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CALCIUM PYROPHOSPHATE CRYSTAL DEPOSITION: A KINETIC STUDY USING A TYPE I COLLAGEN GEL MODEL

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Abstract

Calcium pyrophosphate dihydrate (CPPD) crystal deposition disease is characterized by deposits of triclinic (t) and monoclinic (m) CPPD crystals in articular and fibrocartilage. Many investigators have attempted to model CPPD crystal growth using both solution and a variety of gel systems. We have investigated the effect of type I collagen fibrils on CPPD crystal nucleation and growth using an ionic diffusion model. Collagen was isolated from porcine menisci using a pepsin solubilization procedure and gelled in three layers, with one containing 10 mM pyrophosphate (PPi) plus physiologic ions, the middle containing only the ions, while the third contained 25 mM Ca plus physiologic ions. Initially, amorphorous calcium pyrophosphate formed at the Ca-PPi interface. Monoclinic CPPD crystallized in 6 weeks when the [Ca] was between 2 and 3 mM and the [PPi] was between 50 and 75 μM. At 13 weeks, t-CPPD formed when the [Ca] was also between 2 and 3 mM, but the PPi was less than 25 μM. One of the most striking differences between this system and all previous solution and gel model systems is the total absence of orthorhombic calcium pyrophosphate tetrahydrate (o-CPPT) from the gels made of collagen fibrils in near native conformation. Further, crystals of t-CPPD appear as large single crystals with the classic prismatic growth habit observed *in vivo*, and crystals of m-CPPD also evidence the *in vivo* rod habit. In contrast, the crystal growth habits of t-CPPD, m-CPPD, and o-CPPT grown in all of the other model systems never matched that observed *in vivo*. When compared to the previous studies, these results, particularly the crystal growth habit data, suggest that the native collagen fibrils themselves can nucleate CPPD crystal formation.

Key Words: Calcium pyrophosphate crystal deposition, Model systems, Collagen.

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Introduction

Calcium pyrophosphate dihydrate (CPPD) crystal deposition disease is characterized by CPPD crystal deposits in articular and fibrocartilage, especially knee menisci [26]. To date, there is no way to halt the progressive deposition of CPPD crystals nor is there a clinically suitable method of removing those already deposited [26]. The pathogenesis of cartilage degeneration in this arthritic disease is thought to include an amplification loop [12]; damaged cartilage is predisposed to CPPD crystal deposition which then induces further enzymatic or mechanical damage to the cartilage. In familial CPPD crystal deposition disease, changes have been noted in the cartilagenous matrix which antedate the formation of CPPD crystals [4]. CPPD crystals induce both synoviocytes [10] and chondrocytes [11] in cell culture to release collagenase and neutral protease. This self-perpetuating loop could explain many of the clinical observations seen in patients with CPPD crystal deposition disease.

The initial deposits of CPPD appear to be located at the lacunar margins of degenerating chondrocytes in areas of reduced safranin O staining, indicative of reduced acid mucopolysaccharide concentrations [15], whereas larger deposits are associated with cellular necrosis in the periphery [3]. CPPD deposits have also been observed on collagen bundles [2]. Although some crystals occur in areas of degeneration, others are found in cartilage possessing normal histology. The apparent *in vivo* preference of CPPD crystals for collagen denuded of proteoglycan suggests that the native collagen fibrils (or their accompanying macromolecules) may serve as nucleating templates for crystal formation.

The mechanism of CPPD crystal deposition in cartilage is not understood, in part because CPPD deposition apparently occurs in an undersaturated environment. There does not appear to be any gross disturbance in the systemic metabolism of inorganic pyrophosphate (PPi) in patients with CPPD crystal deposition disease [25]. Even in the elevated state, the [Ca] and [PPi] in the synovial fluid are undersaturated [1]. Thus, the need for an *in vitro* model for CPPD crystal deposition has long been recognized.

Although almost 30 different Ca and mixed Ca pyrophosphates have been synthesized [16], only two distinct crystallographic dimorphs of CPPD have been observed *in vivo*: triclinic (t) and monoclinic (m) CPPD. In menisci, which is the most common crystal deposition site [1], t-CPPD predominates in a ratio of 3:1 [21]. Since *in vitro* preparations of t-CPPD and m-CPPD necessitate a pH of 3.0 and high concentrations of Ca and PPi [20], solution studies modeling CPPD deposition have attempted to determine the

[Ca] and [PPi] necessary for crystal nucleation and growth at physiologic pH [7, 8, 9, 14].

CPPD crystal growth has also been modeled using biological grade gelatin [23], silica gel [23], polyacrylamide gel [13], and whole cartilage [24], however none of these models were able to reproduce t-CPPD and m-CPPD crystal growth at physiologic conditions. We successfully grew t-CPPD and m-CPPD at physiologic ionic concentrations using biological grade gelatin in an ionic diffusion model [18, 19]. However, other crystalline species were also formed and both t-CPPD and m-CPPD grew in non-physiologically observed crystal growth habits, i.e. as long needles. These studies did, however, indicate that the time of incubation of Ca and PPi ions was a critical parameter in determining the type of crystal formed, and that the formation of the two *in vivo* crystals was mediated by the formation of intermediate crystalline materials and the subsequent dissolution of those species. The formation/dissolution/reformation mechanism allowed for a very localized ionic concentrating process to occur.

Although the biological grade gelatin system allowed for a much better understanding of the possible kinetics of CaPPi crystal growth, the question arises as to whether native collagen fibrils act differently than the denatured form of collagen in mediating the crystal growth process. Therefore, we investigated the effect of type I collagen fibrils on CPPD crystal nucleation and growth using our ionic diffusion model.

Materials and Methods

Collagen purification

Type I collagen was isolated from porcine meniscus using a pepsin solubilization procedure [22]. Briefly, the menisci were dissected from the knee joints within 24 hours of slaughter of 4 to 6 month old pigs (Patrick Cudhay, Cudhay, WI). Standard surgical procedures were used to separate the medial and lateral menisci from each joint. The menisci were trimmed of surrounding fat, ligaments, and the opalescent anterior edge which is composed of both type I and II collagens. The menisci were thinly sliced, rinsed with a 0.9% saline solution at 4 °C, and freeze milled (Spex Industries, Metuchin, NJ) for a short time. In order to extract the proteoglycan prior to the collagen isolation procedure, cartilage fragments were then extracted twice with 20 volumes to weight of 4 M guanidine, 0.05 M Tris-HCl at pH 7.4 at 4 °C for 48 hours. Centrifugation between extractions was carried out at 30,000 x g. The precipitated fragments were then stirred for 15 hours at 4 °C in a sufficient volume of cold 0.5 M acetic acid containing 1 mg/ml pepsin to maintain a collagen to pepsin ratio of 10:1. The suspension was centrifuged at 35,000 x g for 1 hour at 4 °C. The extraction procedure was repeated, and the supernatants pooled. The soluble collagen was precipitated by the addition of sufficient crystalline NaCl to give a final concentration of 2.0 M and then collected by centrifugation at 35,000 x g for 2 hours at 4 °C. The precipitated type I collagen was then redissolved in 1.0 M NaCl, 0.05 M Tris-HCl at pH 7.5 and dialyzed overnight against this same solution. Any precipitated material was removed by centrifugation at 35,000 x g and discarded. The type I collagen was again precipitated overnight by the addition of crystalline NaCl to a final concentration of 4.0 M. Then, the solution was centrifuged for 2 hours at 35,000 x g, redissolved in 0.5 M acetic acid, dialyzed against 0.05 M acetic acid and then lyophilized. SDS gel electrophoresis [5] showed the collagen to be pure type I.

Gel Preparation

The gels were prepared as 3 layers in glass tubes (8 mm i.d. x 100 mm, total volume 5 ml). The ends of the tubes were stoppered with serum caps. Three collagen solutions were prepared in 0.05 M Hepes buffer (pH 7.4) at a concentration of 2.5 mg/ml. The bottom layer consisted of 1 ml of the Ca solution (25 mM $CaCl_2$, 78 mM NaCl, 4.5 mM KCl, 1.0 mM $MgSO_4$), the middle layer consisted of 2 ml of only the secondary ions (128 mM NaCl, 4.5 mM KCl, 1.0 mM $MgSO_4$,), and the top layer consisted of 1 ml of the PPi solution (10 mM $Na_4P_2O_7$, 88 mM NaCl, 4.5 mM KCl, 1.0 mM $MgSO_4$). Sodium azide (15 mM) was included in all layers to prevent fungal growth. The solutions were layered separately while cold and incubated at 37° C to gel before the next layer was poured. The total length of the gel was 8 cm.

The gel tubes were prepared in triplicate, incubated at 37° C, and harvested at 14 preset times over a 6 month period. In order to facilitate their handling at harvest, the gel tubes were immersed in an acetone-dry ice bath for 20 seconds. This short cold treatment kept the collagen gels from melting while they were being separated but did not induce any crystal growth. The gel was then pushed from the tube in 1 cm increments which were sliced off and placed into clean tubes for the analyses described below.

Crystal Characterization

The collagen gels were allowed to melt at room temperature and the crystalline material was isolated by centrifugation, washed twice with doubly distilled water, and air-dried. The supernatants from the gels were saved for Ca and PPi analyses as described below. All crystalline samples were characterized using copper radiation generated by a 12 kW high brilliance rotating anode x-ray generator (Rigaku, Japan). Samples were diffracted using a germanium crystal monochromated Guinier powder diffraction camera (Huber Instruments, West Germany). Diffraction patterns were compared to those of known pyrophosphate structures [16]. The term amorphous calcium pyrophosphate (a or a-CaPPi) was operationally defined as the presence of a significant amount of precipitate with a diffraction pattern containing a broad diffuse background but with no distinct diffraction lines. Representative samples were characterized further with scanning electron microscopy (Model 1200, Advanced Metals Research Corp., Bedford, MA).

Pyrophosphate assay

Pyrophosphate determinations were carried out by using the assay kit (P-7275, Sigma Chemical, St. Louis, MO). Aliquots of the meniscus gel were diluted 1:10 before analyses. In addition to determining the [PPi] at each point within the gel, we also determined the amount of PPi hydrolysis which had occurred as the experiment progressed by assaying the PPi level in standard collagen gel tubes, which contained only the PPi solution and were prepared at the beginning of the experiment. Hydrolysis was not a significant problem.

Calcium assay

Calcium was determined by atomic absorption analysis (Model AA-1475, Varian Associates, Palo Alto, CA). A 0.30 ml aliquot of the gel was diluted with a solution containing 1% $LaCl_3$ in 5% HCl according to standard procedures. Concentrations were determined using a 1000 µg/ml Ca standard in 2% HCl (Fisher Scientific, Itasca, IL) diluted as above.

Preparation of Ion Gradient Contour Plots

The [Ca] was plotted as a function of tube position and time. Contours were drawn at levels of 1 mM up to 10 mM, after which they were contoured at 5 mM intervals. The [PPi] contour plot was constructed in the same manner, except that the contour levels were 0.025, 0.05, 0.10, 0.25,

0.50, 0.75, 1.0 mM and then at 1 mM increments up to 10 mM, and the two plots superimposed photographically. The crystals which had been identified at each tube position and time point were then added to the composite ionic gradient plot.

Results

Figure 1 shows the ionic gradient plots of the [Ca] and [PPi] and the crystals formed in the gels. A diffuse amorphous band in the center of the gel was observed in 2 days which corresponded to the Ca/PPi ionic interface. Amorphous CaPPi formed when [Ca] was between 1 and 3 mM and when [PPi] was between 0.1 and 1 mM. This diffuse band spread throughout the tube within 2 weeks, after which time the [Ca] and [PPi] fell to physiologic levels.

At 3 to 4 months, most of the diffuse band had disappeared, leaving a clear gel which contained large clumps of crystals which were identified as t-CPPD and m-CPPD. Monoclinic CPPD crystallized first in 6 weeks when the [Ca] was 2 mM and the [PPi] was between 50 and 75 μM. Triclinic CPPD first formed at 13 weeks when the [Ca] was also 2 mM, but the [PPi] was less than 25 μM. As shown in Figure 2, the t-CPPD crystals which were isolated at 15 weeks had the very distinctive prismatic growth morphology, while the crystal growth habit of m-CPPD was rod shaped. There were numerous t-CPPD and m-CPPD crystals seen in the sample viewed and no other growth habits were observed. The primary crystal growth faces of t-CPPD are the {100}, the {011}, and the {001} [16,17], while those of m-CPPD are the {100}, the {010}, and the {001} [16].

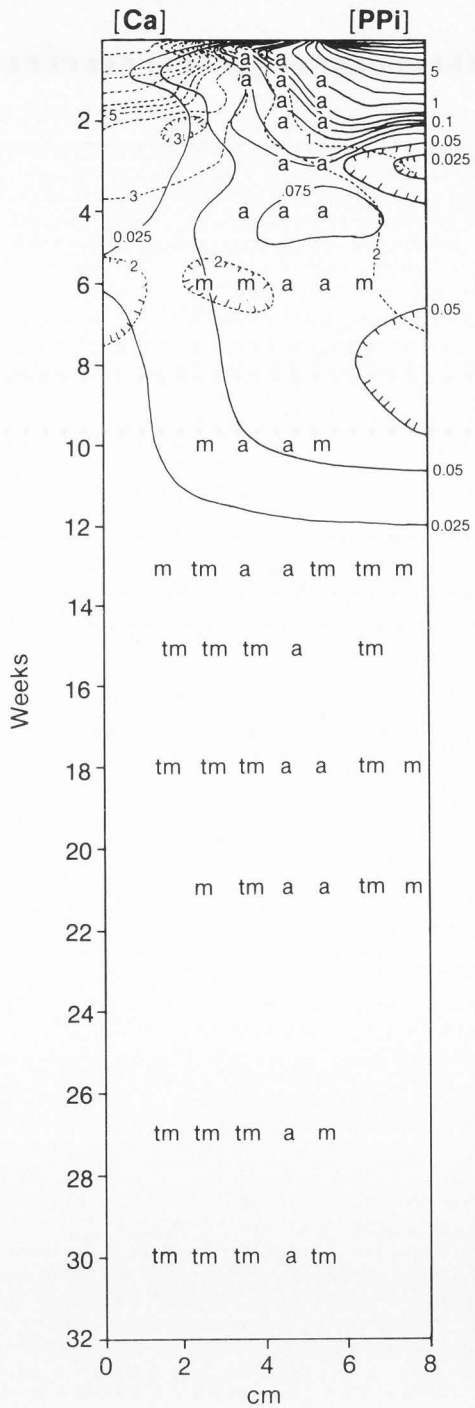
Discussion

Previous solution studies have shown that, although other calcium pyrophosphates would grow at physiologic ionic concentrations, t-CPPD and m-CPPD would not. Hearn and Russell [14] found by optical microscopy that at physiologic [Ca] and [PPi], the first species to form were an amorphous material when physiologic [Mg] was present and orthorhombic calcium pyrophosphate tetrahydrate (o-CPPT) when there was no Mg. Cheng and Pritzker [7] found that for t-CPPD and m-CPPD crystal formation in solution, the PPi concentration must be greater than 1 mM and 3.2 mM, respectively. The mean PPi level in normal synovial fluid is 3 μM [26] and 10 μM in patients with CPPD crystal deposition disease [27]. At physiologic levels of magnesium and phosphate, a-CaPPi formed, but neither t-CPPD nor m-CPPD would form [7, 8].

In our previous biological grade gelatin gels [18, 19], the diffuse band at the Ca/PPi interface was present at early times and always transformed from a-CaPPi to o-CPPT within the first few days of incubation. In contrast, in this study using meniscus collagen gel, this band remained amorphous throughout the entire experiment. Similar to the biological grade gelatin studies, m-CPPD was identified earlier than t-CPPD.

One of the most striking differences between this system and previous solution and gel model systems is the total absence of o-CPPT from the native collagen gels as compared to all the other model studies. Further, crystal morphology is more sensitive to subtle changes in the crystallization conditions than is crystal form. Using the native type collagen gels (Figure 2), crystals of t-CPPD appear as large single crystals with the classic prismatic growth habit observed *in vivo* [6], and crystals of m-CPPD

Figure 1: The kinetic map of CPPD crystallization in pepsin solubilized porcine meniscus type I collagen gels. The [Ca] and [PPi] ionic gradients have been contoured as a function of time and tube position and have been overlayed with the crystal types observed. The [Ca] has been contoured with dashed lines and the [PPi] is denoted with solid lines. The [Ca] has been contoured at 1 mM levels below 10 mM and at 5 mM levels above 10 mM. The [PPi] has been contoured at 0.025, 0.05, 0.075, 0.10, 0.25, 0.50, 0.75, 1.0 mM and then at 1 mM increments up to 10 mM. Tubes were prepared at $[Ca_{initial}]$ = 25 mM and $[PPi_{initial}]$ = 10 mM.



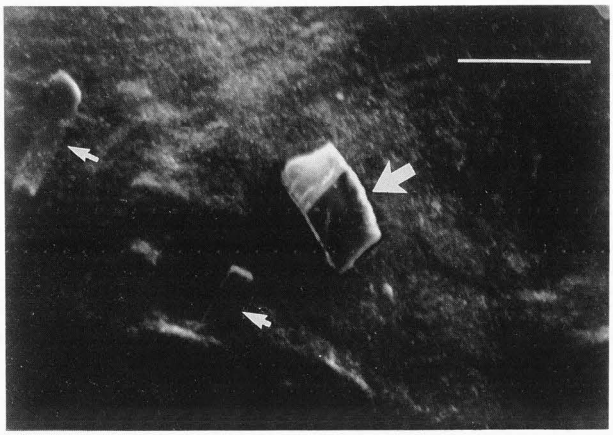


Figure 2: A scanning electron micrograph showing the prismatic crystal growth habit of t-CPPD crystals (large arrow) and the rod crystal growth habit of m-CPPD crystals (small arrows) observed in layer 3 after 15 weeks of incubation. These crystal growth morphologies are identical to those observed *in vivo*. Bar is 25 μm.

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have the classic rod habit. In contrast, the crystal growth habits of t-CPPD, m-CPPD, and o-CPPT grown in biological gelatin model systems were always needles [18, 19].

The ionic diffusion in collagen model system described in this study appears to be the most reasonable model system to date in that it has produced only crystals of a-CaPPi, t-CPPD and m-CPPD. Although the pepsin solubilizing procedure removes the telopeptides from the collagen molecules, it maintains near native conformation of the collagen fibrils themselves. The crystals were grown in the most nearly physiologic environment of all the model systems. Finally, the crystal growth habits of both t-CPPD and m-CPPD grown in the native collagen gel system match those observed *in vivo*. When compared to the previous studies, these results, particularly the crystal growth habit data, would seem to indicate that the native collagen fibrils themselves can nucleate CPPD crystal formation.

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Discussion with Reviewers

K.P.H. Pritzker: This paper describes the formation of m-CPPD and t-CPPD in an *in vitro* collagen gel system. Although the system is different, the results appear to be substantially similar to those recently published (Hunter GK, Grynpas MD, Cheng P-T, Pritzker KPH (1987) Effect of glycosaminoglycans on calcium pyrophosphate crystal formation in collagen gels. Calcif Tissue Int **41**, 164-170).
Authors: We disagree that these are similar to those of Hunter, et. al. First, their experiments were conducted at a Ca:P ratio of 2:1, rather than at the physiological ratio which is in the range of 150:1. The [Ca] and [PPi] which we have investigated in this study not only covered physiological ratios, but also included physiological concentrations.

Hunter, et.al. did not publish the crystallographic identification of the crystalline precipitates formed in their study. Using the three x-ray diffraction patterns which they published instead, we have determined that the crystals that they grew at 1.6 mM Ca and 0.8 mM PPi were, in fact, pure o-CPPT. Also, based on their published patterns, an unknown calcium pyrophosphate crystal was the sole crystalline product formed both at 1.6 mM Ca, 0.8 mM PPi, 0.32 mM Mg and at 40 mM Ca, 25 mM PPi, 8 mM Mg. We have previously reported and characterized this same unknown calcium pyrophosphate crystal, labelling it as Unknown B [19]. Using energy dispersive x-ray microanalysis, we found that this crystalline unknown has an atomic ratio of 0.69:0.08:1.0 for Ca:Na:P.

Finally, Hunter, et. al. described the crystal growth habit as "spherical clusters of needle-shaped crystals". Thus, their results form a very striking contrast to those presented here. There are two possible explanations for the differences in the results between their study and ours even though both experiments were conducted with type I collagen in near-native fibril conformation. One is the differences in experimental conditions and set-ups. Their super physiologic ionic concentration and Ca:PPi ratios has already been discussed. Another experimental difference is in the geometry of the diffusion. In their system, high PPi concentrations were incorporated into a collagen gel and a solution containing high Ca concentrations was layered directly on top of the gel. Another possible contributing factor accounting for these differences may be related to differences in the conformation of collagen isolated from rat tails by acid solubilization versus the conformation of collagen isolated from fibrocartilage by pepsin solubilization.

K.P.H. Pritzker: The authors suggest that type I collagen nucleates crystal formation. The evidence for this inference is not clear. It is possible to grow t-CPPD and m-CPPD in solution and gels by other methods. In these systems, the CPPD crystals can have similar morphology to *in vivo* crystals.
Authors: We agree that the evidence which suggests that type I collagen nucleates CPPD crystal formation is primarily negative, i.e. it is based on the absence of a result rather the presence of concrete data. However, it is a very compelling argument when all the data are reviewed together. It is significant that only t-CPPD, m-CPPD, and a-CaPPi were formed by ionic diffusion of physiological levels of Ca and PPi in the presence of type I collagen, while other non-physiologic calcium pyrophosphates were identified in all of the other previous crystal growth experiments. This fact, coupled with the fact that the characteristic crystal growth habits of t-CPPD and m-CPPD only have been observed in this study and in no other studies, has formed the basis for this inference. We are currently conducting experiments to characterize and quantitate the possible nucleating potential of type I collagen in a more positive way.

R. Terkeltaub: Is there any difference from the results in Figure 1 when different procedures are used to isolate type I collagen from porcine meniscus?
Authors: There is no other way to isolate type I collagen from meniscus. The more gentle isolation procedures used to isolate type I collagen from rat tails will not yield any collagen when applied to cartilagenous tissues. However, we are currently investigating the differences in CPPD crystal growth in gels made of pepsin solubilized meniscus collagen and acid solubilized rat tail collagen.

R. Tawashi: The fact that different faces of the m-CPPD and t-CPPD crystals are developed in the native collagen fibril gel system suggests that the structure of collagen fibrils as well as the presence of other impurities (e.g. sodium azide) are responsible for the direction of growth. I wonder how the growth process will proceed in the absence of 15 mM sodium azide, if growth was conducted under asceptic conditions.
Authors: All of the gel studies conducted in references 18 and 19 were also conducted in the presence of sodium azide. The only previously published report on the formation of t-CPPD and m-CPPD at physiologic levels of calcium and pyrophosphate was in one of our previous ionic diffusion experiments using biological gelatin [18]. In this experiment, the sodium azide concentration was 30 mM. This sample of t-CPPD and m-CPPD also contained o-CPPT. In the biological gelatin experiment, the crystal growth habits of all three crystals were indistinguishable from each other, as they all grew either as needles and as clumps of needles radiating from a single point.

R. Terkeltaub: Have you evaluated collagen isolated from animals with experimental degenerative joint disease? What are the effects of proteolytic collagen peptides or synthetic collagen oligopeptides on CPPD crystal nucleation in this model? Can proteoglycans native to articular cartilage or the extent of proteoglycan polymerization influence CPPD crystal nucleation in this model?
Authors: All of these questions are very important to our understanding of the pathogenesis of CPPD crystal deposition disease. Currently, we are investigating the possibility that it is the method of collagen extraction, i.e. the

nature of the collagen degradation, which may play a role in the apparent potential for type I collagen to nucleate CPPD crystals. These other questions will be addressed in future studies.