Dissolution of calcium pyrophosphate crystals by polyphosphates: an in vitro and ex vivo study

R Cini, D Chindamo, M Catenaccio, S Lorenzini, E Selvi, F Nerucci, M P Picchi, G Berti, R Marcolongo

Abstract

Objective—To determine the dissolving ability (DA) of linear pentasodium tripolyphosphate (PSTP), cyclic trisodium metaphosphate (TSMP), polymeric sodium metaphosphate (SMP) on synthetic crystals of calcium pyrophosphate dihydrate (CPPD) and on crystalline aggregates of menisci from patients with chondrocalcinosis (CC).

Methods—Synthetic CPPD crystals were mixed with phosphate buffered saline (PBS), which contained the different polyphosphates, for one hour at 37°C. The calcified menisci were obtained from the knees of four female patients with CPPD disease who underwent total arthroscopic meniscectomy for degenerative meniscal lesions. Meniscal cryosections and fragments were incubated in SMP (15 mg/ml PBS) at 37°C for one hour and 24 hours, respectively. Histological evaluation on meniscal samples after polyphosphate incubation was carried out by ordinary transmitted light microscopy and polarised light microscopy. The dissolution of CPPD crystals by polyphosphates was assessed by atomic absorption spectrophotometry, which determined the amount of calcium liberated from synthetic crystals and meniscal fragments. Cytotoxicity of SMP was evaluated by tetrazolium salt assay and by an ultrastructural study on cultured chondrocytes.

Results—SMP and PSTP showed higher DA on CPPD crystals than TSMP. Analysis of the DA values at increasing concentrations of SMP showed that a concentration of 15 mg/ml completely dissolved 2.0 mg CPPD crystals. The solution of meniscal CPPD crystals showed a significant increase of calcium concentration after three hours and 24 hours of SMP incubation (p=0.0001; Kruskal-Wallis analysis of variance) compared with fragments incubated in PBS control solution. Macroscopic and microscopic evaluation of meniscal specimens showed a notable reduction of CPPD deposits. A 50% inhibitory dose on cultured chondrocytes was reached at the maximum concentration of SMP used in this work (15 mg/ml); ultrastructural analysis did not show morphological alterations in the treated cells.

Conclusion—The results of this study indicate that linear polyphosphates are effective in dissolving both synthetic and ex vivo CPPD crystal aggregates. This suggests a potential therapeutic use for these molecules in the treatment of symptomatic CC.

Chondrocalcinosis (CC) is a disease characterised by crystalline deposits of calcium pyrophosphate dihydrate (CPPD; [Ca$_2$(PO$_4$)$_2$]·2H$_2$O), which tend to form solid aggregates within the articular cartilage, the synovial membrane, the articular capsule, the tendons, and the ligaments. Crystal shedding in the synovial cavity is thought to trigger acute arthritis (pseudogout). CPPD crystals were first discovered by McCarty some 40 years ago in the synovial fluid of a patient who had recurrent arthritis. Other reports confirmed the role of CPPD crystalline aggregates in setting off an inflammatory response. A number of studies have been performed to evaluate the dissolution of CPPD crystals both in vitro and in vivo, but an effective and safe treatment is yet lacking. Bennett and colleagues observed that joint lavages with calcium chelating agents including sodium EDTA could dissolve CPPD crystals in patients with CC and recurrent bouts of pseudogout; nevertheless, all the experiments provoked a pseudogout attack. Oral magnesium has been reported to lessen the inflammatory manifestations of CPPD crystal related arthropathy, and there was no evidence of decreased deposits.

Our study aimed at estimating the dissolving ability (DA) values of some phosphate-containing molecules, particularly linear and cyclic phosphates, on CPPD crystals in aqueous systems under physiological conditions (pH 7.4 and osmolality in the range 280–300 mmol/kg). These compounds were chosen because their chelating ability towards metal ions has been known for decades and because previous research showed that chain polyphosphates (such as the Graham salts) reduced aortic calcifications in rats.

Materials and methods

Materials

Cyclic Na$_5$(PO$_4$)$_3$·linear Na$_8$(PO$_4$)$_6$·6H$_2$O, CaCl$_2$, MgCl$_2$, NaCl, Dulbecco’s calcium-magnesium free phosphate buffered saline (PBS), Dulbecco’s modified Eagle’s medium (DMEM), clostridium collagenase, tetrozolum salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulphoxide were all Sigma (Italy) analytical grade products. Linear Na$_5$(PO$_4$)$_3$ was from C...
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963
times and the mean (SD) values were expressed
eter. All experiments were performed three
al absorption spectrophotom-
µm). The filtrates, stored in polythene test tubes,
membrane surfactant-free cellulose acetate
filtration, which was measured by a Perkin-Elmer
alculisation tests on the calcified menisci.

EX VIVO TESTS
To evaluate the DA of the polyphosphate on
the tissue aggregates of CPPD we carried out
solubilisation tests on the calcified menisci.

The menisci were obtained from four female
patients (mean (SD) age 60 (3) years) with

Table 1 Analytical data for the dissolution of 2 mg calcium pyrophosphate dihydrate crystals in different polyphosphate samples

<table>
<thead>
<tr>
<th>Dissolving solution*</th>
<th>DA values† (SD) (Ca²⁺ µg/ml)</th>
<th>Dissolution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 mg SMP*/ml</td>
<td>24.1(3)</td>
<td>21.9</td>
</tr>
<tr>
<td>1.5 mg TSMP*/ml</td>
<td>1.6(0.5)</td>
<td>1.5</td>
</tr>
<tr>
<td>2.3 mg PSTP*/ml</td>
<td>21.7(4.3)</td>
<td>19.7</td>
</tr>
</tbody>
</table>

*The amount of the polyphosphates corresponds to a concentration of 14.7 mM phosphate in phosphate buffered saline (280–300 mmol/kg, pH 7.4).
†Dissolving ability (DA) values were expressed as concentrations of calcium ions present in solution after one hour of incubation at 37°C.
§SMP = sodium metaphosphate; TSMP = tetrakisodium tripolyphosphate.

Figure 1 Structural formulas for (A) pyrophosphate, O(PO₃)²⁻; (B) a linear triphosphate, O(PO₃)₃⁻; (C) cyclic trimetaphosphate, (PO₄)₃⁻; (D) polymeric metaphosphate (PO₄)(₅⁻)

Figure 2 Effect of increasing concentrations of polymeric sodium metaphosphate (SMP) on the solubilisation of synthetic CPPD crystals (2 mg) after one hour of incubation at 37°C. DA = dissolving ability. The bars show the mean (SD) of DA values obtained by three separate experiments. Values in brackets show the percentage of dissolution.

Erba (Italy). All compounds were used as pur-
chased without any further purification. Figure 1 shows selected structural formulas.

IN VITRO TESTS
CPPD synthesis
Triclinic and monoclinic CPPD crystals were prepared as described elsewhere. As the dissolving rate of crystals in fluids is related, among other things, to the size of the solid particles, all synthetic CPPD crystals were prepared rigorously under the same conditions to ensure size uniformity. The triclinic and monoclinic crystal phase and the quality of each CPPD crystal preparation were determined by powder x-ray diffraction analysis; the average crystal sizes were checked with a polarised light microscope. The crystals (needle-rhomboïd shaped) had average dimensions of 1–30 µm for all preparations.

Determination of the DA values for CPPD crystals
The solutions of the polyphosphates were prepared by mixing a weighed amount of the compounds with PBS. The pH and osmolality were checked before the addition of the CPPD crystals for all the solutions.

Samples of the air dried CPPD crystals (2.0 mg each) were mixed with PBS (5.0 ml each), which contained the polyphosphate. The mixtures were allowed to remain at 37°C for one hour with continual stirring. The mixtures were then filtered through syringes equipped with membrane surfactant-free cellulose acetate Nalge Nunc International NALGENE filters (disk diameter 25 mm; mean hole diameter 0.22 µm). The filtrates, stored in polythene test tubes, were then analysed for the calcium concentration, which was measured by a Perkin-Elmer Analyst 100 atomic absorption spectrophotometer. All experiments were performed three times and the mean (SD) values were expressed as µg Ca²⁺/ml after correction for the calcium contents in the dissolving solutions which were not mixed with CPPD crystals.

Microscopic study
Transverse 7 µm sections were observed under ordinary transmitted and polarised light microscope and photographed before and after incubation in polymeric sodium metaphosphate (SMP) (15 mg/ml PBS) for one hour at 37°C. The negative controls were obtained by incubating a section of each sample in PBS.

Macrosopic study
This study was carried out on the other two fragments. These were photographed, incubated in SMP (15 mg/ml PBS) and photographed again after one hour, three hours, and 24 hours of treatment. At the same time intervals, a 1 ml aliquot from each solution was filtered and subsequently tested for calcium concentration by atomic absorption spectroscopy. Statistical analysis of the data was carried out by Kruskal-Wallis analysis of variance on ranks. Significance was established at p<0.05.
At the end of the 24 hours’ incubation, the fragments were cut at the mid-line and the resulting two fragments of each sample were snap frozen. Cryosections (7 µm each) were collected on slides and observed under transmitted and polarised light microscopy. Finally, the incubating solutions were centrifuged at 700 g for 10 minutes, and the resulting pellets were observed under polarising microscopy to evaluate the presence of CPPD crystals liberated from meniscal fragments. Negative controls were obtained by incubating fragments of each sample in PBS.

Chondrocyte cytotoxicity determinations

Human articular cartilage was obtained from the femoral heads of four osteoarthritic subjects (mean (SD) age 67.5 (2.5) years) undergoing surgery for total hip prostheses. Immediately after surgery, macroscopically healthy cartilage was removed in aseptic conditions and minced into 2 mm³ pieces. The cartilage fragments were washed in saline solution (140 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM HEPES, pH 7.4) containing 200 U/ml penicillin and 200 µg/ml streptomycin. The cartilage was then digested by clostridial collagenase 1 mg/ml in PBS containing the same concentration of antibiotics. The collagenase digestion was carried out at 37°C for 14–18 hours with moderate stirring. The solution was then filtered, washed in saline solution, and centrifuged for 10 minutes at 700 g. As shown by the trypan blue viable stain, 90–95% of the cells recovered were alive. Chondrocyte cells were plated out in 200 µl of DMEM supplemented with l-glutamine (2 mM), fetal calf serum (10%), penicillin (200 U/ml), and streptomycin (200 µg/ml) in 96 well microtitre plates (10⁴ cells/well) and allowed to attach for 24 hours (37°C, 5% CO₂). Cells were then incubated with an increasing concentration of SMP in PBS (pH 7.4; 280–300 mmol/kg) for 24 hours (six wells for each SMP concentration). The control culture was obtained by incubating cells with PBS for 24 hours. Cytotoxicity was determined two days after exposure to SMP by the MTT assay, which measures the number of metabolically active cells by a colorimetric technique, as reported elsewhere.¹¹ Results were expressed as mean (SD).

Transmission electron microscopy

The human chondrocytes, treated for 24 hours with increasing concentrations of SMP, were mechanically detached from the wells, rinsed in PBS, centrifuged at 700 g for 10 minutes, fixed for two hours at 4°C in cold Karnovsky fixative, rinsed overnight in 0.1 M pH 7.2 cacodylate buffer, and post-fixed for one hour at 4°C in 1% buffered OsO₄, dehydrated in a graded series of ethanol, and embedded in Epon-Araldite. Ultrathin sections cut with an LKB III ultramicrotome were collected in copper grids, stained with uranyl acetate and lead citrate, and then photographed with a Philips CM10 electron microscope. For the ultrastructural examination about 30 chondrocytes for each patient were observed by transmission electron microscopy.

COMPLEX FORMATION ENERGIES

All density functional calculations were performed using the Gaussian94/DFT package on an Origin 2000 SG machine.¹³ Geometry
optimisations and energy calculations were performed using the B3LYP method, and the LANL2DZ basis set; the 6–31G** like functions were used for P atoms (for the methods, basis sets, and other details see Cini et al.14–16 and the references cited therein).

Results

IN VITRO TESTS

Linear phosphates (SMP and linear penta-sodium tripolyphosphate (PSTP)) showed higher DA values on CPPD crystals than cyclic phosphates (table 1). These data pertain to solutions of polyphosphates in PBS 14.7 mM in PO₃ unit: this allows a direct comparison between different polyphosphates (the solutions have pH 7.4 and osmolality in the 280–300 mmol/kg range). The ionic strength of these solutions is due mostly to PBS, and is not significantly influenced by the equilibria which involve the ligands and the dissolution of CPPD crystals. Analysis of the DA values at increasing concentrations of SMP carried out after one hour of incubation showed that a concentration of 15 mg/ml PBS completely dissolved 2.0 mg of CPPD crystals (fig 2).

EX VIVO TESTS

The CPPD crystalline aggregates in all meniscal sections from patients with CC disappeared within one hour from the onset of the incubation in SMP (15 mg/ml PBS), whereas no CPPD crystal dissolution was noted in the sections treated with PBS only (fig 3). The macroscopic appearance of the meniscal fragments before and after 24 hours of treatment with SMP (15 mg/ml PBS) showed a net reduction of these crystalline aggregates (fig 4). Furthermore, mid-line sections showed numerous empty lacunae as compared with control PBS treated samples in the superficial and sublining meniscal layers. Transmitted and polarised light microscopy of the pellets from each incubating solution did not disclose birefringent positive CPPD crystals.

The calcium concentration liberated from the calcified meniscal fragments significantly increased after one hour, three hours, and 24 hours of SMP incubation (p=0.0001, Kruskal-Wallis analysis of variance; fig 5).

Chondrocyte cytotoxicity

The results of preliminary cytotoxicity tests on human chondrocytes at increasing concentrations of SMP in PBS (pH 7.4, 280–300 mmol/kg)

Table 2  Effect of increasing the concentration of polymeric sodium metaphosphate (SMP) on human chondrocytes by tetrazolium salt (MTT) assay

<table>
<thead>
<tr>
<th>SMP solution* (mg/ml)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>6</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Metabolically active cells (mean (SD))†</td>
<td>100</td>
<td>95 (3.2)</td>
<td>91 (3.6)</td>
<td>59.4 (4.3)</td>
<td>50 (7.6)</td>
</tr>
</tbody>
</table>

*SMP was dissolved in phosphate buffered saline (PBS; 280–300 mmol/kg, pH 7.4).
†Values were expressed as a percentage of metabolically active cells as compared with controls.
MTT test measures the number of metabolically active cells by a colorimetric technique. The data represent the mean results of four separate experiments. The control cultures were obtained by incubating cells with PBS for 24 hours.
kg), showed that the 50% inhibitory dose was reached at the maximum concentration (15 mg/ml) used in this work (table 2).

Morphological evaluation by transmission electron microscopy showed no structural abnormalities at any site, including the capsule, the pericellular matrix, the cellular membrane, the mitochondria, the inclusions, the smooth and rough reticulum, the Golgi, the lysosomes, and the nucleus (fig 6).

**Complex formation energies**

The structures for pyrophosphate $O(PO_3)^{2-}$, linear pentasodium tripolyphosphate (PSTP) $O(PO_3)^{5-}$, cyclic trisodium metaphosphate (TSMP) $O(PO_3)^{3-}$, anions, of the respective complexes with calcium $[Ca(OPO_3)^{2-}]^2$, $[Ca(OPO_3)^{3-}]^3$, and $[Ca(OPO_3)^{4-}]^4$ were all fully optimised. A detailed analysis of selected structures has been reported elsewhere. The agreement between observed and computed structures was satisfactory. Table 3 reports the energies for selected formal reactions. The absolute value of computed molar formation energy for calcium pyrophosphate ($-865.951$ kcal, table 3; for the formal reaction at the gas phase) is much smaller than that for the formation of calcium PSTP ($-982.342$ kcal). This is related mostly to the larger overall charge of the $O(PO_3)^{3-}$ compared with that of $O(PO_3)^{2-}$.

**Discussion**

An examination of our in vitro tests (table 1) showed that the linear or branched chains built up by at least three PO$_3$ units had large DA values for the CPPD crystals. In fact, DA values in the range 21.7–24.1 Ca$^2+$ µg/ml were measured for linear SMP and PSTP at 14.7 mM PO$_3$ in PBS. Thus there is no significant influence on DA values when the phosphate chain is lengthened beyond three PO$_3$ units. Interestingly, the DA value for the cyclic TSMP (1.6 (0.5) Ca$^2+$ µg/ml), is much smaller than that of linear PSTP (21.7 (4.3) Ca$^2+$ µg/ml). In addition, the presence of the phosphates contained in PBS decreases the hydrolysis of the phosphate-containing ligands.

The reason for the trend in the DA values lies, at least in part, in the complexing ability of the ligand molecules towards the calcium ion. The linear triphosphate chain seems to be particularly suited to linking a Ca$^{2+}$ ion, whereas the very small DA values for cyclic TSMP suggest that the rigidity of the (PO$_3$)$_3$ molecule, combined with its lower negative charge (when

<table>
<thead>
<tr>
<th>Reaction</th>
<th>$-\Delta E$ (kcal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca$^{2+}$ + O(PO$_3$)$_2$ $\rightarrow$ [Ca(OPO$_3$)$_2$]$^{2-}$</td>
<td>865.951</td>
</tr>
<tr>
<td>Ca$^{2+}$ + (PO$_3$)$_2$ $\rightarrow$ [Ca(PO$_3$)$_2$]$^-$</td>
<td>637.331</td>
</tr>
<tr>
<td>Ca$^{2+}$ + O(PO$_3$)$_3$ $\rightarrow$ [Ca(OPO$_3$)$_3$]$^3-$</td>
<td>982.342</td>
</tr>
</tbody>
</table>

Table 3 Complex formation energies (kcal/mol) for selected formal reactions

Figure 6 Transmission electron microscopy of human chondrocytes cultured in vitro before (A, C) and after (B, D) treatment (24 hours of treatment with 15 mg sodium metaphosphate (SMP) in phosphate buffered saline (PBS)); cells did not show structural abnormalities, including the capsule, the pericellular matrix, the cellular membrane, the mitochondria, the inclusions, the smooth and rough reticulum, the Golgi, the lysosomes, and the nucleus. (Original magnification figs A, C ×6000; figs B, D ×20 000.)
Dissolution of calcium pyrophosphate crystals by polyphosphates

compared with O(P\text{O}_3\text{H})_2^- and O(P\text{O}_2\text{H})_4^2-). This observation suggests that treatment carried out with SMP or other linear metaphosphate polymers/oligomers should maintain a high DA, even though a significant hydrolytic dissociation process takes place in vivo.

Of all these ligands, linear SMP has been shown to be the most efficacious dissolving agent because, compared with the others, it was possible to increase PBS concentrations while remaining in the range of physiological conditions. In particular, the PBS concentration of 15 mg/ml can be considered the highest that can be used, as it reaches the limit value of pH (7.4) and osmolality (300 mmol/kg).

This has led to the speculation that acute attacks may be the result of crystal shedding, promoted by the partial dissolution of superficial calcifications but also more deeply, apparently without altering the normal histological structure of the meniscal fibrocartilage. It has been noted that knee lavage with potent calcium chelating agents, such as disodium EDTA, may induce attacks of pseudogout. This observation highlights the greater potency of SMP as compared with O(P\text{O}_3\text{H})_2^- and O(P\text{O}_2\text{H})_4^2-). This observation suggests that treatment carried out with SMP or other linear metaphosphate polymers/oligomers should maintain a high DA, even though a significant hydrolytic dissociation process takes place in vivo.

Hence, our findings lend support to the idea that phosphates could be used to remove both free and tissue bound crystals without promoting acute pseudogout attacks. Hence, our data may have therapeutic implications, as at least some of the molecules studied could potentially be used for intra-articular treatment in CC, which so far can only be treated symptomatically. Further data are needed to investigate the feasibility of treatment of CC with these compounds.

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