Inhibition of Calcium Pyrophosphate Dihydrate Crystal Formation in Articular Cartilage Vesicles and Cartilage by Phosphocitrate*

(Received for publication, March 13, 1996, and in revised form, August 6, 1996)

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Articular cartilage vesicles (ACV), isolated by differential centrifugation of adult hyaline articular cartilage collagenase digests, mineralized in the presence of calcium and ATP. Mineral analysis by microscopy, chemical analysis, energy-dispersive analysis, and infrared spectroscopy revealed crystals resembling calcium pyrophosphate dihydrate (CPPD). Adult articular cartilage also underwent ATP-dependent mineralization, supporting the contention that vesicles in situ fostered adult articular cartilage mineralization.

Phosphocitrate (PC) is a recognized in vitro inhibitor of hydroxyapatite and calcium oxalate monohydrate crystal formation, but it is not known whether PC can similarly restrict CPPD crystal development. In the present study we examine the effect of PC, citrate, and n-sulfo-2-amino-tricarballylate (SAT, a PC analogue) on the ATP-induced CPPD crystal formation in both ACV and articular cartilage models.

Only PC (10–1000 μM) blocked both the ATP-dependent and -independent mineralization in ACV in a dose-dependent fashion. At 1 μM, SAT and citrate blocked the ATP-independent mineralization. Similarly, only PC blocked both the ATP- and non-ATP-dependent mineralization in native articular cartilage slices. PC, SAT, and citrate had no effect on ACV nucleoside triphosphate pyrophosphohydrolase activity, suggesting that none of these agents blocked mineralization through the inhibition of nucleoside triphosphate pyrophosphohydrolase activity, which generates inorganic pyrophosphate from ATP.

Crystalline calcium pyrophosphate dihydrate (CPPD) and basic calcium phosphate (BCP; a term including carbonate-substituted apatite, octacalcium phosphate, and tricalcium phosphate) are the two common forms of pathologic articular mineral. Each occurs frequently in degenerative joints, and each may be phlogistic, causing acute attacks of pseudogout, in the case of CPPD crystals, and acute calcific periartthritis, in the case of BCP crystals (1, 2). Both crystal species appear in cartilage (chondrocalcinosis) and can engender enzymatic damage to the cartilage matrix. Pathways by which this damage occurs have been studied in our laboratory and have been found to particularly involve the induction of collagen and stromelysin synthesis and secretion (3). Formation of these crystals is poorly understood.

Articular cartilage vesicles (ACV), isolated by differential centrifugation of adult hyaline articular cartilage collagenase digests, mineralized in the presence of physiologic concentrations of calcium when exogenous ATP was added. Analysis of mineralized ACV by microscopy, chemical analysis, energy-dispersive analysis, and infrared spectroscopy revealed crystals resembling CPPD (4). Subsequent electron beam diffraction studies confirmed CPPD formation (5). Crucial to the formation of CPPD crystals by ACV is the ACV enzyme nucleotide triphosphate pyrophosphohydrolase (NTPPPH), which generates inorganic pyrophosphate (PPi) from ATP (6). ACV are enriched in NTTPPPH compared with cell membrane (7).

Using differential centrifugation, Derfus et al. (7) separated ACV into a “heavy vesicle fraction” (HVF) and a “light vesicle fraction” (LVF). HVF consists of two populations of vesicles measuring 120 ± 24 nm and 50 ± 11 nm. LVF is composed of a homogeneous population of vesicles measuring at 57 ± 13 nm. Both fractions supported ATP-dependent calcium pyrophosphate precipitation and formation of CPPD crystals.

Ryan et al. (8) extended these studies by demonstrating that native adult articular cartilage also underwent ATP-dependent mineralization, as had isolated ACV, supporting the contention that vesicles foster adult articular cartilage mineralization in situ. This in vitro system may serve as a model for the study of chondrocalcinosis caused by BCP and CPPD.

Our present understanding of the mechanisms of pathological calcification is limited; therefore, no reliable method has been devised to prevent calcium crystal deposition. Phosphocitrate (PC) is a naturally occurring compound that has been identified in mammalian mitochondria (9) and crab hepatopancreas (10). It has been speculated that PC may have an important role in preventing calcium phosphate precipitation in cells or cellular compartments maintaining high concentrations of calcium and phosphate (10). PC prevents soft tissue calcification in vivo and does not produce any significant toxic side effect in rats or mice when administered through intraperitoneal injections in doses up to 150 μmol/kg/day (11, 12).

Although PC is a potent in vitro inhibitor of BCP (11, 13) and calcium oxalate monohydrate crystal formation (14, 15), it was not known whether PC had any inhibitory effect on CPPD crystallization. In the present study, we examine the effect of citrate, PC, and n-sulfo-2-amino-tricarballylate (SAT, a PC analogue) (Fig. 1) on the ATP-induced CPPD crystal formation in both ACV and native articular cartilage models.

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*This work was supported in part by National Institutes of Health Grants AR38421-06 (to H. S. C.) and AR38856-07 (to L. M. R.) and a grant from National Health and Medical Research Council, Australia (to J. D. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: CPPD, calcium pyrophosphate dihydrate; BCP, basic calcium phosphate; ACV, articular cartilage vesicles; NTPPPH, nucleotide triphosphate pyrophosphohydrolase; HVF, heavy vesicle fraction; LVF, light vesicle fraction; PC, phosphocitrate; SAT, n-sulfo-2-amino-tricarballylate; DMEM, Dulbecco’s modified Eagle’s medium; HBSS, Hank’s balanced salt solution without calcium and magnesium; CSS, calcifying salt solution.
Dulbecco’s modified Eagle’s medium (DMEM), Hanks’ balanced salt solution without calcium and magnesium (HBSS), fetal bovine serum, penicillin/streptomycin/fungizone, and HEPES buffer were obtained from Life Technologies, Inc. 5Ca-labeled CaCl2 was from Amersham Corp. [32P]ATP was from obtained from DuPont NEN. Collagenase, trypsin, hyaluronidase, ATP, sodium pyrophosphate decahydrate, AMP, citrate, and yeast inorganic pyrophosphatase were from Sigma. Protein assay reagents for cell protein measurements were from Bio-Rad (Richmond, CA).

PC was prepared as the sodium salt by phosphorylation of tribenyl citrate followed by deprotection through hydrogenation. The monosodium salt was crystallized from water (16). SAT was synthesized according to the procedure of Brown et al. (12).

ACV isolation and mineralization were done according to the procedure outlined by Derfus et al. (4, 7). Briefly, hyaline articular cartilage was obtained from femoral condyles and patellae of freshly slaughtered pigs. Cartilage slices were sequentially treated with 0.1% (w/v) hyaluronidase, 0.05% (w/v) collagenase overnight.

The cartilage digest was serially centrifuged in a Beckman L-8-80M ultracentrifuge (Beckman, Irvine, CA) according to the following protocol: 300 x g for 10 min to remove intact cells; 20,000 x g for 10 min to remove larger cell fragments; 50,000 x g for 60 min to pellet the HVF; and then 200,000 x g for 40 min to pellet LVF. The ACV fractions were washed twice and resuspended in HBSS to a final concentration of 3–6 mg of protein/ml.

ACV mineralization was assayed in the presence or absence of test inhibitors (PC, SAT, or citrate) in a calcifying salt solution (CSS) containing 2.2 mM CaCl2, 1.6 mM KH2PO4, 1.6 mM MgCl2, 85 mM NaCl, 15 mM KCl, 10 mM NaHCO3, 50 mM n-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, and 1 mM ATP, pH 7.6. Twenty-five microliters of ACV suspension was added to 500 μl of CSS in 1.5-ml polypropylene tubes, vortexed, and incubated in a 37°C water bath. CSS was labeled was trace-labeled with 45Ca (1 Ci/ml). ATP-dependent mineralization in both ACV fractions was assayed in the presence or absence of test inhibitors in CSS as outlined under “Experimental Procedures.” 1 mM of citrate (C), PC, and SAT were used in the experiments outlined in both panels A and B. B, effect of citrate, PC, and SAT on mineralization of HVF. Experimental procedures were identical to that for panel A. Effect of citrate, PC, and SAT on mineralization of “light vesicle fraction.” Experimental procedures were identical to that of Fig. 2a.

A native articular cartilage mineralization assay was performed according to Ryan et al. (8). Briefly, pig hyaline articular cartilage slices were cultured in a 20-fold (v/wt weight) volume of 50 mM HEPES-buffered DMEM, pH 7.4, containing 10% fetal bovine serum. Medium was trace-labeled with 4Ca (1 μCi/ml). ATP-dependent mineralization in the presence or absence of test inhibitors was measured as cartilage retention of 4Ca counts after incubation with ATP. A concentrated solution of ATP was added to the explant cultures to bring the media to 100 μM with respect to ATP. After 48 h the incubations were terminated by transferring cartilage to a separate plate and washing extensively with buffered DMEM. Cartilage was then hydrolyzed by heating to 70°C in 6 N HCl for 1 h. Aliquots of the digest were counted for 45Ca.

To determine the chemical form of phosphate precipitated, triplicate preparations of porcine cartilage and porcine chondrocyte monolayers were trace-labeled with [32P]ATP instead of 4Ca. The resultant mineral was isolated by centrifugation after digestion with collagenase (2 mg/ml in DMEM) overnight. The pellet was washed with DMEM, resuspended, and dissolved in 200 μl of 2 N EDTA in water. Dissolved mineral containing 32P was then diluted in DMEM to 500 μl. An aliquot was assayed for content of 32P inorganic phosphate (P) using the method of Sugino and Miyoshi (17). Another aliquot was treated for 1 h at 37°C with bakers’ yeast pyrophosphatase (2 units/ml) and then analyzed for [32P]Pi content by the same method. The amount of PPi was determined as the difference between P, present before and after digestion with pyrophosphatase.

Twice washed ACV mineral from the 48-h incubation was examined by guest on June 23, 2017 http://www.jbc.org/ Downloaded from

FIG. 1. The formulas of phosphocitrurate, citrate, and SAT at physiological pH.

FIG. 2 A, Effect of citrate, PC, and SAT on mineralization by heavy matrix vesicle fraction. ACV were incubated by differential centrifugation of adult hyaline cartilage collagenase digested into “heavy vesicle fraction” [HVFP] and “light vesicle fraction” [LVF], ATP-dependent (●) and independent (■) mineralization assays were performed in the presence or absence of test inhibitors in CSS as outlined under “Experimental Procedures.” 1 mM of citrate (C), PC, and SAT were used in the experiments outlined in both panels A and B. B, effect of citrate, PC, and SAT on mineralization of HVF. Experimental procedures were identical to those for panel A. Effect of citrate, PC, and SAT on mineralization of “light vesicle fraction.” Experimental procedures were identical to that of Fig. 2a.

using a compensated polarized light microscope (Leitz, Midland, Ontario, Canada). Samples from similar incubation but in the presence of PC were examined for comparison. The presence of PPi was confirmed by testing positively birefringent crystals for susceptibility to dissolution with bakers’ yeast pyrophosphatase (10 μl of a 0.2 unit/ml stock solution of bakers’ yeast pyrophosphatase in HBSS containing 0.5 mM MgCl2 and 0.4 mM MgSO4 for 1 h at 37°C).

The NTPPPH enzyme activity assay was performed by combining a 100-μl ACV suspension with 100 μl of 2 mM thymidine monophosphate n-nitrophenyl ester, 50 mM HEPES-buffered HBSS, pH 7.4. The mixture was incubated at 37°C for 2 h before terminating the reaction by the addition of 800 μl of 0.1 N NaOH. The p-nitrophenol product was determined by spectrophotometer at 410 nm. Triple determinations were made on all samples. NTPPPH activity is expressed as nmol of p-nitrophenol formed/mg of protein (18, 19).

Measurements are expressed as the means ± S.D. All experiments were repeated at least 3 times. The Wilcoxon rank sum test was used to compare paired samples.

RESULTS

The effects of PC, SAT, and citrate on the mineralization of HVF and LVF in the presence or absence of ATP are summarized in Fig. 2, A and B, respectively. Only PC (1 mM) blocked both the ATP-dependent and -independent mineralization in both HVF and LVF. At 1 mM, SAT and citrate only blocked the ATP-independent mineralization.

FIG. 3 shows that PC (10 μM to 1 mM) inhibited ATP-dependent mineralization in both ACV fractions in a significantly (p <
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FIG. 3. Dose response of PC on mineralization of HVF (■) and LVF ([ ]). Mineralization assay was done in similar fashion as in Fig. 2 except in the presence of various concentrations of PC. A, control, no PC; B, 10 μM; C, 20 μM; D, 100 μM; E, 200 μM; F, 1 mm.

FIG. 4. Effect of PC and citrate on native articular cartilage mineralization. The mineralization assay was performed according to Ryan et al. (6) and outlined under “Experimental Procedures.” In a typical experiment, seven groups of explants were studied: one with no added ATP (0); another with ATP added at time 0 (1); and with ATP added twice (0 and 8 h) (2); 3 times (0, 8, and 16 h) (3); 4 times (0, 8, 16, and 24 h) (4); 5 times (0, 8, 16, 24, and 32 h) (5); and 6 times (0, 8, 16, 24, 32, and 40 h) (6). After 48 h, the incubations were concluded by transferring cartilage to a separate plate and washing extensively with buffered DMEM. Cartilage was then hydrolyzed by heating to 70°C in 6 M HCl for 1 h. Aliquots of the digest were counted for 45Ca. ■, HVF; □, LVF; + inhibitor; , LVF; [], LVF + inhibitor.

FIG. 5. Effect of citrate (C), PC, and SAT on NTPPPH activity. Enzyme activity was measured by combining 100-μl ACV suspensions with 100 μl of 2 mM thymidine monophosphate n-nitrophenyl ester, 50 mM HEPES-buffered HBSS, pH 7.4. The mixture was incubated at 37°C for 2 h, and then the reaction was terminated by the addition of 800 μl of 0.1 N NaOH. The p-nitrophenol product was determined by spectrophotometer at 410 nm. Triple determinations were made on all samples. NTPPPH activity is expressed as nmol of p-nitrophenol formed/h/mg of protein. ■, HVF; □, HVF + inhibitor; , LVF; [], LVF + inhibitor.

FIG. 6. HVF ATP-dependent mineralization as a function of delayed addition of PC (1 mM). Mineralization assays were performed in CSS as outlined under “Experimental Procedures.” A, control (no PC added); B, PC added at time 0; C, PC added at 2 h; D, PC added at 4 h; E, PC added at 6 h; F, PC added at 24 h; G, PC added at 30 h. Incubation time for all the samples was 48 h.

As previously shown by Derfus et al. (7), HVF possessed approximately 3–4-fold higher NTPPPH specific activity than LVF. PC, SAT, and citrate had no effect on NTPPPH activity in either ACV fraction in the presence or absence of ATP (Fig. 5), suggesting that none of these agents blocked mineralization through the inhibition of NTPPPH activity (7, 20).

When mineral was trace-labeled with 32P derived from [γ-32P]ATP, most of the 32P moiety was in the form of PPi. Mineral produced in the control cultures contained 95% [32P]PPi, and 5% [32P]Pi (monolayers) and 97% PPi, and 3% P, (cartilage). In the presence of PC, although the total amount of mineral was greatly reduced, mineral phase was still predominantly PPi (97% PPi in monolayer and 96% in cartilage).

Compensated polarized light microscopy of similarly prepared samples incubated in the absence of PC confirmed a population of strongly positively birefringent, rod-shaped crystals. This appearance is very similar to that of CPPD crystals (data not shown). Exposure of ACV mineral to pyrophosphatase eliminated all of these birefringent crystals, thereby confirming their PPi content.

HVF ATP-dependent mineralization as a function of delayed addition of PC (1 mM) is summarized in Fig. 6. PC similarly inhibited mineralization (~70%) when added up to 6 h after the incubation began. With a delayed addition of PC at 24 and 30 h of incubation, the inhibitory effect of PC on mineralization diminished to 42 and 34%, respectively, suggesting that PC may have slowed crystal growth subsequent to nucleation.

DISCUSSION

In the present study, both the ACV and the native adult cartilage models were used to examine the effect of PC, SAT (PC analogue), and citrate on the ATP-dependent and -independent mineralization. The native cartilage model complements the ACV model, because it eliminates the effects that ACV release and isolation procedures may have on mineralizing capacity. It also allows mineralization to occur in unaltered cartilage matrix. Since PC blocked both ATP-dependent and -independent mineralization in both systems, diffusion of PC through the cartilage matrix to block mineralization in cartilage is likely.

Recently, Kranendonk et al. (21) reported that CPPD-like crystals were the predominant species formed in the presence of ATP, while BCP crystals were the primary crystals formed in the absence of ATP in the ACV mineralization model. Since PC, SAT, and citrate are known inhibitors of BCP crystal formation (11, 13), our present results (Figs. 2–4) would support the...
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Notion that in the absence of ATP, BCP crystal formation would be blocked by all three compounds. Since only PC blocked ATP-induced mineralization in ACV (both HVF and LVF) and cartilage explants, this provides the first evidence suggesting that PC can specifically inhibit the formation or growth of CPPD crystals.

As in the ACV system, the cartilage explant mineralization requires hydrolysis of ATP to produce PPi. NTPPPH is the enzyme responsible for generation of PPi from extracellular ATP in cartilage (22, 23). It is a membrane-associated ectoenzyme found in cell membranes and vesicle fraction of adult articular cartilage (6, 24). Since PC, SAT, or citrate had little effect on the NTPPPH enzyme activity in both HVF and LVF (Fig. 5), the possibility that PC inhibits CPPD crystal formation through inhibition of NTPPPH activity is unlikely (7, 20).

Numerous studies with PC have established that it powerfully restricts transformations involved in the nucleation, growth, and aggregation of many calcium salts including phosphate, oxalate, and carbonate (13–15, 25–27). The strong binding affinity that PC possesses for growing crystals accounts for the superior inhibitory capacity compared with citrate and SAT and is believed to result from both its multinegative charges (Fig. 1) and natural stereochemistry (28). Recent insights into the mechanism of PC action have been gained by exploring its interaction with the crystal faces of calcium oxalate monohydrate using both experimental evidence and computer modeling (14, 15). PC with both its PO4 and carboxylate groups contributing has been shown to bind more favorably than citrate (possessing carboxylates only) with calcium ions distributed on the (−1 0 1) and (0 1 0) surfaces of calcium oxalate monohydrate crystal, thus blocking growth. We speculate that the inhibitory action of PC on the CPPD unit cell lattice, although different from calcium oxalate monohydrate or BCP, would be achieved by a similar inhibitory mechanism, and this aspect is currently being explored. SAT is not as efficient an inhibitor as PC for calcium salts examined so far, which probably reflects its stereochemistry and the fact that both a sulfate moiety (less one charge than PO4) and the presence of a nitrogen atom reduce its capacity to position itself correctly for crystal face interaction.

Our earlier studies had shown that PC specifically inhibits CPPD and BCP crystal-induced metalloproteinase synthesis and mitogenesis in human fibroblasts in vitro, while PC has no effect on similar biologic responses induced by epidermal growth factor, platelet-derived growth factor, and serum (29). PC prevents disease progression in murine progressive ankylosis (an animal model of crystal deposition diseases), a condition marked by extensive BCP deposition (30). Taken together with the present data, PC may be considered a potential therapeutic agent for both CPPD and BCP crystal deposition diseases. Our working hypothesis is that PC will have dual beneficial effects of blocking the degeneration-promoting effects of crystals e.g. metalloproteinase synthesis and mitogenesis (30–32), and of inhibiting further BCP and CPPD crystal formation in articular tissue (11, 12).

REFERENCES


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