCP crystal activation of p44/42 MAPK (Phospho p44), showing that neither external calcium influx nor internal calcium release is required for the activation of this signal transduction pathway by BCP crystals in HF. Similar studies have previously shown p44/42 activation to be independent of PKC, extracellular calcium, and intracellular calcium (43, 45). Other studies have also shown p44/42 MAPK activation to be independent of PKC and extracellular calcium but dependent upon intracellular calcium levels (42, 46).

**Fig. 13.** Proposed model of BCP crystal-induced signal transduction in human fibroblasts. The p44/42 MAPK and PKC signal transduction pathways activated upon BCP crystal stimulation initially function independently, ultimately leading to an increase in mitogenesis and MMP synthesis. The pathways may converge downstream of p44/42 MAPK and PKC to mediate BCP crystal-induced cellular responses. A question mark (?) indicates that this component of the signal transduction pathway is currently unknown.
We have hypothesized that BCP crystal induction of MMP synthesis involves the up-regulation of activating protein-1 (AP-1) DNA binding activity (16, 17, 25). AP-1, a dimeric transcription factor typically composed of the protein products of c-fos and c-jun, recognizes a consensus DNA binding sequence present in the promoters of various AP-1-responsive genes, including MMP-1 and MMP-3 (47). Indeed, we have previously demonstrated that BCP crystal stimulation of HF results in the up-regulation of both c-fos and c-jun mRNA and in the activation of nuclear AP-1 DNA binding activity (17, 18, 25). The signal transduction pathways involved in the transcriptional regulation of c-fos itself may have differential requirements for calcium, p44/42 MAPK, and PKC, depending upon the cell type and the stimulus being assessed. Our laboratory has shown that BCP crystal up-regulation of c-fos mRNA expression in HF occurs via a PKC-dependent mechanism, because co-treatment of BCP crystal-stimulated cells with staurosporine greatly attenuated c-fos mRNA expression (25). Our previous work has also demonstrated that removal of calcium from the cell culture medium results in the reduction of BCP crystal-induced c-fos mRNA expression, indicating that an influx of extracellular calcium is required for maximal c-fos induction (18). These results are similar to the work of others showing PKC and extracellular calcium requirements for c-fos induction (48, 49).

The up-regulation of c-fos and c-jun mRNA expression induced by BCP crystals is also blocked by PC, a specific inhibitor of BCP crystal-mediated biological effects (17). PC may have an important protective role in preventing calcium phosphate precipitation in cells or cellular compartments maintaining high concentrations of calcium and phosphate. We have demonstrated that PC interferes with many biological effects of calcium-containing crystals. Crystal-induced MMP synthesis and mitogenesis (17) and p44/42 MAPK activation (15) in HF are specifically inhibited by PC, although it has no effect on similar processes induced by growth factors or serum. Additionally, PC prevents BCP crystal deposition and disease progression in murine progressive ankylosis, an animal model of BCP crystal-induced c-jun and c-fos induction (48, 49). These facts and our present observations, therefore, lead us to the proposed model shown in Fig. 13 and to the hypothesis that the PKC and p44/42 MAPK signal transduction pathways, activated by BCP crystals in HF, initially function independently, ultimately leading to an increase in mitogenesis and MMP synthesis, and may converge downstream of PKC and p44/42 MAPK to mediate BCP crystal-induced cellular responses.

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