Phosphocitrate, A Potential Therapeutic Agent for Calcium Crystal Deposition Diseases

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Abstract: The deposition of calcium-containing crystals in articular tissues is probably an under-recognized event. Clinical observations indicate that exaggerated and uniquely distributed cartilage degeneration is associated with these deposits. Perhaps the most compelling argument favoring a role for crystals in Osteoarthritis (OA) stems from their *in vitro* effects on articular tissues. Therapeutic options are limited and of compromised value for controlling and/or eliminating calcium crystal salt diseases. This review highlights past and present studies related to phosphocitrate (PC), a relatively unheralded compound with an ability to inhibit crystal nucleation, growth and aggregation of calcium salts, including basic calcium phosphate (a term including carbonate-substitute apatite, octacalcium phosphate, and tricalcium phosphate) and calcium pyrophosphate dihydrate. In addition, cell culture studies reveal that specific calcium phosphate-induced cellular events associated with osteoarthritis also are retarded by PC. Interest in the tetra-sodium PC form and the new Calcium/Sodium/PC (CaNaPC) salt has stemmed from their chemical characteristics and biological actions. In two instances, the CaNaPC has displayed superior inhibitory properties to that of the tetra-sodium salt. Using a calcergy animal model, a chemically-induced calcifying skin plaque in rats has been ameliorated while in cell culture studies, strong inhibition of calcium phosphate-DNA co-precipitates induced cell death has been noted. The assessed data indicate that either of the PC salts through their modes of action, could be useful as adjunct therapeutic treatment of crystal associated OA.

Keywords: Phosphocitrate, review, chemistry, inhibition, osteoarthritis, therapeutic options.

BACKGROUND

The multiplicity and diversity of roles for mineral elements ensures that essential metabolic reactions are maintained for an organism's life cycle. The importance of calcium, for example, is exemplified by its essentiality not only to the development and maintenance of the skeleton, but also by its role in specific enzyme activation and other biochemical and physiological parameters. Deposition of calcium salts can be triggered by tissue trauma or other signals such as abnormal fluctuations in intracellular calcium ion concentrations. Initially, it may accumulate in an amorphous state but, under continuing favorable environmental conditions, nucleation and transformation to a hard, crystalline salt with minimum solubility is sustained to activate further events for the development of the specific pathological disease state. This scenario prevails in a broad range of diseases such as renal calcinosis, urinary lithiasis, arteriosclerosis, heart valve calcification, soft tissue and tumor calcification, chondrocalcinosis and in selected arthropathies. It is no surprise then, that investigators are continuing to seek therapeutic solutions for calcium crystal deposition diseases. From perhaps an over-simplistic, even controversial viewpoint, this review will focus on two aspects in respect to examine the role of calcium-containing crystals in OA and the potential future therapeutic role offered by phosphocitrate ([§]PC) as an inhibitor of crystal induced events. In consideration of possible PC therapeutic benefit, data accumulated during the past 4 decades from numerous *in vitro* and *in vivo* studies with PC together with new findings is reviewed.

PHOSPHOCITRATE(S) CHEMISTRY

A chemical synthesis for citric acid with a phosphate moiety was first published in 1959 [1]. Subsequent studies with this compound were not pursued nor its importance recognized by others, perhaps because the required equipment for preparation of the compound was too specialized and soon obsolescent. However, in the period 1960-1980, studies were in progress to isolate and identify compounds that might be capable of inhibiting the development of urinary stones. As a result, in 1976 a component "factor X" was postulated to be present in urine with both phosphate and citrate moieties [2]. This led to speculation that "factor X" might be PC and perhaps an important controller of unwanted mitochondrial calcification [3]. An impetus to investigate more thoroughly the reality of such a claim, was given a significant boost when two groups simultaneously published synthetic strategies to enable chemical and biological characterization of PC [4,5]. The

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[§]PC abbrev. indicates a neutral tetra-sodium salt of phosphocitrate. The CaNaPC salt will be otherwise specified where appropriate.

1573-3971/06 $50.00+.00

ability of PC to prevent crystal nucleation was confirmed when it was demonstrated that its presence prevented amorphous calcium phosphate from transforming into the hard crystalline hydroxyapatite form. Of further interest, small quantities of PC were detected in studies exploring mammalian soft tissue mitochondria [5,6]. It was therefore clear, that this compound was worthy of more intense scrutiny, particularly, with respect to ameliorating the severity of calcium crystal deposition associated pathological diseases. These early observations coupled with availability of pure compound then, have provided the foundation stones for our subsequent studies.

Today, we have extensive knowledge regarding the chemical characteristics and ability of PC both *in vitro* and *in vivo* to influence calcium salt driven events. In a physiological pH range, PC in solution possesses multiple negative ionic charges as a result of the deprotonation of 4 out of 5 available ionizable groups *i.e.* two –OH protons from $-OPO_3H_2$ and two protons from the -COOH groups (Fig. **1**). This feature enables strong binding to selected, calcium rich, positively charged ionic crystal faces.

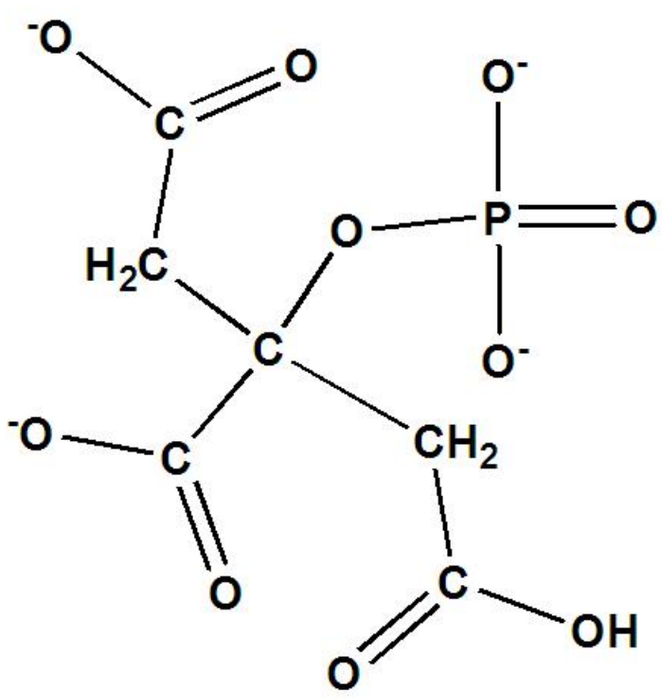

Fig. (1). Ionized form of PC at neutral pH.

In addition, the proximity of the PO_4 group to the central C atom of the citric acid backbone confers configurational specificity to its action. Any reduction in charge or disruption to this configuration then leads to a reduction of inhibitory power [7]. The majority of studies have used the tetrasodium salt of PC as neutrality is a particular issue with *in vivo* animal and cell culture research.

Crystallographic studies employing scanning electron microscopy combined with computer simulation have assisted in exposing the mechanism of PC action on several calcium salt crystals. Hydroxyapatite, calcium pyrophosphate and calcium oxalate all can be grown *in vitro* in the presence of controlled amounts of PC. Under such conditions, total inhibition is avoided to permit the mechanisms of PC action to be exposed [8,9]. Once binding of the inhibitor occurs, image analysis reveals the changing shape of the crystal as its normal growth pattern is changed. Computer modeling can then simulate the changes and input images of the crystal lattice atoms and the molecular configuration required for optimized contact of PC with the crystal face [9]. These approaches have delineated and confirmed the unique characteristics inherent in the PC molecule.

Another interesting feature only recently documented, is that the nature of the cations in the PC salt can augment the inhibitory action of the molecule. A new salt, a CaNaPC form has been developed [10] which, in limited studies, has raised the possibility that in defined situations it can be more potent than its parent PC [10,11]. The exact chemical formula of this form of PC is "$CaNa(PC)_2(H_2O)$". The structure of this salt is most unusual and differs in several aspects from PC (see Fig. **2**, right).

It is polymeric with $Ca(PC)_2(H_2O)$ "monomers" connected through Na^+ bridges (these are not shown in Fig. **2** for simplicity). The Ca^{2+} cation occupies the center of an irregular polyhedron defined by 4 phosphate, 4 carbonyl and 1 water oxygen atom. Accordingly, it has a coordination number of 9 for Ca^{2+} which is a relatively rare phenomenon in nature.

ROLE OF CRYSTALS IN OSTEOARTHRITIS

Crystalline calcium pyrophosphate dihydrate (**CPPD**) and basic calcium phosphate (**BCP**) are the two most common forms of pathological articular mineral. Each occurs frequently in OA joints, and each may be phlogistic, causing acute attacks of pseudogout in the case of CPPD crystals and acute calcific periarthritis in the case of BCP crystals [12-14].

Evidence for a causal role of crystals in cartilage degeneration is primarily inferential, based on correlative data. However, clinical observations and experimental evidence of their *in vitro* effects support the thesis that articular crystals promote cartilage degeneration. More definitive investigations of causality are impeded by the lack of a suitable animal model for studying non-inflammatory aspects of crystal deposition, and the slow pace of degeneration [15].

Correlative data indicate that CPPD and BCP crystals are more common in degenerative joints than in normal joints or joints affected with inflammatory forms of arthritis. Conversely, OA is both more common and more severe in patients with calcium-containing crystals. One or both crystals are present in up to 60% of fluid from knees of patients with advanced OA [16,17]. The presence of CPPD crystals predicts a poor clinical and radiographic outcome [18].

Studies of synovial fluid containing CPPD crystals suggest associated chondrolytic activity in the involved joints, usually of a greater magnitude than seen in fluids from patients with OA but no crystals. Patients with acute "pyrophosphate arthropathy" (CPPD in joint fluids and OA) had the highest synovial fluid proteoglycan fragment concentrations of six disease categories evaluated [19-21]. Moreover, these CPPD-containing fluids also had the highest levels of matrix metalloproteinase (**MMP**)-1, MMP-3, and the highest ratio of MMP3:tissue inhibitor of metalloproteinase (**TIMP**), all factors, which would support increased matrix catabolism [21]. Although these studies do not prove a causative role of the crystals in producing OA, they strongly support an association of accelerated matrix degradation with the presence of calcium-containing crystals.

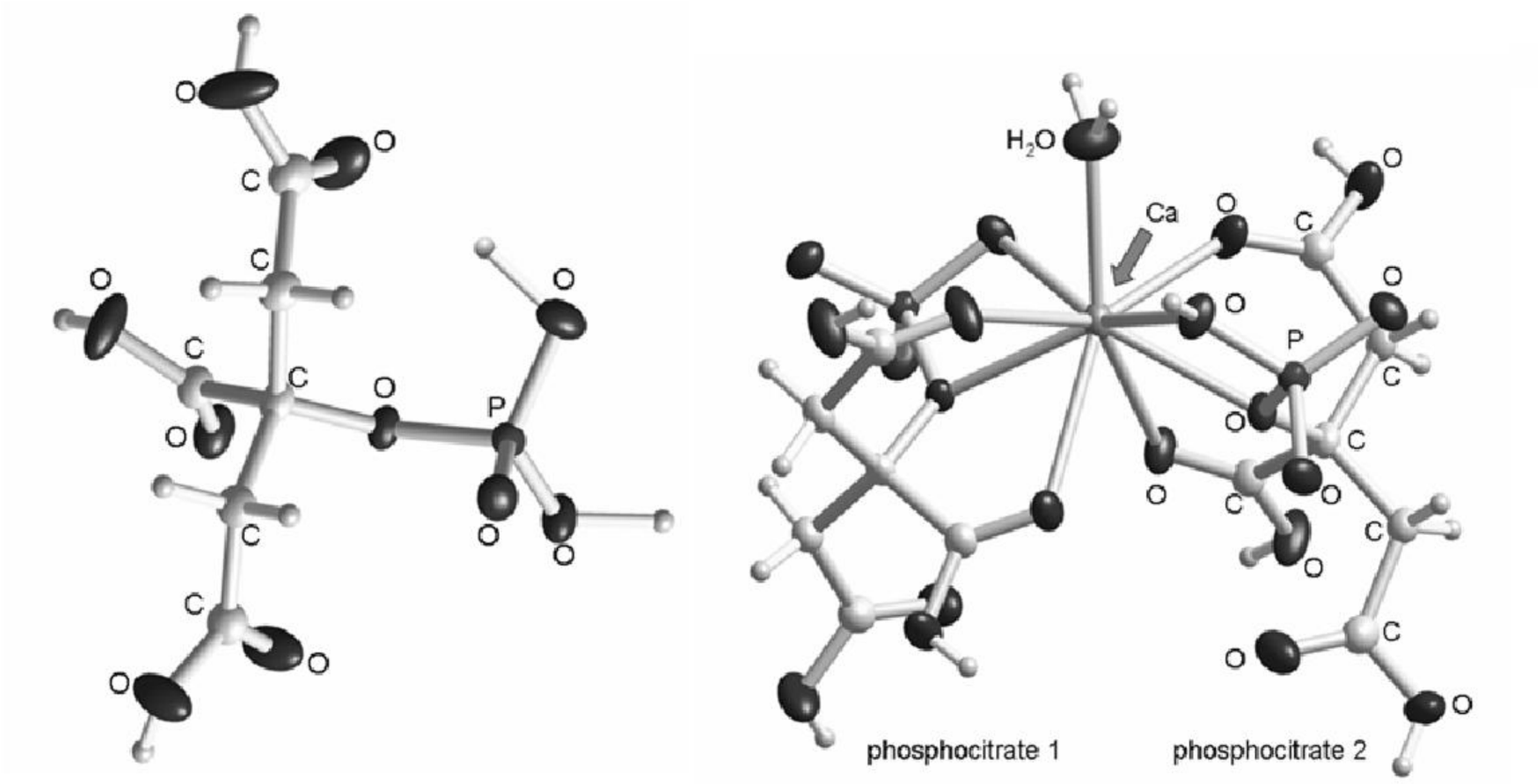

Fig. (2). A comparison of the molecular structures of PC (in its acid form, left) and CaNaPC, right.

Perhaps the most compelling argument favoring a role for crystals in causing osteoarthritis stems from their *in vitro* effects on articular tissues. Crystals can cause the degeneration of articular tissues in two separate pathways. In the "**Direct**" pathway, crystals directly induce fibroblast-like synoviocytes to proliferate and produce metalloproteinases and prostaglandins (PGE_2). The other "**Paracrine or Indirect Pathway**" centers on the interaction between crystals and macrophages/monocytes which leads to synthesis and release of cytokines which can reinforce the action of crystals on synoviocytes and/or induce chondrocytes to secrete enzymes and which eventually cause the degeneration of articular tissues [22].

The potential value of PC then for inclusion as adjunct therapy in the management of crystal associated OA is certainly worthy of consideration. As discussed below, this compound not only prevents crystal generation but also exerts a strong associated influence on intracellular events known to be active in calcium crystal deposition.

PREVIOUS AND PRESENT RESEARCH WITH PC

In considering PC for therapeutic use for crystal associated OA, it is pertinent to reflect on observations which have emerged from using this compound in a broad range of calcium crystal-induced situations. Pertinent to arteriosclerosis development for example, studies have revealed that uptake of calcium into aortic smooth muscle cells and the adhesion of monocytes to the aortic endothelium is remarkably reduced [23]. Also, receptor bound low density lipoproteins are released and calcification of aortic implants is inhibited [24].

The control of these important events is paramount to slowing the progression of the disease. Studies both *in vitro* and *in vivo* in the field of Urolithiasis have indicated the positive control exerted by PC to restrict nucleation and growth of calcium oxalate crystals [8]. These calcium salt crystals are prominent in the majority of kidney and urinary tract stone. Interesting studies relating to calcification in breast cell cancer also have been reported [25]. PC has been shown to inhibit hydroxyapatite induced mitogenesis and the upregulation of a number of important cell factors in human breast cell lines. These encouraging findings are creating a stimulus to seek additional therapeutic options for management of such a disease.

As a further example of the wide-ranging potential value of this compound toward pathological calcification, a study has been reported on its ability to restrict murine progressive ankylosing spondylitis. In mice bred with the genetic variant leading to the disease, continuous treatment with PC over a 6-week period resulted in a significant improvement in reducing the severity of conditions [26].

The overall conclusion reached from all the broader calcification studies employing PC is that it certainly has potential to be of ultimate benefit in an OA situation, where crystals of calcium phosphate and pyrophosphate are intimately associated with many aspects promoting joint degradation. Studies to date in respect to established events in crystal associated OA have all been encouraging. Cell culture studies using chondrocytes and other cell types have noted positive benefits with PC present [27]. Specifically, calcium phosphate and calcium pyrophosphate induction of metalloproteinase synthesis can be inhibited by PC [28]. Other crystal induced mitogenic effects associated with the Protein Kinase C induction pathway also are inhibited and there is a decreased ability to secrete matrix metalloproteinase, collagenase and stromolysin [29]. Gene expression of the degradative enzymes is a key target for control. PC also is known to block ATP dependent and ATP independent mineralization in articular cartilage vesicles [30].

A report this year, using an *in vitro* model of osteoarthritis human synovial fibroblasts and human foreskin fibroblasts describes PC inhibition of crystal induced prostaglandin E2 production with influence on the Cylco-oxygenase pathway [31]. This new observation confirms speculation offered some 20 years earlier when indomecin was found to block crystal-induced PGE2 production, that

the Cylco-oxygenase pathway indeed is a key player in helping develop the degenerative disease [32].

Aside from its demonstrated influence on crystal development and the many sequential intracellular reactions, the induced trauma arising from crystal contact with membranes is not to be ignored. Unrelated studies with polymorphonuclear leucocytes have revealed that PC can intervene to limit breakdown of membrane traumatized by crystals [33]. The protection mechanism of PC's action is not clear but it could be as a result of PC binding to the membrane by virtue of its charge or simply not allowing crystals to penetrate it. It is important to point out that PC has never been observed to provoke any toxicity to animals or cells at any of the concentrations used [10,23,26,34]. This is perhaps not surprising given that it exists in mammalian mitochondria.

PC VERSUS CaNaPC

Although studies of the CaNaPC salt are in their infancy, the data emerging suggest that in some instances, the CaNaPC may be a more effective therapeutic agent. An animal model using rats was selected to compare the inhibitory power of both salts to diminish dystrophic calcification of an chemically-induced skin plaque [10]. Treatment by intra-peritoneal injection of the respective PC salt (equivalent phosphate content) was given on alternate days for a designated 10 day period. In comparison to non-drug treated animals, the PC-reduced plaque size and its embedded calcium phosphate by 30% whereas the CaNaPC effected a 95% reduction. Higher dosage of PC given more frequently is required to achieve the same goal *i.e.* a 95% reduction.

Any interpretation of the apparent superior inhibitory actions of the CaNaPC is complex and at this stage, speculative. From a chemical viewpoint, as depicted in the molecular structure derived from crystallographic data, the CaNaPC molecule is quite bulky in size. It might be anticipated that such a feature would reduce the opportunity for maximum binding of all charged groups to a crystal's surface. On the other hand, if it carries more negative ionic charge and it configures the groups better to the crystal surface, it could be more potent. Just how the CaNaPC binds to crystals or indeed influences other crystal-induced biological events currently is being investigated. One can safely assume that CaNaPC is not broken down to its constituents (Ca^{2+}, Na^{+} and PC), as such a process would result in identical inhibitory activities between PC and CaNaPC. Then too, the inhibitory powers of the two salt forms may also relate to the Na cation lability in an aqueous environment. In the original PC molecule, the Na^{+} ions are probably removed in solution setting up an equilibrium between complexed and uncomplexed PC. When a Ca^{2+} center is now included in CaNaPC, such equilibrium will certainly be disturbed. Most likely, the Na^{+} ion is removed (at least partially), but the Ca^{2+} cation is probably maintained in the ligand "cavity", mainly due to the strong interactions between Ca^{2+} and the carboxylates/phosphonate ligand environment. Perhaps this allows a slower release of "active" PC from tissues at the injection site.

Incomplete knowledge of the actions of CaNaPC does not in any way detract from the advantages that are gained. The key issue is that both compounds exert a powerful inhibitory influence against calcium salt crystal development and their subsequent targets whether the assessment is made *in vivo* or *in vitro*. More recently, inhibitory comparisons of the two compounds have been evaluated through cell culture experiments. Key parameters relating to OA were measured [11]. The potency of both salts appeared similar toward inhibiting BCP crystal–stimulated induction of MMP1 mitogenesis and endocytotic activity. However, the CaNaPC was distinctly more effective in the inhibition of calcium phosphate DNA co-precipitates-induced cell death. Since amorphous calcium phosphate precipitates coexist with BCP crystals in calcified tissues, these findings are of considerable interest with crystals associated OA.

CONCLUSIONS

Currently, there is no single treatment to cure the various arthropathies, particularly those with associated crystal salt involvement. General management is normally focused on relief of pain and inflammation using acetaminophen, non-steroidal anti-inflammatory or corticosteroidal drugs. A few compounds attempt to target other issues such as increasing fluid viscosity (hyaluronan), providing a chondroprotective function (glucosamine) or attempting to inhibit cell Cylco-oxygenase activity (celebrex, Vioxx). The latter compounds are under a cloud because of secondary side effects. There are no compounds reported which specifically attempt to control crystal development and associated crystal-induced reactions seen in some of the arthropathies. The phosphocitrates then, would have a unique niche as a treatment option through their ability to exert a dual control action *i.e.* by inhibiting new crystal formation and preformed crystal stimulated cellular reactions. Extensive chemical, biological and cell culture studies in related fields as discussed earlier, have revealed that: (a) the compound can prevent new crystals of calcium salts from forming (b) the growth of developing crystals is inhibited (c) calcium salt crystal aggregation is discouraged (d) calcium phosphate induced stimulation of cellular directed events, such as, the proliferation of fibroblast-like synoviocytes, chondrocytes, metalloproteinases and COX-2 activity is retarded and (e) the compound has displayed a membrane protective effect against the presence of calcium salt crystals. These characteristics of PC would suggest then that the compound could serve in valuable adjunct therapy specifically for crystal associated osteoarthritis. The PC salts (tetrasodium PC and CaNaPC) are not yet being produced commercially to allow extension of studies to clinical trials. Nevertheless, research using small animals as models with laboratory synthesized compounds can continue to explore and build a comprehensive picture of their capabilities, which would be considered mandatory before any human trial evaluation.

ACKNOWLEDGEMENTS

This work is supported by NIH R0-1 AR38421-15, VA Senior Research Career Scientist Award and a VA Merit Review.

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Received: December 1, 2004 Accepted: April 19, 2005 Revised: July 25, 2005