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Calcium and disease: molecular determinants of calcium crystal deposition diseases

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Abstract. Deposition of basic calcium phosphate (hydroxyapatite, octacalcium phosphate and tricalcium phosphate) (BCP) and crystalline calcium pyrophosphate dihydrate (CPPD) is associated with a variety of aging-related pathologies, including osteoarthritis, cartilage degeneration and pseudogout. These diseases of calcium deposition serve as some of the best-studied examples of how calcium-regulated changes in gene expression can directly lead to pathogenic consequences. Tissue damage can result when crystals stimulate cells to release matrix-degrading molecules or secrete cytokines that stimulate the release of matrix-degrading molecules. Exposure of cultured cells to crystals induces expression of cellular proto-oncogenes such as c-*fos*, c-*myc* and c-*jun*, by a calcium-dependent mechanism, and this response can be blocked by a potential therapeutic compound, phosphocitrate. Activation of the c-*fos* and c-*jun* genes is directly involved in expression of metalloproteinases such as collagenase and stromelysin, suggesting that crystal-mediated activation of these genes is directly involved in pathogenesis. In this review recent advances in the molecular mechanisms responsible for crystal-mediated cell activation are discussed.

Key words. BCP; CPPD; calcium deposition disease; signal transduction; gene expression.

Calcium-containing crystals BCP and CPPD are responsible for mediating tissue destructive effects

Changes in intracellular calcium levels can regulate gene expression to affect a wide variety of processes ranging from cell growth to apoptosis. While considerable progress has been made in understanding how changes in calcium levels affect new gene expression, examples of calcium-regulated pathogenesis are not well documented. One paradigm where calcium is involved in mediating the disease process, based directly on its ability to activate new gene expression, is in the diseases of calcium deposition. Calcium-containing crystal deposition diseases are a group of clinically heterogeneous arthropathies which are a significant source of morbidity in the elderly (reviewed in [1–5]). The etiology of these diseases is unknown. Monosodium urate, calcium pyrophosphate dihydrate (CPPD) and basic calcium phosphate (carbonate-substituted hydroxyapatite and octacalcium phosphate) (BCP) crystal aggregates are the most common forms of calcium crystals found. All three types of crystals are more common in older persons. Pathologically, these crystals are associated with gout (monosodium urate), pseudogout (CPPD), and cartilage degeneration and acute calcific periarthritis (BCP). Secondary deposition of BCP crystals has been observed in chronic renal failure [6], in patients with collagen vascular diseases, following neurologic injury and after local corticosteroid injection. Both BCP and CPPD crystals also appear in cartilage diseases (chondrocalcinosis).

Clinical observations and serial radiographic surveys support the thesis that crystal deposition causes tissue degeneration. The degeneration accompanying crystal deposition differs from that of primary osteoarthritis (OA), although the prevalence of BCP crystals from patients with OA ranges from 30 to 60% [7]. The basis of tissue damage by crystals is a matter of conjecture. Theoretically, crystals in cartilage may directly injure chondrocytes. However, in pathologic specimens crystals are rarely seen in immediate contact with chondrocytes and even less frequently are found engulfed by chondrocytes. It is more likely that cartilage damage

results when synovial lining cells ingest crystals then release matrix-degrading molecules or secrete cytokines that stimulate chondrocytes to generate matrix-degrading molecules. Milwaukee shoulder syndrome serves as a human model from which many concepts of crystal-related cartilage damage have been generated [8, 9]. Milwaukee shoulder syndrome epitomizes noninflammatory joint destruction associated with crystal deposition. In all cases BCP crystals are identified in fluid from affected joints, and 50% of the time these fluids also contain CPPD crystals. In vitro studies have shown that in concentrations found in pathologic human joint fluids BCP and CPPD can stimulate fibroblast, synovial cell and chondrocyte mitogenesis in vitro by a process similar to that of platelet-derived growth factor (PDGF) [10].

Based on clinical and synovial fluid findings and in vitro data [11], a hypothesis, diagrammed in figure 1, 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 (i) protease synthesis and secretion, which in turn releases additional crystals and collagen from the surrounding tissue; (ii) prostaglandin E_2 (PGE_2) production; and (iii) DNA synthesis as a result of protein kinase C activation and crystal dissolution. Proteases and PGE_2 result in the 'degeneration' of the periarticular tissue. DNA synthesis then leads to an increase in synovial cells that generate more proteases and PGE_2 [12].

BCP-mediated responses can be inhibited by phosphocitrate

Phosphocitrate (PC) is a naturally occurring compound which has been identified in mammalian mitochondria, crab hepatopancreas and human urine [13]. PC is a potent in vitro inhibitor of BCP [14] and crystal formation [15]. PC also prevents soft tissue calcification in vivo and does not produce any significant toxic side effects in rats or mice when given in doses up to 150 μmol/kg/day [14, 16]. Studies have shown that PC specifically inhibits CPPD and BCP crystal-induced proto-oncogene (c-*fos* and c-*jun*) expression, metalloproteinase synthesis and mitogenesis in human fibroblasts in vitro, while having no effect on similar biologic responses induced by epidermal growth factor (EGF), PDGF and serum [17, 18].

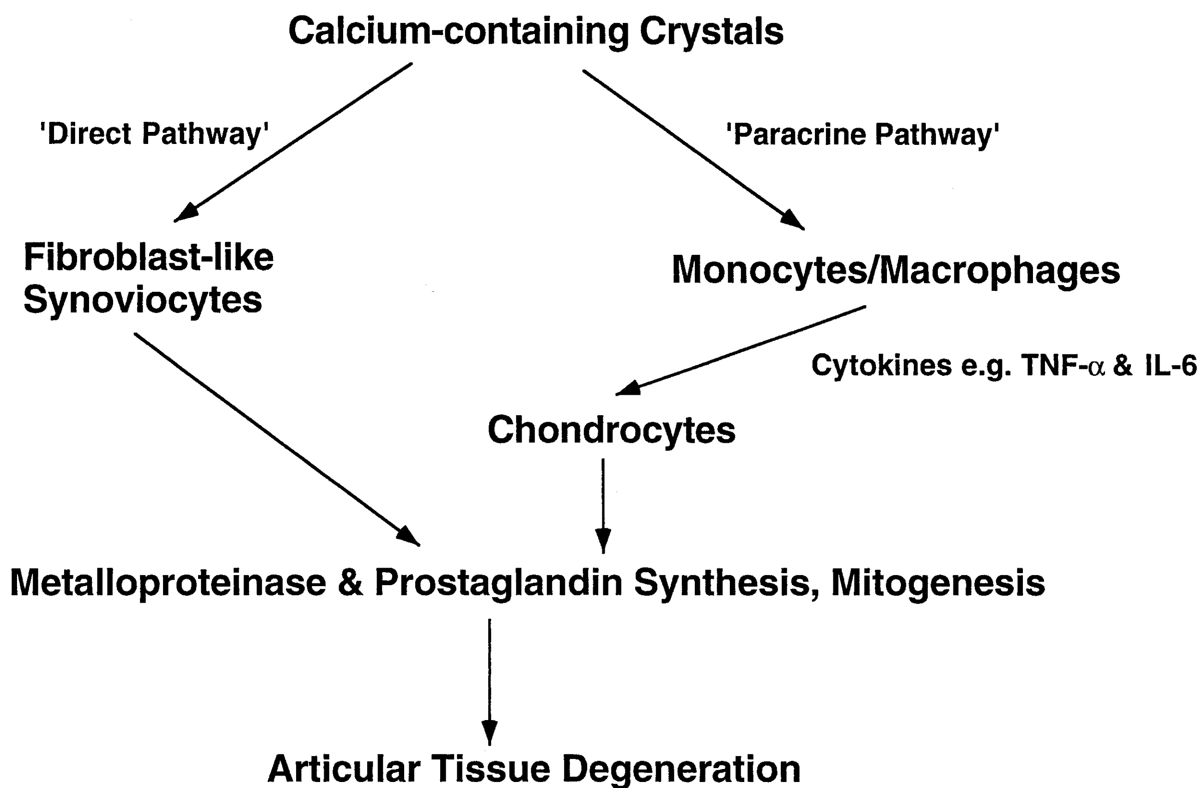

Figure 1. Roles of calcium-containing crystals in articular tissue degeneration. See text for discussion.

PC also has significant promise as a therapeutic compound based on its effects in a murine progressive ankylosis (MPA) model. MPA is a manifestation of an autosomal recessive mutation, which produces an inflammatory joint disorder associated with intraarticular BCP crystal deposition and culminates in fusion of the joints [19]. One study showed that PC treatment significantly inhibited disease progression in peripheral joints uninvolved at the start of treatment. Histologically, there was inhibition of synovial proliferation, chondroid metaplasia, and ankylosis in peripheral and spinal joints of PC-treated animals. It was concluded that PC inhibits disease progression in MPA when given before disease is established [20].

It has been shown that calcium crystals can activate a wide variety of cell types, including chondrocytes [21], fibroblasts [22], synoviocytes [23] and immune cells [24–26], which are likely to contribute to the pathologic response. The remainder of this review, however, will focus mainly on the underlying mechanisms by which calcium crystals activate mitogenesis and metalloproteinase induction in fibroblasts, and fibroblast-like cells. BCP will be used as a paradigm, since BCP is one of the most prevalent crystals found in vivo, and BCP-mediated intracellular mechanisms have been the most well studied.

Mitogenic effects of calcium-containing crystals

Calcium-containing crystals have a number of growth factor-like effects. BCP crystals can substitute for PDGF as a competence growth factor in vitro [10]. BCP crystals and PDGF exert similar biologic effects on cultured cells, such as stimulation of PGE_2 production via the phospholipase A_2/cyclo-oxygenase pathway, activation of phospholipase C and inositol phospholipid hydrolysis [27], and induction of collagenase and neutral protease synthesis [25, 28]. Proto-oncogenes c-*fos* and c-*myc* are induced with similar kinetics [29, 30]. Crystal-induced mitogenesis is preceded by calcium crystal interaction with the cell plasma membrane, followed by ingestion, dissolution and activation of a number of cellular signaling pathways. BCP, CPPD, calcium urate and calcium carbonate crystals are mitogenic to a variety of cell types [21, 22]. Addition of noncalcific particulates, such as latex beads, diamond dust or MSU crystals does not induce mitogenesis. Lysosomotropic agents, chloroquine and NH_4Cl, which raise the lysosomal pH, significantly inhibit the mitogenic effect of BCP crystals but not that of serum [31]. This suggests that mitogenesis and intracellular crystal dissolution are related phenomena [31, 32].

The role of calcium in crystal-mediated intracellular signaling

Calcium-containing crystals can induce mitogenesis in cultured fibroblasts [22], however, the mechanisms by which this occurs are not completely understood. In cell culture studies crystal deposition on the cell membrane leads to an acute response which is characterized by an initial rapid rise in intracellular calcium concentration. This is then followed by a sustained elevation in intracellular calcium levels that is due to crystal ingestion and dissolution. Endocytosis and intracellular dissolution of crystals resulting in elevated intracellular calcium concentration are important for cellular activation. The transient increases in intracellular calcium concentrations seen in the acute phase are also important for crystal action and can apparently occur independently of crystal dissolution. Experiments in which cells are treated with crystals that are too large to endocytose [33] show transient rises in intracellular calcium, suggesting that crystal deposition causes an influx of extracellular calcium or a release of calcium from internal stores. The mechanism by which crystal deposition leads to calcium transients is unclear, although it has been proposed that calcium crystal shape and charge effects may be involved in activating membrane calcium channels. Alternatively, although calcium-containing crystals are highly insoluble in solution [34, 35], it is also possible that solubilization at the cell surface under local physiological conditions may be playing a role.

The signal transduction pathway for mitogen-stimulated cell proliferation in cultured fibroblasts involves a sequence of events which begins at the cell surface. The earliest of these events is a dramatic increase in intracellular Ca^{2+}. The increased cytosolic Ca^{2+} is believed to be an important signal for later events which result in proto-oncogene expression, metalloproteinase synthesis and initiation of DNA synthesis. Mitogenesis and metalloproteinase synthesis may not necessarily be regulated by the same mechanism, since it has been shown that while intracellular dissolution of crystals is important for mitogenesis, it does not seem to be necessary for metalloproteinase induction [32], although in another report uptake of crystals was shown to be necessary for metalloproteinase induction [33].

The mechanisms involved in crystal-mediated changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) are poorly understood. In response to many extracellular signals, mammalian cells can trigger an elevation of $[Ca^{2+}]_i$ by increasing entry of Ca^{2+} from the extracellular space and/or by releasing Ca^{2+} from intracellular stores, which is likely to be due to 1,4,5-inositol trisphosphate (IP_3) [27, 36]. Consistent with this mechanism, phosphatidylinositol turnover has been shown to occur upon treatment of cells with BCP crystals [23].

As measured by Fura 2 imaging in human fibroblasts, in response to treatment of cells with BCP crystals, $[Ca^{2+}]_i$ is transiently induced 10-fold, from baseline levels within seconds, followed by a slow but sustained increase in $[Ca^{2+}]_i$ for 60 min after stimulation [37]. Pretreatment of cells with NH_4Cl to prevent intracellular crystal dissolution in phagolysosomes eliminates the second sustained rise of $[Ca^{2+}]_i$ level but has no effect on the early transient peak. This is in contrast to another mitogenic agent epidermal growth factor (EGF), which also induces an early 8-fold transient rise of $[Ca^{2+}]_i$ over baseline level but not the second sustained increase. In cells stimulated by crystals in Ca^{2+}-free HBBS media, the transient rise in Ca^{2+} is reduced by over 95%. This suggests that influx of extracellular Ca^{2+} and not the IP_3 generated by 4,5-phosphatidyl inositol bisphosphate (PIP_2) hydrolysis by PLC is primarily responsible for the early transient rise of $[Ca^{2+}]_i$, whereas the second sustained increase is due to crystal dissolution. The consequences of Ca^{2+} influx in crystal-induced early events are unknown, although as discussed below recent evidence suggests that early Ca^{2+} transients can at least in part contribute to the initiation of early events in the cell such as activation of Ras-dependent signaling pathways and subsequent activation of proto-oncogene expression, as well as the activation of NF-κB [38].

PKC activation also appears to be important for the mitogenic effects of calcium crystals. The effects of BCP crystals and PDGF on proto-oncogene activation and DNA synthesis in control and protein kinase C (PKC)-deficient 3T3 fibroblasts have been examined [30]. Stimulation of DNA synthesis by BCP crystal and tumor-promoting phorbol diester (TPA) was inhibited after downregulating PKC. No effect on PDGF-stimulated DNA synthesis was observed under the same conditions in control cells. Consistent with this result, in PKC downregulated cells both TPA- and BCP-crystal stimulations of c-*fos* and c-*myc* were inhibited markedly, whereas the induction of these transcripts by PDGF was unaffected. These data suggest that BCP crystals induce c-*fos* and c-*myc* expression and DNA synthesis, at least in part, through a PKC-dependent mechanism.

Induction of metalloproteinases by calcium-containing crystals

Most connective tissue cells produce low levels of collagenase and stromelysin in culture. However, in response to various factors, e.g. cytokines, latex beads and collagen fibrils, they can synthesize high levels of metalloproteinases. As discussed above, BCP has also been postulated to stimulate metalloproteinase expression. One hypothesis is that BCP crystals shed from synovial depots into the joint space from where they may be phagocytosed and thereby stimulate synoviocytes to secrete metalloproteinases. These secreted metalloproteinases may then be responsible for joint destruction and release of additional crystals and particulate collagen into the joint space. This hypothesis was tested by determining whether calcium crystals could activate cells to secrete metalloproteinases. Upon adding natural and synthetic BCP, and CPPD crystals to cultured synoviocytes, collagenase activity in the ambient media was significantly augmented in a dose-related fashion over untreated cultures [17, 25, 39].

It has been shown that metalloproteinase induction and secretion also occur in human fibroblasts in response to BCP crystals. A dose-dependent accumulation of collagenase and stromelysin messenger RNA (mRNA) is evident by 4 h and continues for at least 24 h in crystal-stimulated cultures compared with the unstimulated controls. Increased collagenase activity and protein in the conditioned media of stimulated cultures confirmed the synthesis and secretion of collagenase. Synthesis and secretion of stromelysin was also confirmed by immune precipitation [28, 40]. Thus, the mitogenic response to BCP crystals is accompanied by coordinate induction and secretion of collagenase and stromelysin [40], which can be inhibited by agents that raise cyclic adenosine menophosphate (cAMP) levels [41].

Signaling pathways in crystal-mediated cell activation

Treatment of cultured cells with calcium crystals can activate a variety of cellular signaling molecules associated with cell growth and differentiation. The signaling pathways by which crystals activate cells have recently been the object of a number of studies. These studies have revealed that a wide variety of signaling intermediates are activated when cells are treated with calcium crystals. These include tyrosine kinases [unpublished observations], phospholipase C (PL-C) [23], protein kinase C (PKC) [30], Ras small molecular weight guanosine nucleotide triphosphatases (GTPases) [unpublished observations] and members of the mitogen-activated protein kinase (MAPK) family [18].

Phospholipase C can be induced in synovial fibroblasts by BCP. As mentioned above, Mitchell et al. [42] have shown in mouse fibroblasts that when PKC is downregulated by TPA, mitogenesis is inhibited, suggesting that PL-C may be involved in BCP-mediated proliferation by activation of PKC. Consistent with this idea, PKC translocation from the cytoplasm to the nucleus is also stimulated by BCP [43]. Therefore, one possibility is that BCP may signal to the nucleus to stimulate proliferation by a PL-C/PKC-mediated signaling mechanism.

BCP crystals can also activate RAS/MAPK-dependent signaling pathways. It has been shown that BCP treatment of fibroblasts in culture can specifically activate the ERK1 and ERK2 MAPKs, but not p38 MAPK [18]. This activation mostly appears to occur through a RAS-dependent pathway, although there may be a small contribution from Ras-related pathways, including the Rho and Rac pathways [unpublished observations]. Whereas the BCP response is calcium-dependent, 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 [unpublished observations].

Consistent with its role in inhibiting BCP-mediated responses, phosphocitrate can specifically inhibit BCP-dependent activation of MAPK pathways [18]. In one study, the effects of PC, *n*-sulpho-2-amino-tricarballylate (SAT, a PC analog [43]) and citrate on this signal transduction pathway were examined [18]. It was shown in this report that BCP and CPPD crystals activate an ERK, and not a p38 protein kinase, pathway, and activation of the ERK pathway occurred with a 5-min delay in induction, compared with serum- and IL-1β-induction. It was further shown that PC (10^{-3}–10^{-6} M) specifically blocks BCP and CPPD crystal activation of MAPK and mitogenesis in a dose-dependent manner. This inhibition is specific for PC, although SAT and citrate in high concentration (10^{-3} M) can partially block MAPK and mitogenesis [18]. The mechanism of action of PC is unclear. One hypothesis is that it competes with BCP and/or CPPD for binding sites on the cell surface.

A recent report by McCarthy et al. [38] also investigated the role of tyrosine kinases in BCP-mediated activation of human fibroblasts. Using anti-phosphotyrosine (pTyr) antibodies, these authors did not detect any induction of tyrosine phosphorylation. In contrast, we have recently found in cultured mouse 3T3 fibroblasts that BCP treatment induced pTyr phosphorylation on a Ca^{2+}-dependent tyrosine kinase related to the Pyk2/FAK family of kinases. Phosphorylation occurs in a rapid, robust and transient manner [unpublished observations]. Since Pyk2/FAK have been implicated in activating RAS-mediated signaling pathways, this may suggest that BCP can activate RAS/ERK signaling through this pathway. The reason for the discrepancy between our results and those of McCarthy et al. [38] is unclear. It may be due to differences in cell type or in the reagents used to detect pTyr. In this context, it should be pointed out that so far with the exception of metalloproteinase secretion, all signaling events that have been observed in human fibroblasts have also been shown to occur in cultured mouse 3T3 fibroblast cell lines.

Nuclear targets of BCP-mediated activation: activation of the c-*fos* gene may be a triggering event for metalloproteinase induction

Since crystal treatment of cells can induce new gene expression, crystal-induced signaling pathways must target nuclear factors to effect new transcription. The nuclear response to calcium crystals, however, has not been well studied. Recently published studies have shown that BCP can target the CREB transcription factor, as well as NF-κB. Both these factors are important regulatory molecules for a wide variety of genes. In murine fibroblasts, NF-κB can be activated in response to BCP treatment of cells [38]. The mechanism by which BCP activates NF-κB has not been characterized, although it appears that PKC is not involved, since the PKC inhibitor staurosporine has no specific ability to inhibit NF-κB activation. In the case of CREB, treatment of rodent fibroblasts with BCP rapidly induces phosphorylation on serine-133, an activating event for CREB-mediated gene expression [18]. We have recently shown that BCP targets both CREB as well as another transcription factor complex, the serum response factor (SRF)/TCF complex, for maximal activation of c-*fos* gene expression [unpublished observations]. The SRF/TCF complex consists of a dimer of SRF and at least a monomer of a member of the Elk-1 subfamily of the ETS oncoproteins, termed ternary complex factor (TCF) (reviewed in [44]). The TCF can be activated by serine phosphorylation by members of the MAPK family of kinases. In response to treatment of cells with a variety of mitogenic factors, the RAS/ERK pathway has been shown to phosphorylate TCF. We have also recently shown that BCP-mediated activation of the c-*fos* gene is primarily regulated by activation of the RAS/ERK signaling pathway. The RAS/ERK pathway has as one of its targets a SRF/TCF binding site in the c-*fos* promoter [unpublished observations]. Our results suggest that BCP is likely targeting the TCF component of the SRF/TCF complex for phosphorylation.

The collagenase promoter is regulated by the AP-1 transcriptional activator. AP-1 consists of a heterodimer of the Fos and Jun proteins. In unstimulated cultured cells, c-*fos* RNA and protein are virtually undetectable; however, upon stimulation, expression of c-Fos protein is dramatically induced [45]. Newly synthesized protein can complex with Jun proteins, leading to assembly of the AP-1 activator protein. Therefore, it is reasonable to conclude 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 (fig. 2). In this scenario, an early transient rise in $[Ca^{2+}]_i$ would lead to RAS/MAPK-dependent activation of the c-*fos* gene, which would be a triggering event that could directly

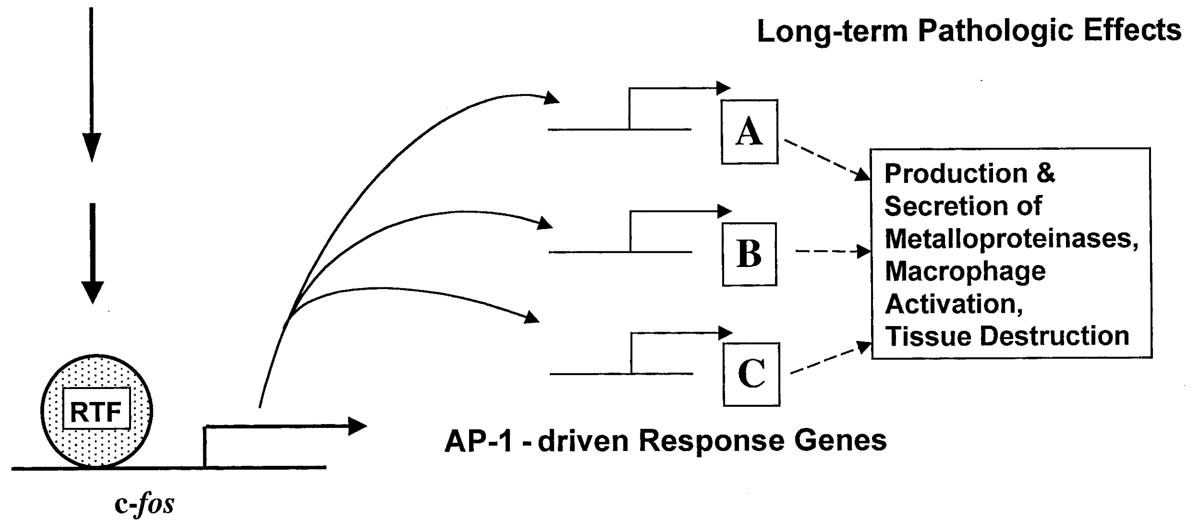

Figure 2. Calcium-containing crystals activate signaling pathways that activate programs of gene expression, via regulatory transcription factors (RTF), which lead to activation of the c-*fos* gene. The product of the c-*fos* gene can then activate AP-1-driven response genes whose products can elicit a variety of pathological long-term effects.

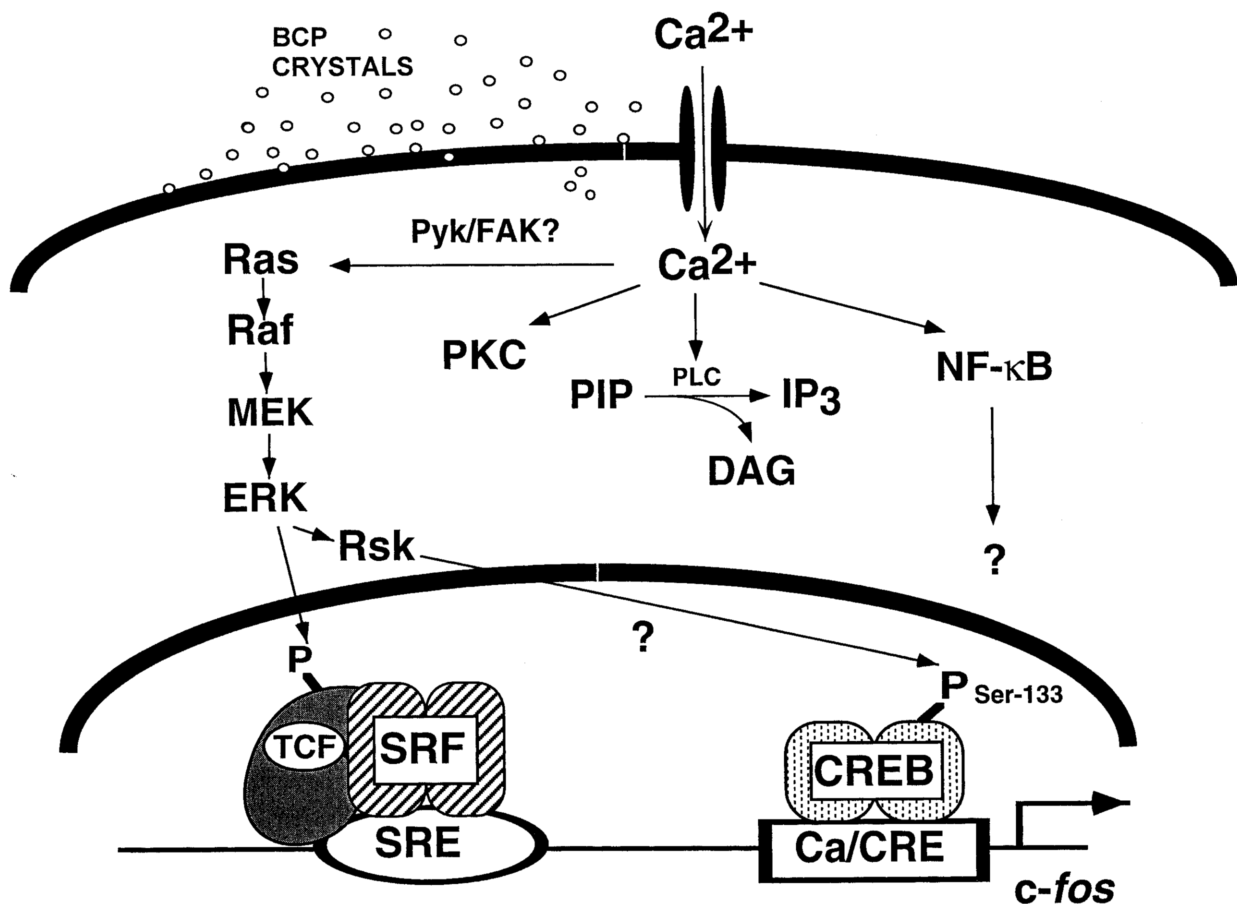

Figure 3. The acute cellular response to crystal deposition can activate a variety of intracellular signaling molecules. Depicted are (i) signaling intermediates that are activated by the calcium crystal response, and (ii) a putative RAS/ERK signaling pathway responsible for BCP-dependent activation of the c-*fos* gene.

lead to pathological consequences. Known and potential nuclear signaling mechanisms in response to BCP crystals are schematically depicted in figure 3.

Outstanding issues

Understanding the underlying molecular mechanisms involved in crystal-mediated activation of cells is still in the rudimentary stages. While significant progress has been made in understanding intracellular mechanisms involved in activation of cultured fibroblasts, a number of issues remain. It will be important to determine whether the intracellular mechanisms that are being worked out for crystal-mediated activation of cultured fibroblasts are also similar in other cell types. Similarly, it will be important to determine whether the mechanisms of activation that have been determined by studying cells growing in culture are relevant to mechanisms at mineralization sites where crystal deposition takes place in vivo. Developing in vitro articular cartilage mineralization mixed cell culture model systems that can be used to complement already existing in vitro articular mineralization techniques [46–48] will be helpful in addressing these issues. Another significant area that has been particularly difficult to address is the mechanism by which crystals interact with the cell surface and how this is involved in cell activation. Development of additional mouse genetic model systems to investigate calcium crystal mineralization propensity and susceptibility to calcium deposition would also be informative.

Acute symptoms of diseases of calcium deposition such as pseudogout and acute calcific periarthritis are readily treated medically, but the chronic effects of crystals containing calcium are not. Hopefully, with increasing research and with the advent of new in vivo methodologies to study calcium deposition disease mechanisms, new therapies that target the biologic effects of these crystals at the molecular level will be developed.

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