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Basic Calcium Phosphate Crystals Activate *c-fos* Expression Through a Ras/ERK Dependent Signaling Mechanism

Michael L. Major, Herman S. Cheung⁺, and Ravi P. Misra^{*}

Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI 53226

⁺ *Geriatric Research Education and Clinical Center, VA Medical Center and Department of Medicine, University of Miami School of Medicine, Miami, FL 33101*

Abstract

Diseases caused by calcium pyrophosphate dihydrate (CPPD) and basic calcium phosphate (BCP) crystals occur frequently in osteoarthritic joints. Both crystals induce mitogenesis, metalloproteinase synthesis and secretion by fibroblasts and chondrocytes, promoting degradation of articular tissue. We investigated the mechanism by which BCP activates the *c-fos* proto-oncogene, which has been shown to activate various *matrix* metalloproteinases (*MMPs*). We demonstrate that BCP crystals induce *c-fos* expression primarily through a Ras/ERK dependent signaling mechanism targeting two highly conserved regulatory binding sites, the serum response element (SRE) and the cAMP response element (CRE). These results establish a calcium crystal induced, calcium/Calmodulin independent, signaling pathway in which BCP crystals activate Ras/MAPK, which can directly target an SRF-containing transcription factor complex, to induce fibroblasts to secrete metalloproteinases.

OA in humans can result from various factors including genetic disposition, joint trauma or joint disuse. A potentially important aspect of articular cartilage and other articular tissue is their tendency to form calcium-containing crystals. Crystalline CPPD and BCP are the two commonest forms of pathologic articular mineral. Each occurs frequently in osteoarthritic joints, and each may be phlogistic, causing acute attacks [1,2].

There is compelling in vitro evidence that these crystals engender multiple biological effects promoting joint degeneration, and clinical observations support a relationship to OA[2]. Both crystals stimulate fibroblast, synoviocyte and chondrocyte mitogenesis in vitro[2], stimulate the production of prostaglandin (PGE₂) via the phospholipase A₂/cyclo-oxygenase pathway (ref in [2], activate phospholipase C and inositol phospholipid hydrolysis ref in [2], induce the expression of proto-oncogenes, *c-fos* and *c-myc*, ref in[2], induce the synthesis and secretion of MMPs [2–4] and down-regulate tissue Inhibitors of metalloproteinase [3].

The collagenase-1 (MMP1) promoter can be directly regulated by the AP-1 transcriptional activator [5], and AP-1 binding sites are found in all three human collagenase genes implicated in calcium-mediated tissue destruction [6–8]. AP-1 consists of a heterodimer of Fos and Jun proteins. Crystal-mediated stimulation dramatically induces Fos leading to collagenase expression suggesting calcium-containing crystals may activate expression of matrix degrading enzymes by directly regulating expression of the *c-fos* gene [9].

^{*}To whom correspondence should be addressed: Tel. # 414-456-8433, Fax # 414-456-6510, Internet: rmisra@mcw.edu

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The *c-fos* gene is regulated by two major elements in the promoter region, a 22 bp region of imperfect dyad symmetry which lies approximately 300 base pairs upstream from the start site of transcriptional initiation, termed the serum response element (SRE) (reviewed in [10]); and another centered 60 bp from the start site of initiation termed the cyclic AMP response element (CRE), also termed the calcium response element (CaRE) [11]. The SRE is sufficient to mediate activation of *c-fos* by nearly all agents that have been shown to activate the gene, except cAMP. The major SRE binding protein is the serum response factor (SRF) [12]. p21^{Ras} Ras-dependent signaling pathways target a promoter complex consisting of SRF and members of the Elk-1 subfamily of ETS oncoprotein transcription factors termed Ternary Complex Factors (TCF). TCFs bind the SRE in association with SRF (reviewed in [13]), and become transcriptionally permissive upon phosphorylation by various Mitogen-activated Protein Kinase (MAPK) family members including ERKs, whose activation is indirectly regulated by p21^{Ras}. In neuronal systems where calcium is mobilized by neurotransmitters or membrane depolarization agents a calcium-mediated Ras/Elk independent pathway, which likely involves Ca²⁺/calmodulin kinase family members, operates through SRF to activate *c-fos* transcription [14,15,16]. The -60 CaRE/CRE is sufficient to mediate activation of the *c-fos* gene in response to elevation of intracellular cAMP or calcium levels [11] through the cAMP response element binding (CREB) protein, which activates transcription upon inducible phosphorylation of Ser-133. CREB Ser-133 phosphorylation can also be stimulated by a growth factor activated Ras/MAPK pathway [17], thus linking SRE and CaRE/CRE mediated activation.

In murine and human fibroblasts BCP crystals activate the ERK subfamily of MAPKs, indicating that ERKs are central regulators of BCP signaling to the nucleus [18,19]. Here we show that a Ras/ERK pathway is primarily responsible for activating the *c-fos* gene through an SRE/CRE dependent mechanism. Together with the results from studies by Sun et al. that show a Ras/MAPK/AP-1 mechanism activates the hMMP1 gene in response to BCP treatment of fibroblast-like synoviocytes [5], and Brogley et al. [19] that show an ERK-dependent mechanism activates hMMP-1 and stromelysin, our results define a mechanism of signaling that underlies the pathogenesis of calcium crystal deposition diseases.

Material and Methods

Cell Culture

NIH3T3 cells were maintained in 10% calf serum/DMEM (Dulbeccos Modified Eagle Media) (Corning) at 37° C/5% CO₂.

Transfection/Stimulation

NIH3T3 cells were seeded 24 hours prior to transfection, and stimulated by addition of a sonicated solution of 100µg/ml BCP/DMEM for 30 minutes for message accumulation studies, and 15 minutes for phosphorylation studies. See Supplement for additional details.

DNA constructs

Constructs pFos711, pFos711ΔCRE, pFos711ΔSRE, pFos711ΔCREΔSRE, pFosΔTCF, and pFosΔCREΔTCF were described in [20]. Dominant/negative constructs DN-RasN17, DN-Rac1 N19, and DN-RhoA N19 are described in [21]. HA-ERK2 was a gift of A. Ganesan and J. Barbieri, and consists of the coding region of ERK fused to an N-terminal hemagglutinin (HA) epitope tag.

Inhibitor studies

Inhibitors U0126 (Promega), KN62 (Calbiochem), and Calmidazolium Chloride (Calbiochem) were added concentrations of 1.0, 10.0, and 25.0 μ M for 30 minutes, as described in Figure legends.

RNase Protection Assay

RNase protections were as previously described [22]. See Supplement for details.

Immune Complex Kinase Assay

For Dominant/Negative experiments cells were transfected with 100ng HA-ERK2 and either 250, 750, or 1500 ng of DN-RasN17, DN-Rac1N19, or DN-RhoAN19. See supplement for additional details.

Detection of Phosphorylated Signaling Intermediates

Cells were made quiescent in 0.5% serum for 30–36 hours at 37°C, 5% CO₂. Media was removed, cells were washed with PBS, and DMEM containing PD98059, KN62, or calmidazolium chloride was added to each dish and incubated 30 minutes prior to stimulation. See supplement for additional details.

Results

BCP activates the *c-fos* proto-oncogene by targeting the SRE and CRE

To determine the role of the SRE and CRE in BCP mediated activation of the *c-fos* gene we used reporters that encoded either the wild type human *c-fos* reporter (pFOS-711myc) or mutant derivatives of pFOS-711myc, depicted in Figure 1. The SRE or the CRE were mutated either singly or in combination such that their ability to bind their cognate transcription factors was abrogated. SRE mutant constructs consist of either point mutations that abolish SRF and p62^{TCF} binding (Δ SRE), or point mutations that abolish p62^{TCF}/SRF complex formation but leave SRF binding intact (Δ TCF) [20]. Since the transfected mutant *c-fos* reporters corresponded to the human *c-fos* gene, using a radioactively labeled probe perfectly complementary to the human gene but imperfectly matched to the mouse gene allowed us to use RNase protection assays to distinguish between transcripts generated from the exogenous constructs and the endogenous murine gene. An example of this assay using wild type, SRE mutant, CRE mutant, or double mutant *c-fos* promoters is shown in Figure 1.

Mutations in any of the three cis acting elements significantly diminishes responsiveness compared to the wild type promoter, and maximum inhibition occurs when the CRE and either the p62^{TCF} binding site or SRE are mutated (Figure 1). The p62^{TCF} and SRE mutations inhibit the response to virtually identical levels, and mutations in the SRE abolish p62^{TCF} binding indicating that the CRE and TCF site mediate virtually the entire BCP *c-fos* response. Furthermore, it can be seen that the sum of the activation of the TCF (or SRE) and CRE single mutants is significantly less than the total activation of the wild type constructs. In the absence of the other, the TCF and CRE sites together only account for approximately 60% of the wild type response. This suggests that the CRE and TCF sites functionally cooperate to mediate the full BCP response.

BCP activates *c-fos* through a p21^{Ras}-dependent signaling pathway

We previously showed that BCP can activate ERKs [18,19]. Since members of the Ras family are major upstream regulators of ERK activation we investigated the role of Ras family members in mediating BCP-dependent *c-fos* activation using dominant negative (DN) forms of Ras, Rac1 and RhoA that act by competitively inhibiting the endogenous GTP-binding

proteins for their respective GEFs [23]. NIH3T3 fibroblasts were transfected with wild-type *c-fos* reporter plasmid pFOS-711myc together with increasing amounts of plasmid expressing dominant negative forms of p21^{Ras} (DN-RasN17), Rac1 (DN-Rac1N19), or RhoA (DN-RhoN19). Figure 2 shows all three constructs diminish the response to BCP. DN-RasN17 shows a dose response with increasing concentrations of plasmid and blocks BCP-mediated activation greater than 90% at 750 ng of construct, and nearly 99% inhibition at 1500 ng. In contrast DN-Rac1N19 and DN-RhoAN19 only show between 40% and 50% inhibition at 750 ng. At higher concentrations both DN-Rac1N19 and DN-RhoA N19 constructs cause extensive cell death (data not shown). These results indicate that BCP activates *c-fos* expression primarily by a pathway that is regulated by Ras family members, with p21^{Ras} being the most important regulator, with some contribution from Rho-related pathways Rac and RhoA.

BCP activates ERKs through a Ras/MEK1/2 dependent pathway

Since BCP preferentially activates the ERKs in NIH3T3 cells [18,19], to determine whether activation of ERKs is regulated by a Ras dependent pathway we investigated whether DN-Ras family members could block ERK activation using an HA epitope tagged ERK co-transfected with increasing amounts of DN-Ras family members. Immunocomplex kinase assays using a myelin basic protein derived peptide as a substrate showed in Figure 2 that DN-RasN17 significantly blocks ERK activation. whereas, DN-Rac1N19 and DN-RhoAN19 fail to significantly block ERK activation.

Major upstream activators of ERKs in response to a wide variety of stimuli are MEK1/2. To determine whether MEK1/2 are involved in BCP activation we used a highly specific inhibitor for MEK1/2, U0126 [24]. Cells were transfected with HA-ERK and then preincubated with increasing concentrations of U0126 inhibitor, and stimulated with BCP. As seen in Figure 2 ERK activity was inhibited in a dose dependent manner, which was specific since activation of another MAPK signaling kinase p38 by serum was not affected at these concentrations (not shown). Similar results were seen with another MEK inhibitor PD90859 (not shown). Together results presented in Figure 2 indicate BCP activates ERK through a p21^{Ras}/MEK pathway.

BCP activates *c-fos* through an ERK-dependent pathway

To determine the role of ERKs in activating BCP mediated gene expression, we looked at whether MEK1/2 inhibitors U0126 and PD90859 inhibit endogenous *c-fos* gene activation. Cells were pretreated with increasing concentrations of either U0126 or PD90859. Figure 2 shows both U0126 and PD90859 drastically inhibit *c-fos* activation in a dose-dependent manner. In contrast, SB203580, a specific inhibitor for p38 MAPK, shows no effect when used at a concentration that effectively inhibits p38 MAPK [25]. These results indicate that *c-fos* gene activation occurs primarily through a MEK1/2-ERK-mediated signaling pathway. Approximately 10% activation of *c-fos* occurs in the presence of inhibitor, suggesting a non-ERK pathway may also partially contribute to the BCP response.

Calmodulin inhibitors fail to block BCP-dependent signaling

BCP dependent activation of cells and of the *c-fos* gene occurs in a Ca²⁺-dependent manner, suggesting calcium activated signaling molecules may contribute Ras activation. We investigated the role of Ca²⁺/calmodulin dependent BCP activation of ERK and *c-fos*. Figure 3 shows BCP stimulated cells treated with either vehicle control or increasing concentrations of either calmidazolium or KN-62, a Ca²⁺/calmodulin kinase II inhibitor, activates ERK to a similar extent.

Previously, we have shown that BCP is capable of inducing phosphorylation of the CREB transcription factor on a critical activating serine residue, serine-133, and Figure 1 indicates CREB is a critical activator required for the full transcriptional response of the *c-fos* promoter

to BCP. To investigate whether BCP activates a CREB through a Ca^{2+} /calmodulin dependent mechanism we determined whether calmodulin inhibitors could block BCP-mediated activation of CREB. Figure 3 shows both calmidazolium and KN-62 fail to appreciably inhibit CREB serine 133 phosphorylation. To determine whether calmodulin may be involved in BCP-mediated activation by other mechanisms we directly measured BCP-mediated activation of the endogenous *c-fos* gene in the presence of calmidazolium or KN-62. Figure 3 shows that increasing concentrations of calmidazolium or KN-62 have no effect on BCP dependent activation.

Together, the above results indicate that BCP-mediated activation of *c-fos* gene expression proceeds by a pathway that does not operate through a calmodulin dependent mechanism.

Discussion

Various studies reveal that numerous signaling intermediates are activated when cells are treated with calcium crystals including PL-C, PKC α and μ [26,27], Ras small molecular weight GTPases [5], and the ERK1 and ERK2 members of the mitogen activated protein kinase (MAPK) family [18]. Our experiments indicate activation ERK1/ERK2 mostly occurs through a p21^{ras} -dependent pathway. Activation of the ERKs does not appear to occur through a calmodulin-dependent mechanism since inhibitors of calmodulin and calmodulin/dependent kinases do not affect BCP mediated activation of ERKs in mouse 3T3 fibroblasts .

Phospholipase C can also be induced in synovial fibroblasts by BCP. Mitchell et al. ref. in [2] have shown that in mouse fibroblasts that when protein kinase C is down regulated by TPA mitogenesis is inhibited. Inhibition of PK-C also attenuates BCP dependent *c-fos* activation and blocks BCP-mediated metalloproteinase induction [28]. These observations, together with the observation that calcium dependent isoforms of PKC are involved in activation MMP-1 [2] indicate that BCP signals to the nucleus by a PL-C/PKC-C mediated signaling mechanism. However, the role of PK-C in MAPK dependent BCP-mediated activation of *c-fos* is unclear. Similarly the relationship between Ras activation and PK-C mediated activation is unclear. Experiments from Brogley and Cheung indicate that inhibition of PK-C does not block MAPK activation by BCP [2]. These observations suggest that PK-C and MAPK pathways independently converge downstream of MAPK to target *c-fos* activation (Figure 4). Consistent with this idea PKC translocation from the cytoplasm to the nucleus is also stimulated by BCP [2].

McCarthy et al. ref in[2] investigated the role of tyrosine kinases in BCP mediated activation of human fibroblasts. Using anti-pTyr antibodies they did not detect induction of tyrosine phosphorylation. In contrast, we see that in mouse 3T3 fibroblasts BCP treatment induces pTyr on a FAK tyrosine kinase related-epitope (supplementary data). Phosphorylation occurs in a rapid and robust manner. Since FAKs have been shown to be involved in activating Ras-mediated signaling pathways [29] this suggests that BCP may activate Ras/ERK signaling through this pathway, although the mechanism by which this occurs, and whether this is a primary pathway for BCP mediated activation remains to be investigated.

The nuclear response to calcium crystals has not been well studied. Some studies show BCP targets the CREB transcription factor [18] as well as NF- κ B. The mechanism by which BCP activates NF- κ B has not been characterized, although it appears that PKC is not involved. In the case of CREB, BCP rapidly induces phosphorylation on serine-133, an activating event for CREB mediated gene expression [18]. We have shown in the present study BCP targets both CREB as well as the SRF/TCF complex, for maximal activation of *c-fos* gene expression. We also show that BCP-mediated activation of the *c-fos* gene is primarily regulated by activation

of the RAS/ERK/SRF/TCF signaling pathway, and that BCP signals require the TCF component of the SRF/TCF complex.

Our results suggest a model for BCP-dependent cell stimulation, Figure 4, in which activation of the *c-fos* gene is a triggering nuclear event for downstream pathological responses that include activation of matrix degrading metalloproteinases. It is likely that calcium-containing crystals may activate expression of matrix degrading enzymes that are regulated by AP-1 sites, by directly regulating expression of the *c-fos* gene. In this scenario an early transient rise in $[Ca^{2+}]_i$ upon crystal treatment would lead to RAS/MAPK dependent activation of the *c-fos* gene leading to activation of metalloproteinases. Strong support for this model comes from studies carried out by Sun et al. who showed that in BCP treated fibroblasts activation of the MMP-1 gene promoter is RAS dependent and mediated by AP-1 binding sites [5].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Mapping BCP Response Elements

i- Promoter mutants used in this study. The Δ CRE mutant promoter replaced the -60 CRE with a Gal4 DNA binding site, the Δ SRE mutant promoter replaced the wild type SRE with a MCM1 yeast DNA binding site, and the Δ TCF mutant replaced the *c-fos* TCF binding site flanking the SRE with a LexA half site. **ii-** Multiple promoter regulatory elements regulate BCP responsiveness. **(A)** Representative RNase protection analysis. Transfected NIH 3T3 fibroblasts were serum-starved and stimulated with BCP, total RNA isolated, and RNase protection assays performed. The positions of the protected RNA species are indicated (transfected *c-fos*^H = 251 nt., globin = 133 nt., endogenous *c-fos*^M = 65 nt.). The film purposely overexposed to show the weak bands generated from the mutant reporters. The levels of expression of the reporters were normalized for transfection efficiency against the level of expression of a co-transfected constitutively expressed β -globin gene. **(B)** BCP responsiveness of various mutants expressed as percentage of the response of the wild type promoter. All transfections were carried out at least three independent times. Data were quantitated by PhosphorImager analysis, and normalized against the internal transfection control and stimulation of the endogenous *c-fos* gene.

Figure 2. BCP Signaling Mediated by RAS/ERK Pathways

i- Inhibitory Ras blocks BCP-signaling. NIH3T3 fibroblasts transfected with the wild type p711/c-fos reporter, and i increasing amounts of inhibitory (A) Ras, (B) Rac1, or (C) RhoA, serum-starved for 36 hours and stimulated with BCP. RNase protection data quantitated by PhosphorImager analysis and normalized for transfection efficiency. Data were normalized between each set of transfections for a given inhibitory construct. Activation of the wild type plasmid alone was set at 100 percent. Percent activation for each set containing inhibitory plasmid was determined by comparing the fold activation upon BCP stimulation of that set to the fold activation of set with no inhibitory construct. Results presented from the average of at least three independent determinations. The wild type transfection with no inhibitory construct was performed in duplicate and the expression varied by less than 6% between experiments.

ii- Ras/MEK inhibition blocks BCP-mediated ERK activation. (A) NIH3T3 fibroblasts transfected with HA-tagged ERK expression construct and increasing amounts of dominant inhibitory forms of Ras, Rac1, or RhoA. Cells were serum-starved for 36 hours and stimulated with BCP for five to ten minutes. Immunocomplex kinase assay were performed. Activation in the absence of inhibitory construct was set at 100 percent. Percent activation for each set containing inhibitory plasmid was determined by comparing the fold-activation of that set to the fold activation of the set with no inhibitory construct. The results presented for the inhibitory constructs are the average of at least three independent determinations. (B) Increasing concentrations from 1–25 μ M of the MEK inhibitor U0126 were added to NIH3T3 cultures

transfected with the HA-ERK construct and cells stimulated with BCP for 5–10 minutes. Percent activation was calculated based on the fold activation of kinase activity of cells treated with U0126 compared to those untreated. The results presented are the average of three independent determinations.

iii- MEK Inhibition blocks BCP-mediated *c-fos* activation. Increasing concentrations to **(A)** 100 μ M of the MEK inhibitor PD98059 and/or the p38 MAPK inhibitor SB203580 at 30 μ M, or **(B)** 1–25 μ M MEK inhibitor U0126 were added to serum starved cultures of NIH3T3 fibroblasts that had been transfected with the wild type p711/*c-fos* reporter. Cultures were stimulated with BCP and RNase protections performed. Percent activation was calculated based on the fold activation of *c-fos* gene expression in cells treated with inhibitor compared to untreated. Transfections in **(A)** were performed in duplicate and the expression levels varied by less than 5% between duplicates. In **(B)** transfections were performed in triplicate.

Figure 3. Role of CaM kinase in BCP activation

Calcium calmodulin inhibitors do not block BCP-mediated ERK or CREB activation. Serum starved NIH3T3 cells treated with the calmidazolium or KN-62 were stimulated with either a final concentration of 15% fetal calf serum or BCP for five to ten minutes, and cellular lysates were immunoblotted with (A) antiphospho ERK antibody, or (B) antiphospho CREB Ser-133 antibody.

Figure 4. Schematic depiction of the signaling mechanism involved in BCP crystal mediated activation of the *c-fos* gene
See text.