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2 **Role of calcium-phosphate deposition in vascular smooth**
3 **muscle cell calcification.**

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10 Running title: Calcium phosphate deposition in vascular cells

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18 ABSTRACT

19 In this work we are studying if calcium phosphate deposition (CPD) during vascular calcification is a
20 passive or a cell-mediated mechanism. Passive CPD was studied in fixed vascular smooth muscle cells
21 (VSMC), which calcify faster than live cells in the presence of 1.8 mM Ca^{2+} and 2 mM Pi. CPD seems to
22 be a cell-independent process that depends on the concentration of calcium, phosphate, and hydroxyl
23 ions, but not on Ca x Pi concentration products, given that deposition is obtained with 2x2 and 4x1 Ca x
24 Pi mM^2 but not with 2x1 or 1x4 Ca x Pi mM^2 . Incubation with 4 mM Pi without CPD (i.e., plus 1 mM Ca)
25 does not induce osteogene expression. Increased expression of bone markers such as Bmp2 and Cbfa1
26 is only observed concomitantly with CPD. Hydroxyapatite is the only crystalline phase in both lysed and
27 live cells. Lysed cell deposits are highly crystalline while live cell deposits still contain large amounts of
28 amorphous calcium. High-resolution transmission electron microscopy revealed a nanostructure of
29 rounded crystallites of 5-10 nm oriented at random in lysed cells, which is compatible with spontaneous
30 precipitation. The nanostructure in live cells consisted of long fiber crystals, 10-nm thick, embedded in an
31 amorphous matrix. This structure indicates an active role of cells in the process of hydroxyapatite
32 crystallization. In conclusion, our data suggest that CPD is a passive phenomenon, which triggers the
33 osteogenic changes that are involved in the formation of a well organized, calcified crystalline structure.

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35 Keywords

36 Vascular calcification; hyperphosphatemia; crystal structure; calcium phosphate deposition; vascular
37 smooth muscle cell.

38

39 INTRODUCTION

40 Considerable efforts have been made over the last decade to understand the pathogenesis of vascular
41 calcification (VC). It is now generally accepted that medial mineralization of the arteries (i.e.,
42 Mönckeberg's sclerosis) is an orchestrated phenomenon that resembles the ossification processes of the
43 bone (4, 30). The phenomenon includes the transdifferentiation of vascular smooth muscle cells (VSMC)
44 to a phenotype with osteogenic characteristics as a response to uremic toxins and hyperphosphatemia in
45 chronic kidney disease (CKD) or in diabetes (9, 17).

46 Despite the significant amount of knowledge in the field, important questions remain to be answered. For
47 example, one of the key questions that is still unclear refers to whether calcification is an actively
48 promoted phenomenon or an actively inhibited phenomenon (27, 28). Regarding the former possibility,
49 physiological (e.g., bone) and ectopic calcifications take place after the expression of a mineralizing
50 extracellular matrix (ECM) and after stimulation by hormonal and non-hormonal factors (28). Calcification
51 is therefore an active process. Regarding the latter possibility, calcification could occur as a consequence
52 of the absence (bone) or loss (VC) of calcification inhibitors, and therefore calcium phosphate deposition
53 occurs as a passive phenomenon (28). In this case, mineralization is actively inhibited and prevented in
54 the arteries under physiological conditions. A key finding that supports the loss-of-inhibition hypothesis of
55 VC is the observation that a knock-out mouse carrying the VC inhibitor, matrix-Gla protein, shows
56 extensive calcification of soft tissues, even in the presence of normal blood concentrations of Ca^{2+} and Pi
57 (15). This hypothesis is further supported by the fact that the concentrations of Ca^{2+} and Pi exceed the
58 solubility product in the extracellular milieu (19), and therefore in the presence of such metastable fluids,
59 mineralization should start as soon as local calcification inhibitors are eliminated (20). However, the
60 problem of ectopic calcification can apparently not be explained exclusively by either possibility, given
61 that the expression of both mineralization activators during VC and the expression of inhibitors under
62 physiological circumstances have been demonstrated.

63 One of the key players in the pathogenesis of VC in CKD is Pi, but its specific pathogenic role is still
64 unclear despite extensive work over the last decade. In this paper we have done an *in vitro* study of the
65 pathogenesis of Pi-induced calcification by analyzing the extent of cellular activity involvement during

66 initial calcium phosphate deposition (CPD) and by studying the relative role of the osteogenic changes in
67 the process. We have concluded that while CPD is a passive phenomenon that depends on the absence
68 of calcification inhibitors, CPD is also responsible for the osteogenic changes that build a biomineralized
69 deposit with a crystalline nanostructure that is very different from the crystalline nanostructure of passive
70 mineralization.

71

72 MATERIALS AND METHODS

73 *Cell culture*

74 The isolation and culture conditions of rat aorta VSMC, as well as opossum kidney cells (OK), have been
75 described previously (37, 38). The protocols were submitted to and approved by the Ethics Committee of
76 the University of Zaragoza. Cells were grown to confluence and used after an overnight quiescence step.
77 For calcification, cells were plated in 24-well dishes. VSMC were grown in a minimum essential medium
78 (MEM), and OK cells were grown in DMEM/F12. Both MEM and DMEM/F:12 were supplemented with
79 glutamine and 10% fetal calf serum. All cell culture reagents were from Invitrogen (Paisley, UK). For the
80 preparation of customized (synthetic) MEM (sMEM), the inorganic salt, D-glucose (Dextrose), amino acid,
81 and vitamin components of the MEM culture medium were obtained separately from Invitrogen. The final
82 concentrations correspond to the composition of the MEM: 0.814 mM of magnesium sulfate (MgSO_4),
83 5.33 mM of potassium chloride (KCl), 26.19 mM of sodium bicarbonate (NaHCO_3), 117.24 mM of sodium
84 chloride (NaCl), and 1000 mg/L of D-glucose.

85 *Calcification determination*

86 Calcification of VSMC was performed as described, including the preparation and use of lysed cells (39),
87 except for 24-hour preincubation with 2 mM Pi before lysis, which we found to be unnecessary (see
88 Results). Both live and fixed cells were calcified in an identical way, and the same calcification medium
89 was used, which contained 0.5% FCS. In this work, identical results were obtained by paraformaldehyde
90 fixation and overnight drying. As a negative control, a plate without cells was always used in the
91 experiments. The calcification of VSMC was identified by Alizarin red staining and was quantified using a
92 QuantiChrom™ Calcium Assay Kit (BioAssay Systems, Hayward, CA).

93 *Collagen and elastin calcification*

94 A rat type I collagen solution was obtained from Invitrogen, and a soluble human elastin, fraction V bovine
95 albumin, and poly-L-lysine were obtained from Sigma-Aldrich. The collagen, elastin, and albumin were
96 fixed to a plastic cell culture support at $5 \mu\text{g}/\text{cm}^2$ following the collagen manufacturer's instructions

97 (Invitrogen). The poly-L-lysine coating was applied following the classical protocol for cell culture.
98 Calcification was then performed as it was for the cells.

99 *Real time PCR*

100 The relative quantification of Msx2, Cbfa1, Bmp2, and SM22 alpha was performed by real-time PCR
101 using SYBR Green on a LightCycler (Roche Applied Science, Mannheim, Germany), following the
102 manufacturer's instructions for calibrator normalized protocols as described (40). The primers used to
103 amplify Msx2, Cbfa1, and Bmp2 have been listed previously (41). SM22 α was amplified using
104 CAGACTGTTGACCTCTTTGAAG as the upper primer and TCTTATGCTCCTGGGCTTTC as the lower
105 primer. The acidic ribosomal phosphoprotein (Arp) RNA was used as an endogenous reference (40).

106 *Analysis of calcification composition*

107 The composition, microstructure, and crystal structure of the deposits were characterized by X-ray
108 powder diffraction (XRD; Rigaku D-max B), scanning electron microscopy (SEM; JEOL 6400) equipped
109 with an INCA-300 energy dispersive spectroscopy (EDS) system (Oxford Instruments), transmission
110 electron microscopy (JEOL 2000FXII) equipped with an INCA-200 microanalysis system (Oxford
111 Instruments), and high-resolution electron microscopy (FEI TECNAI G2 F30). Samples for XRD were
112 prepared by spreading ground powders on a glass holder. Samples for the structural characterization
113 were prepared by collecting deposits from 6-well plates for each live and lysed cell cultures and then
114 mixing them in a mortar. XRD analysis was performed on ground powders spread on a glass holder. For
115 TEM analysis, powders were dispersed in hexane, and the dispersion was evaporated on a carbon-
116 coated microscope grid. Samples for SEM observations were prepared by gluing pieces of the culture
117 recipient bottom and coating them with gold or carbon. Part of the samples was washed repeatedly with
118 water by decantation to eliminate any soluble substance present in the sample before observation.

119 *Data analysis*

120 The mean effective constants of calcification prevention were calculated by non-linear regression, thereby
121 fitting data to a logarithmic dose-response curve (39). Each experiment was repeated at least three times
122 with similar results. Data are shown as the mean \pm SE. The significances of differences were evaluated
123 by an analysis of variance and a Tukey's multicomparison post-test.

124 RESULTS

125 *Passive versus active calcium phosphate deposition*

126 We studied passive CPD (i.e., in the absence of any cellular activity or metabolism) using fixed VSMC
127 (39). In this case, we did not preincubate the cells with 2 mM Pi for 24 hours before lysis, as initially
128 described (39), because we found that dead cells calcified to the same extent, whether or not the cells
129 were subjected to this previous treatment (data not shown). We compared the amount of CPD obtained
130 using either live or fixed VSMC in the presence of a culture medium (MEM) containing 2 mM Pi (Fig. 1A).
131 Calcium deposition increased significantly in fixed cells (black bars) after 3 days and significantly in live
132 cells (open bars) after 5 days. At 3, 5, and 7 days, the amount of precipitated Ca^{2+} in fixed cells was three
133 times the amount in live cells. This difference was then kinetically analyzed by comparing the dose-
134 response effects of pyrophosphate on the prevention of calcification in both live and fixed cells (Fig. 1B).
135 The Hill slopes of the curves were similar (-2.4 vs. -2.7 for live and fixed cells, respectively), but the EC_{50}
136 of PPI were very different: 2.6 and 8.8 μM for live and fixed cells, respectively. Therefore, more PPI is
137 necessary to prevent calcification in fixed cells. Subtraction of both logarithmic curves provided a log
138 normal distribution curve with a mean of $4.9 \pm 1.0 \mu\text{M}$ and a standard deviation equivalent to the Hill⁻¹
139 slope (2.14). Several interpretations can explain the shift in EC_{50} , but it is most likely due to the amount of
140 calcification inhibitors, such as PPI, which are produced by live cells and are missing in fixed cells. Similar
141 findings were obtained in our previous work using bisphosphonates (39).

142 Next, we attempted to define the nature of the macromolecular matrix required for CPD and adsorption in
143 the cells. VSMC were incubated in MEM with 2 mM Pi for up to 5 days (Fig. 2A), and every day a group of
144 cells were trypsinized or lysed with the indicated detergents at 0.1%. The wells were then kept in the 2
145 mM Pi medium for the remaining days. The cells that were lysed with any of the three detergents calcified
146 at the same intensity as from the first day of lysis. This suggests that calcification takes place
147 predominantly in the protein phase of the cells, but a role by the lipid component cannot be excluded. Fig.
148 2B shows the calcium quantification after 7 days of incubation with 1 or 2 mM Pi, according to the
149 indicated treatments.

150 Next, we directly studied the role of collagen and elastin, two extracellular proteins that are known to
151 induce calcification. Rat collagen type I and human elastin were fixed onto cell culture plates and
152 incubated with MEM containing either 1 or 2 mM Pi for seven days. Staining with Alizarin red revealed an
153 intense calcification of all collagen fibers incubated with 2 mM Pi, as well as intense calcification of the
154 globular elastin that was attached to the plastic surface, which concurs with previous *in vitro* and *in vivo*
155 studies (Fig. 2C). Surface coating with albumin or poly-L-lysine did not induce CPD in the culture dishes.
156 These results indicate that calcification can apparently be obtained with single molecules when specific
157 experimental conditions are attained. However, as shown below, the definite crystal ultrastructure is only
158 obtained when the active cell is present.

159 *Role of the composition of the cell culture medium and of pH*

160 *In vitro* calcification of VSMC is an established model of VC that, in some cases, has yielded significant
161 differences in calcification rates among laboratories, as demonstrated by the use of different Pi
162 concentrations (usually 2-4 mM Pi) in culture media to induce Pi-related VC. Therefore, before continuing
163 with subsequent experiments, we checked to see if the composition of the different culture media could
164 be at least partially responsible for these differences. We obtained identical qualitative results using either
165 live or lysed cells. VSMC were induced to calcify using 2 mM Pi by supplementing the phosphate
166 concentration of three different culture media: MEM, DMEM, and DMEM/F:12. Figure 3A shows that the
167 calcification induced using 2 mM Pi (black bars) was only obtained with MEM or DMEM, while cells
168 incubated with DMEM/F:12 did not calcify. Comparison of the media revealed that MEM and DMEM
169 contain 1.8 mM Ca^{2+} , while DMEM/F:12 contains only 1.05 mM. DMEM/F:12 was subsequently
170 supplemented to reach a final concentration of 1.8 mM Ca^{2+} , after which calcification was successfully
171 induced using 2 mM Pi (Fig. 3B) at pH 7.5 or higher (pH 8.0). In this study we only used fixed VSMC to
172 maintain a constant pH. No calcification was observed at pH 7.0 or in cells incubated using 1 mM Pi at
173 any of the three pHs. Therefore, while VSMC cultures can be a valuable model for studying cell-mediated
174 calcification, it is essential to observe standardized experimental conditions and the use of similar culture
175 media. In any event, this finding does not invalidate the knowledge obtained with these cultures during
176 the last decade.

177 While the effect of pH on calcification has been extensively studied, we nevertheless performed an
178 additional experiment to understand the pathogenesis of CPD. Opossum Kidney (OK) cells constitute a
179 proximal tubular cell line that is known to quickly acidify culture medium, even below pH 7.0.
180 Consequently, when OK cells were incubated with DMEM/F:12 (the usual culture medium) containing 1.8
181 mM Ca^{2+} and 2 mM Pi, they did not calcify (Figure 3C). In contrast, when we used fixed OK cells, the pH
182 did not change and calcification occurred at a rate similar to the VSMC rate. While there are other
183 possibilities in addition to pH, such as the presence of calcification inhibitors, this finding suggests that the
184 expression of a specific extracellular matrix is not necessary to initiate CPD and that calcification will take
185 place in any cell in the absence of anticalcifying agents such as pyrophosphate or an acidic milieu.

186 *Effects of pH and Ca x Pi products on in vitro calcification*

187 Apart from pH, the Ca x Pi concentration product is another parameter that affects calcification, and while
188 it is generally considered to be a risk factor in hemodialysis patients, there is also growing controversy
189 about its significance (19). We studied the validity of this parameter using the passive CPD model,
190 because fixed VSMC allow the use of a supraphysiological concentration of Ca^{2+} and Pi. VSMC were
191 incubated for six days in a DMEM/F:12 medium supplemented with different concentrations of Ca^{2+} and
192 Pi (1, 2, or 4 mM, Fig. 3D). No calcification was observed when the Ca x Pi molar product was kept below
193 4 mM, in any combination. Calcification was observed when a 4 mM Ca x Pi product was obtained by
194 combining 2 mM Ca^{2+} and 2 mM Pi, or 4 mM Ca^{2+} and 1 mM Pi. No calcification was observed when the
195 4 mM Ca x Pi product was obtained with 1 mM Ca^{2+} and 4 mM Pi.

196 These results point to a predominant role of Ca^{2+} over Pi during calcification, as it has been reported
197 previously (42) and very recently while our work was under review (33). A more detailed analysis of the
198 calcium effect was studied on fixed VSMC so that non-physiological concentrations of Ca and Pi could be
199 used. VSMC were incubated in the presence of a constant concentration of 1 mM Pi, with increasing
200 concentrations of Ca^{2+} (Fig. 4A). Under these conditions, no saturation was obtained, but calcification
201 increased with the increasing Ca^{2+} concentration. Conversely, when the concentration of Ca^{2+} was fixed
202 at 1.5, 1.8, or 2.0 mM, the calcification obtained by increasing the concentration of Pi was saturated
203 (maximal) at 3 mM Pi and 2 mM Ca^{2+} (Fig. 4B). The predominance of Ca^{2+} over Pi can be explained in

204 physiochemical terms (see Discussion), considering that the cells were lysed and therefore any effect by
205 calcium signaling can be discarded.

206 *Calcifying components of the culture medium*

207 Based on the varying extent of the calcification obtained using the different basal culture media and the
208 dependence of calcification on the calcium content, we attempted to determine the involvement of the
209 other components of the culture media in *in vitro* calcification. We once again used lysed VSMC so that
210 non-physiological conditions could be used in the experiments. Cells were incubated in MEM or in the
211 different components of this basal medium, supplemented (or not) with phosphate to finally obtain 2 mM
212 Pi. When the following components were combined, a synthetic MEM (sMEM) was obtained, as explained
213 in Methods: a salt component (SC) and SC plus amino acids, vitamins, or glucose (Fig. 4C). In the
214 presence of 2 mM Pi, calcifications were observed in all combinations, and the addition of amino acids
215 and vitamins did not alter the extent of calcification. The involvement of the MEM salt component in
216 calcification was further analyzed by combining the various components and by incubating fixed VSMC
217 for six days using 1.8 mM Ca^{2+} plus 2 mM Pi (Fig. 4D). NaCl, KCl, and MgSO_4 , either individually or
218 combined, reduced the deposition of calcium induced with Ca and Pi. Conversely, the bicarbonate ion
219 increased calcium deposition as expected, independently of any other ion present in the medium. The pH
220 of the medium was kept at a constant 7.4 using 10 mM HEPES-Tris, and it was checked using a pH-meter.
221 In summary, choosing the correct culture medium is a critical factor when performing *in vitro* calcification
222 assays.

223 *Calcium phosphate deposition induces osteogenic changes*

224 The preceding findings on CPD and Ca x Pi products led us to compare the effects of different conditions
225 on osteogene expression and on the transdifferentiation of VSMC during calcification. Specifically, we
226 could now study the effects of CPD on osteogene expression separately from the effects of a high Pi
227 concentration on osteogene expression. VSMC were incubated for 24 hours with MEM containing 1.8 mM
228 Ca^{2+} , plus either 1 or 2 mM Pi, and then the abundance of Bmp2, Cbfa1, Msx2, and Osx was determined
229 by using real-time PCR. Fig. 5A shows that, after just one day of incubation, the expression of Cbfa1,

230 Msx2, and Osx was significantly increased. The abundance increased thereafter (including Bmp2), and it
231 was very strong after 5 days (Fig. 5B), when calcification was very intense (see Fig. 1A).

232 For a more detailed analysis of the effects of Ca x P, different combinations of Ca²⁺ and Pi were used to
233 study the osteogene expression response in VSMC (Fig. 5C). VSMC were incubated for three days in
234 sMEM in the presence of either 2 mM Ca²⁺ plus 1 mM Pi, 2 mM Ca²⁺ plus 2 mM Pi, 1 mM Ca²⁺ plus 4 mM
235 Pi, or 4 mM Ca²⁺ plus 1 mM Pi. The combinations that induced calcification (2 mM Ca²⁺ plus 2 mM Pi and
236 4 mM Ca²⁺ plus 1 mM Pi) were the only ones to increase the abundance of Bmp2 and Cbfa1, while the
237 expression of the smooth muscle marker SM22 α decreased. When the concentration of Pi was increased
238 to 4 mM and the concentration of Ca²⁺ was decreased to 1 mM to avoid calcification (see Fig. 3D), no
239 significant changes in the abundances of Cbfa1, Bmp2, or SM22 α were observed compared to the control
240 (2 mM Ca²⁺ plus 1 mM Pi). These findings demonstrate that the increased extracellular concentration of
241 Pi is not responsible for changes in osteogene expression in VSMC, but rather the initial deposition of
242 calcium phosphates is responsible for the changes.

243 *Analysis of calcium phosphate deposits*

244 The calcifications obtained in live and lysed cells were analyzed to check whether the final composition of
245 the mineralization process (i.e., after CPD) was similar in both situations. Significant differences between
246 the compositions in both experimental situations would indicate the active involvement of
247 transdifferentiated cells in the process. The crystal structure of deposits of live and fixed cells were
248 analyzed by x-ray powder diffraction. XRD patterns from live and fixed samples showed narrow peaks
249 corresponding to a halite structure (NaCl). Patterns of samples after washing are shown in Fig. 6A. The
250 broad band centered at 22 $^{\circ}$ arises from the glass holder. The rest of the peaks correspond to nanometric
251 crystals, and they can be indexed according to an apatite crystal structure, in both live and lysed VSMC.

252 The composition and microstructure of the deposits in live and lysed VSMC were analyzed by SEM (Fig.
253 6B and C). The surface of the deposits was flat, especially in living cell cultures, and it was formed by
254 compact arrangements of spherulite-shaped particles.

255 EDS analyses showed the presence of P, Ca, Mg, Cl, and Na in both live and lysed cell samples.
256 However, the Ca/P atomic ratios were different in lysed and live cell deposits. In lysed cells the Ca/P ratio

257 was close to 1.7 (mean = 1.72, SD = 0.16), which is the ratio corresponding to the hydroxyapatite
258 compound ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) and which explains the presence of the Mg that is often found in this
259 compound. In live cells this ratio significantly decreased (mean = 1.40, SD = 0.21), whereas the O/P ratio
260 increased, indicating the presence of calcium-deficient compounds with a high degree of hydration [such
261 as amorphous calcium phosphate ($\text{Ca}_9(\text{PO}_4)_6 \cdot n\text{H}_2\text{O}$; Ca/P = 1.5, O/P = 4.8), octacalcium phosphate
262 ($\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$; Ca/P = 1.33, O/P = 4.8), and dicalcium phosphate dihydrate or brushite
263 ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$; Ca/P = 1.0, O/P = 6.0)] (16). The absence of these compounds in XRD patterns can be
264 due to their amorphous character or to a small crystallite size.

265 *Ultrastructural analysis of crystallites*

266 A more detailed view of the microstructure was obtained by TEM and high-resolution TEM (HRTEM).
267 Images at low resolution show planar formations in both types of deposits (Figs. 7A and 7C). However,
268 high-resolution observations revealed that these formations are polycrystalline and that they have a very
269 different degree of crystallization and crystallite shape in the deposits of live versus lysed cells. The insets
270 of panels A and C are at higher magnifications and show granular and fibrillar structures, respectively.
271 The nanostructure in lysed cell deposits consists of highly crystalline regions with a size of 5 to 10 nm and
272 a rounded shape (Fig. 7B). However, live cells showed an amorphous or poorly crystalline background,
273 which is crossed by long filaments that have well-defined crystal planes and a thickness of about 10 nm.
274 The orientation of the crystallites within the sheets was analyzed by direct measurement of the interplanar
275 distances on atomic resolution HRTEM images and by Fast Fourier Transform (FFT) diffraction patterns
276 from the images. Crystallites in lysed cell samples yielded 7 different d_{hkl} distances corresponding to the
277 (300), (002), (112), (210), (211), (222), and (300) crystal planes of hydroxyapatite. All these planes were
278 observed on FFT patterns (Fig 8A) that also showed 10 extra reflections that could be assigned to
279 hydroxyapatite crystal structure. It is therefore apparent that hydroxyapatite is the only crystalline phase in
280 the samples and that the crystallites are oriented at random with respect to the sheet plane. In live cell
281 samples, the distances measured between planes parallel to the direction of elongation of the fiber-
282 crystals were mainly close to 3.42 Å, which corresponds to d_{002} in hydroxyapatite (3.45 Å), although in
283 some defected crystals the distance was slightly larger (up to 3.9 Å). Therefore, the direction of
284 elongation of the fiber-crystals is perpendicular to [001]. This reflection was also present in all the FFT

285 patterns taken on both single-crystal and polycrystalline regions (Fig. 8B). FFT patterns showed an extra
286 reflection at $2.07 \pm 0.04 \text{ \AA}$, forming an angle of nearly 30° with the (001) reflection that could be assigned
287 to d_{113} in hydroxyapatite (2.07 \AA). Therefore, the crystals are oriented with their [-110] direction parallel to
288 the electron beam, and they are elongated along the [110] direction. Apart from the amorphous
289 background and the fiber-crystals, HRTEM images of lysed cell samples showed areas with bent parallel
290 grooves, as shown in Fig. 8C, that could be interpreted as intermediate stages of crystallization.

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296 **DISCUSSION**

297 Medial vascular calcification is a degenerative disease that is highly prevalent among patients who are on
298 hemodialysis or who have diabetes. Recently, many characteristics of ossified arteries have been
299 described, including the activation of a transdifferentiation program of VSMC into cells with osteoblast-like
300 properties and the expression of a calcifying ECM. Nevertheless, neither the pathogenic steps during VC
301 nor the specific roles of some of the agents that are clearly involved (hyperphosphatemia, uremia,
302 hyperglycemia, and aging) have yet to be fully understood (for reviews of these subjects see references
303 3, 5, 11, 14, 15, and 17).

304 For example, it is still unclear whether VC is an active degenerative process or, conversely, it is a passive
305 phenomenon that the tissue attempts to control. In this work we have aimed to clarify this point by using
306 lysed VSMC as a simple model of passive calcification. Our findings point to an intermediate possibility:
307 CPD is a passive phenomenon that seems to take place when the abundance of calcification inhibitors
308 are decreased, and this adsorption to the cells is a major step that initiates specific osteogene expression
309 and trans-differentiation of VSMC into osteoblast-like cells. Such osteogenic changes seem to be
310 responsible for the organized apatite crystal ultrastructure, which consists of an amorphous crystalline
311 background crossed by long, fibrillar crystal planes.

312 *Role of initial calcium phosphate deposition*

313 Our conclusion that CPD is a passive phenomenon is based on several results. First, we have found that
314 lysed VSMC calcify faster than live cells when they are incubated with 2 mM Pi in a MEM culture medium
315 (Fig. 1). One new fact that we have unveiled is that it is not necessary to incubate live cells with 2 mM Pi
316 for 24 hours before lysis so that they calcify after lysis (39). This finding means that the induction of
317 calcifying genes and the formation of a specific ECM (i.e., calcification activators) are not requisites for
318 the *initial* step in VC, i.e., calcium phosphate deposition. Under these *in vitro* conditions, it is necessary to
319 add more PPI to lysed cells than to live cells in order to prevent Pi-induced calcification (Fig. 1B). This
320 finding can be interpreted, but not exclusively, as the inability of lysed cells to produce calcification
321 inhibitors (pyrophosphate, triphosphate, and other polyphosphates), wherefore additional PPI must be
322 added during the assay to prevent calcification. As a whole, the finding that the induction of specific

323 calcification activators (i.e., specific osteogenes) is not necessary to initiate the calcification process, and
324 that additional PPI is necessary in lysed cells to prevent calcification agrees with and supports the loss-of-
325 inhibitors hypothesis of VC.

326 Additional results in this study also support the view that CPD is a passive phenomenon. CPD can simply
327 start after combining a few components of the MEM cell culture medium. As shown in Fig. 4D, the calcium
328 deposition obtained with 1.8 mM Ca^{2+} plus 2 mM Pi in water is decreased by the presence of Na^+ , K^+ , and
329 Mg^{2+} ions in the assay, but it is increased with the addition of a bicarbonate ion. The Na^+ , K^+ , and Mg^{2+}
330 ions can play a role in the onset of calcification, because several ion substitutions have been described
331 regarding hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$): Mg^{2+} , Sr^{2+} , Pb^{2+} , or Na^+ for Ca^{2+} ; carbonate or arsenate for
332 phosphate; and F^- or Cl^- for OH^- (10). Therefore, in the calcification process each of these ions will
333 determine the formation and final composition of calcium-phosphate deposits, and when calcification
334 inhibitors or an acidic pH are neutralized, CPD will even start in non-VSMC (e.g., renal OK cells; Fig. 3C).
335 Deposition depends on many other factors, but only minimally on the calcium-phosphate concentration
336 product (19). In blood, while Ca and Pi concentrations exceed the solubility product for hydroxyapatite,
337 these concentrations are not supersaturated, because blood composition is very complex: other ions and
338 proteins are involved in solubility and deposition. In a quiescent *in vitro* model of VSMC, media
339 composition is not as complex. Nevertheless, we have confirmed that this is also not a valid parameter *in*
340 *vitro*, because the product of 4 mM² (Ca x P) is only critical when calcium is present at ≥ 2 mM and
341 because a medium containing 1 mM Ca and 4 mM Pi does not result in calcium-phosphate deposition
342 (Fig. 3D). This is based on calcium's predominant role over Pi (Fig. 4A and B) and on the relevance of
343 hydroxyl ions (Fig. 4D) during calcium-phosphate deposition, which concurs with previous calcification
344 studies (28) and with some works showing that hydroxyapatite is synthesized from brushite or
345 octacalcium by adding calcium and hydroxyl ions (3, 16).

346 Another remarkable finding in this work is that the induction of osteogene expression and the
347 transdifferentiation of VSMC *in vitro* are not caused by high Pi levels in culture media such as those
348 observed during hyperphosphatemia, but rather they are caused by CPD. As shown in Fig. 3D, we have
349 been able to increase the Pi concentration up to 4 mM without calcium phosphate deposition simply by
350 reducing the calcium concentration to 1 mM. Under these conditions, no calcification was observed, the

351 expressions of Bmp2 and Cbfa1 were not induced, and there was no change in the abundance of the
352 smooth muscle marker Sm22 α (Fig. 5C). The expression of Bmp2 and Cbfa1 and the inhibition of Sm22 α
353 only occur concomitantly with CPD. This finding implies that not only does CPD precede changes in the
354 osteogene expression characteristic of VC but also that deposition itself, rather than the concentrations of
355 free Pi in the cell culture, is most likely responsible for the changes in gene expression and for the
356 transdifferentiation of this *in vitro* model. These findings concur with previous studies that prevented Pi-
357 induced osteogene expression using PFA (2, 11). The results had been interpreted as the inability of Pi to
358 enter the cell and induce gene expression. However, considering that PFA is not an inhibitor of VSMC Pi
359 transport but rather an inhibitor of CPD (39), those previous results (2, 11) can be now reinterpreted,
360 because they suggest that osteogene expression is caused by calcium phosphate deposition. Our
361 findings also agree with previous proposals of Prof. Shanahan, who suggested that calcium-induced
362 microcalcifications in sites of apoptosis precedes the osteogenic differentiation of VSMC, and that this
363 could be a universal initiation mechanism for both medial and intimal calcification (32).

364 *Ultrastructural analysis of calcification crystals*

365 While the initial steps in ectopic calcification (i.e., CPD) seem to constitute a passive phenomenon, an
366 analysis of the calcification crystal structure has revealed that cells participate actively in the final
367 outcome, because there are a series of important differences between the composition and structure of
368 the calcification deposits in lysed and (metabolically active) live cells (Figs. 7-8). In both cases, however,
369 apatite is the predominant phosphate crystalline compound, and at a micrometer level, deposits are
370 composed of polycrystalline planar formations. Hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) is the most stable
371 calcium phosphate phase, and it is the most frequent one in geological and biological mineralizations (5,
372 43). It precipitates directly only when the calcium phosphate ionic product is above the hydroxyapatite
373 saturation point, but below the saturation point of intermediate CaP phases, such as octacalcium
374 phosphate (OCP, $\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$). Otherwise, the hydroxyapatite formation process occurs after an
375 initial precipitating phase of amorphous calcium phosphate, which has a low Ca/P ratio (1.5) and consists
376 of $\text{Ca}_9(\text{PO}_4)_6$ clusters (Polner's cluster) arranged in low density fractal aggregates (31). These aggregates
377 are gradually ordered into a crystalline hydroxyapatite structure with a Ca/P ratio of 1.68. This process
378 can be very slow, especially in biological systems (5,43), but it can be accelerated by high Ca/P ratios

379 and a high pH. The theoretical equilibrium morphology of hydroxyapatite crystals consists of a {101}
380 bipyramid with {100} prismatic lateral faces and {103} and {111} faces at the corners (6). However, the
381 real morphology of crystals grown at nearly equilibrium conditions is that of hexagonal prisms bounded by
382 {100} side faces, with elongation in the [001] direction (24, 35). Given that the solubility of hydroxyapatite
383 in water is very low, crystal growth in aqueous solutions is usually far from equilibrium conditions.
384 Consequently, critical supersaturation for nucleation is very high, the nucleation rate is also very high, and
385 the crystal growth rate is very low. Thus, unless special methods are used, such as hydrothermal or slow
386 diffusion, the morphology of hydroxyapatite crystals grown in aqueous media is far from the equilibrium
387 morphology. Typical hydroxyapatite aqueous precipitates consist of spherulitic agglomerates of a) non-
388 faceted needle crystals elongated along the [001] direction when the pH is abruptly increased (26) or b)
389 (100) platy crystals for slow pH increase (12). Fast growth conditions favor a needle-like morphology (23),
390 and crystallization from intermediate phases favor a planar morphology (43). Biomimetic hydroxyapatite
391 precipitates also show spherulitic formations of platy crystals elongated along [001] (18). In physiological
392 calcium phosphate biomineralizations (i.e., bones and teeth), the main phase is hydroxyapatite, although
393 they may contain other intermediate phases (especially in bones). The main feature that distinguishes
394 physiological crystallizations from pathological ones is that the particles are hierarchically ordered thanks
395 to the templating effect of organic matrixes (14, 44). In pathological calcifications, hierarchical order is
396 lost, although some texturing produced by the presence of an organic matrix is usually found. The same
397 thing occurs in aortic calcifications, which show a stratified microstructure of concentric, inorganic layers
398 bounded by an organic matrix (1, 8, 21, 29). The hydroxyapatite crystals are planar and are arranged in
399 spherulitic formations. The crystallite size, around 2-10 nm, is much smaller than in physiological
400 calcifications.

401 In our experiments of live vs. dead cell calcification, there are considerable differences in the crystal
402 structure. CaP deposits from lysed cells show a hydroxyapatite atomic structure, a slight presence of
403 amorphous calcium phosphate, a high Ca/P ratio (~1.7), spherulitic arrangements of platelets, a rounded
404 crystallite shape, and a crystallite size of around 5 nm. Moreover, there is no preferential growth direction
405 or oriented crystallite assembly. This structure is similar to the structure found in aortic calcifications at
406 atomic/nanometric and microscopic levels, apart from the presence of organic matrix in aortic

407 calcifications. For example, studies of calcifications in the iliac arteries of patients with uremia showed the
408 presence of spherulites and a microstructure of planar formations composed of rounded nanocrystals
409 with a size of 2 to 10 nm (29). The crystalline phase in the referenced study was mostly apatite, together
410 with whitlockite, which was only detected by using a very intense x-ray source such as synchrotron
411 radiation. This crystallite structure is also found in spontaneous calcium phosphate precipitations, and it
412 can therefore be produced without the intervention of foreign substances. The structure of the deposits
413 gives some clues about the precipitation conditions. The small crystallite size suggests intense nucleation
414 and short crystal growth periods; the planar shape is consistent with crystallization from amorphous
415 calcium phosphate precursors; and the high Ca/P ratio indicates a complete amorphous calcium
416 phosphate to hydroxyapatite conversion. Live cell deposits show similar macroscopic and microscopic
417 structures. However, at atomic and nanometric levels, there are significant differences: amorphous
418 calcium phosphate is present in large amounts, the Ca/P ratio is low (~ 1.4), and crystalline domains are
419 strikingly fiber-shaped. Elongated crystals are found in synthetic crystals of various sizes and are very
420 common in physiological biomineralizations. However, the direction of elongation is usually the [001],
421 while here the fibers are elongated along the perpendicular direction (apparently the [110] direction).
422 Moreover, regarding enamel, rod crystals are formed by a templated assembly of spherical crystals
423 induced by the amel protein (44), whereas here the elongated shape appears at the very first instance of
424 nucleation. In fact, the presence of bent grooves suggests the intervention of flexible organic
425 macromolecules that self-assemble with Polner's clusters, thereby forcing the alignment of these clusters
426 and the formation of fibrillar nuclei. Furthermore, as shown in Fig. 1, the total amount of deposited mineral
427 is substantially less in live cells than in lysed cells. On the other hand, the presence of other hydrated
428 calcium phosphate species in live cells, in addition to apatite, contradicts previous *in vitro* works (11) but
429 agrees with some *in vivo* studies (13, 29, 36). These hydrated species are deficient in Ca^{2+} but rich in
430 oxygen, thereby resulting in a Ca/Pi index of about 1.4, and they can be considered to be hydroxyapatite
431 precursors (7, 10). This indicates that the crystallization process in live cells is at an early stage, most
432 likely as a consequence of a reaction by cells to avoid calcification. Indeed, VSMC cells can reduce the
433 free calcium content in the medium by several mechanisms. For instance, they can do so through the
434 matrix-Gla protein (MGP), which acts as a local calcification inhibitor by calcium sequestration (15). In

435 addition, the expressions of transient receptor potential calcium channels (TRPCs) and of vanilloid
436 receptor channels (TRPVs) can assist in this process. In conclusion, live cells have a strong influence on
437 CaP crystallization in three ways: 1) by reducing the precipitation rate, 2) by restraining the amorphous
438 calcium phosphate to hydroxyapatite conversion, and 3) by templating the crystal nucleation process.
439 Promoters, inhibitors, and templates of crystallization are omnipresent in physiological and pathological
440 biomineralization processes (7, 8, 22, 25, 34). In fact, normal human serum is supersaturated with
441 respect to hydroxyapatite, OCP, and carbonate apatite, but normal vascular cells have the capacity to
442 inhibit CaP precipitation (8). Actually, the role of inhibitors in preventing mineralization in noncalcifying
443 tissues is generally assumed (22), whereas the lack of inhibitors is considered an important risk factor for
444 pathological calcifications (34). There may be several types of substances that induce the changes to
445 hydroxyapatite crystallization observed in live cell deposits. Effects 1) and 2) can be produced by calcium
446 ligands such as citrate or pyrophosphate or by macromolecules containing carboxylate, sulfate, or
447 phosphate residues. However, effect 3) requires a high templating capacity that is usually exerted by
448 proteins such as collagen, elastin, osteopontin, etc.

449 In summary, our data point to an intermediate scheme in the calcification process, i.e., between the loss-
450 of-inhibitors hypothesis and the view that calcification is an active, orchestrated process. While calcium
451 phosphate deposition seems to be a passive phenomenon starting with the local loss of calcification
452 inhibitors, CPD actually induces the expression of key osteogenes that modulate the biomineralization of
453 the ECM in a specific crystalline structure. Several important questions, however, remain to be answered.
454 For example, we need to decipher the mechanisms that lead to the initial deposition of calcium
455 phosphates and the loss of local inhibitors that seem to precede calcification. In addition, further research
456 is necessary to make a detailed comparison of the crystallite structure of *in vitro* and *in vivo* aortic VSMC
457 calcification, at a resolution that is at least as high as the one used in this study. Similarly, it is also
458 necessary to understand the specific roles that osteogenes play in the calcification process, because the
459 difference between dead or metabolically active (live) cells is *only* related to the ultrastructure of the
460 nascent crystals. Cells prevent calcification through the production of potent inhibitors (e.g., Fig. 1), while
461 at the same time they can express specific osteogenes as a response to CPD. Only osteogene-specific
462 deletion will help to understand this apparent dichotomy, but at present active prevention of calcification

463 with inhibitors seems to play an initial and mandatory, key role in the process. For example, gene deletion
464 of the local inhibitor MGP induces calcification in arteries under homeostatic Pi conditions (15), and VC is
465 also observed under normal Pi concentrations if other uremic toxins are present (17). Therefore, even
466 under normal Ca and Pi concentrations, CPD seems to be an inevitable phenomenon over time, which
467 arteries attempt to prevent by using mechanisms that need to be deciphered. Whether osteogene
468 expression plays an active role in the pathogenesis of vascular calcification or, conversely, it is only a
469 non-pathological response to CPD, is a point of debate that needs to be definitively clarified.

470

471

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474

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480

481 **DISCLOSURES**

482 None.

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584

585 **FIGURES**

586 **Figure 1.** Calcification of VSMC *in vitro*. A. Incubation of live (open bars) and fixed (black bars, 3%
587 paraformaldehyde) VSMC with MEM containing 2 mM Pi, for the indicated times. B. Kinetic analysis of the
588 effect of PPI on the calcification of live cells (squares) and fixed cells (triangles), showing fits to sigmoidal,
589 dose-response curves. The subtraction of both curves provides a Gaussian fit (bold line). *Significantly
590 different from the corresponding value at 1 day.

591 **Figure 2.** Proteins involved in vascular calcification. A. Effect of detergents on cells incubated with 2 mM
592 Pi for up to 5 days. Every day a group of cells were either trypsinized or treated with the indicated
593 detergents and saponine, all of them at 0.1 %. Calcification was visualized with alizarin red. B.
594 Quantification of the calcium content in cells treated as indicated during seven days in MEM with 2 mM
595 Pi. C. Alizarin red staining of collagen I, elastin, albumin or poly-L-lysine attached to a plastic support and
596 incubated with 1 or 2 mM for seven days.

597 **Figure 3.** Effect of cell culture composition, pH and Ca x P products. A. Effect of 2 mM Pi on the
598 calcification of fixed VSMC using MEM, DMEM, and DMEM/F:12. B. Effect of pH on 2 mM Pi-induced
599 calcification in lysed VSMC cells using DMEM/F:12, supplemented to contain 1.8 mM CaCl₂. C.
600 Calcification using DMEM/F:12 with 2 mM Pi in live (open bars) and fixed (black bars) OK cells for the
601 indicated times. D. Calcification takes place when Ca x Pi products are obtained with 2x2 or 4x1 mM Ca x
602 Pi. Cells were fixed with 3% paraformaldehyde.

603 **Figure 4.** Role of calcium and media components on calcification. A. Calcification of fixed VSMC with
604 increasing concentrations of Ca²⁺ and a constant Pi (1 mM). Cells were fixed with 3% paraformaldehyde.
605 B. Calcification induced with increasing concentrations of Pi, at three fixed concentrations of Ca²⁺: 1.5,
606 1.8, and 2 mM. C. Effect of MEM components on Pi-induced calcification: aa+vit, amino acids plus
607 vitamins; SC, salt component; Glc, glucose. D. Analysis of the effect of the salt component of MEM
608 culture media on Pi-induced calcification in VSMC.

609 **Figure 5.** Osteogene RNA expression during calcification. A. Expression of Bmp2, Cbfa1, Msx2, and Osx
610 in VSMC incubated with sMEM for 24 hours with 1.8 mM Ca²⁺ plus 1 or 2 mM Pi. B. Expression of the four
611 genes as a function of the number of days of treatment with 2 mM Pi. *Significantly different from the

612 corresponding 1 mM Pi ($p < 0.05$). C. Expression of Cbfa1, Bmp2, and Sm22 α in the presence of the
613 indicated combinations of Ca²⁺ and Pi, in mM, for three days. Cbfa1 and Bmp2 are only induced when the
614 combination of Ca²⁺ and Pi leads to CPD, as shown in Fig. 2D. *Significantly different from 2 mM Ca²⁺+1
615 mM Pi ($p < 0.05$).

616 **Figure 6.** Analysis of the crystal composition in mineralized live and lysed cells. A. XRD patterns of lysed
617 and live powdered deposit samples; bars correspond to the hydroxyapatite crystal structure. B and C,
618 SEM images of deposits from lysed (B) and live (C) cell cultures. Cells were lysed with 3%
619 paraformaldehyde.

620 **Figure 7.** Transmission electron microscopy analysis of crystalline domains. A and C, deposits of lysed
621 and live cells; insets show higher magnification revealing granular and fibrillar structures, respectively. B
622 and D, HRTEM images of lysed and live cell deposits, showing the difference between rounded
623 crystalline domains in lysed cells and long fibers in live cells. Cells were lysed with 3% paraformaldehyde.

624 **Figure 8.** Fast Fourier transform of atomic resolution HRTEM images of (A) a polycrystalline area from a
625 lysed cell deposit sample; and (B) a fiber-nanocrystal from a live cell deposit sample. C. HRTEM image of
626 an area of a live cell deposit sample showing parallel bent grooves that can be interpreted as an
627 intermediate stage of fiber-crystal formation process.

628

Figure 1

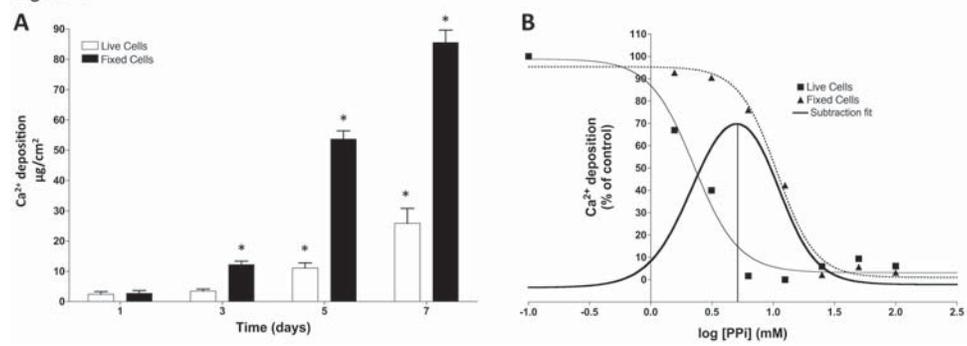


Figure 2

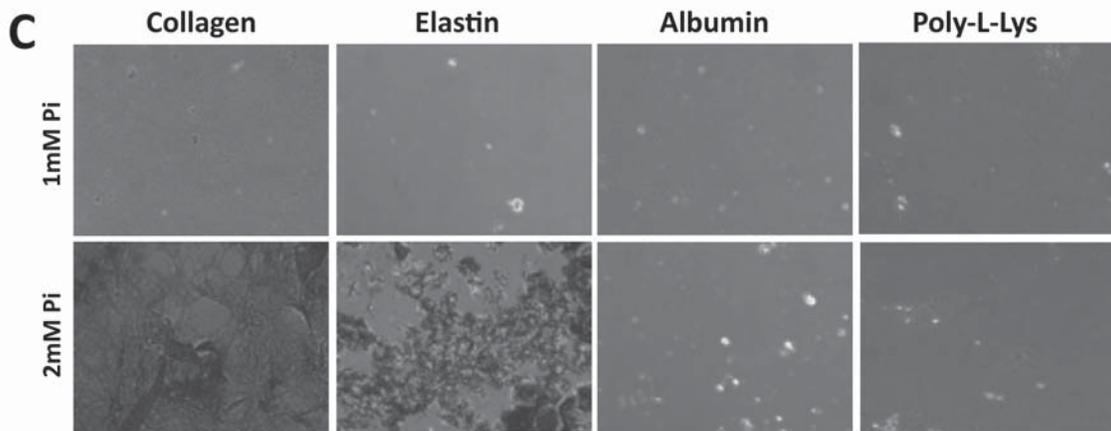
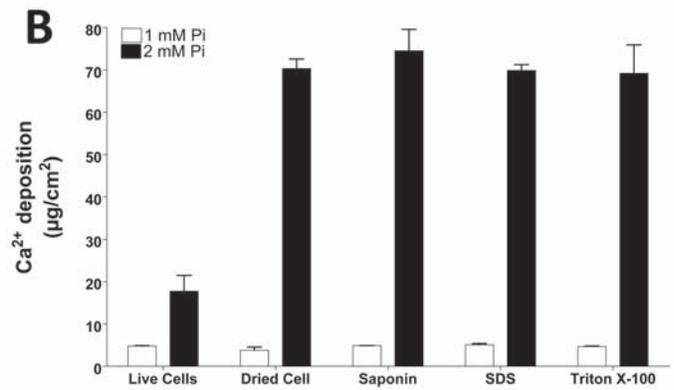
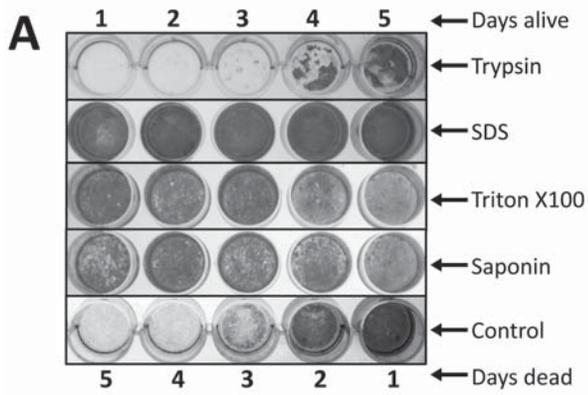


Figure 3

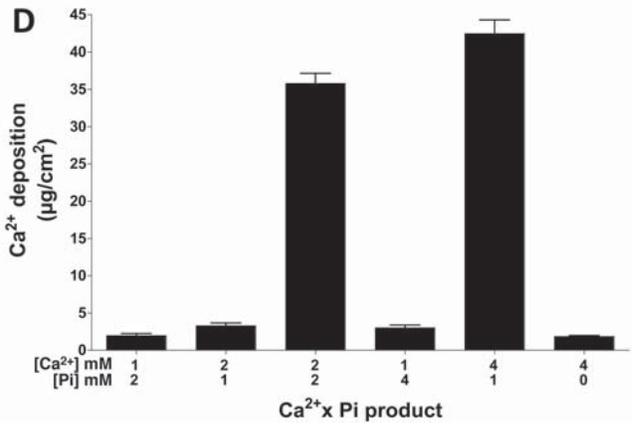
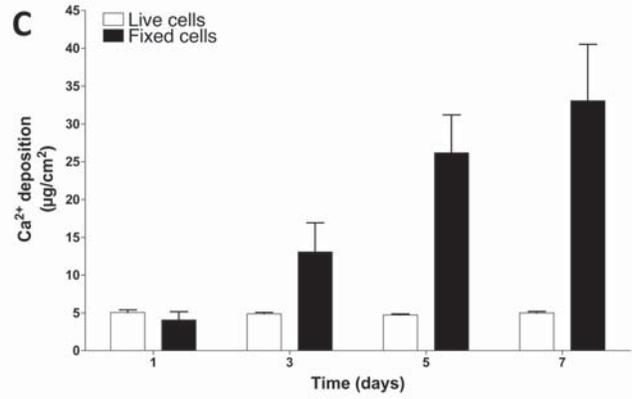
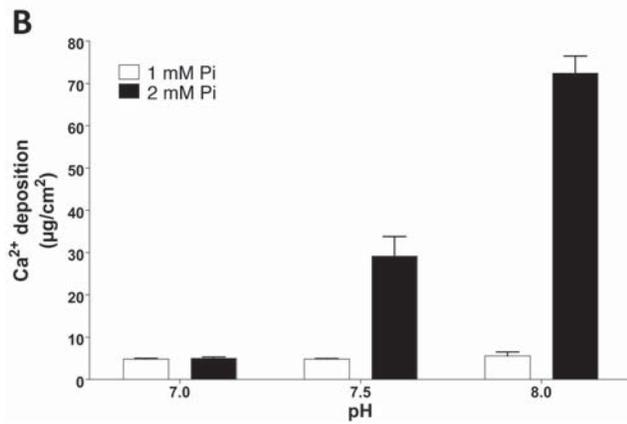
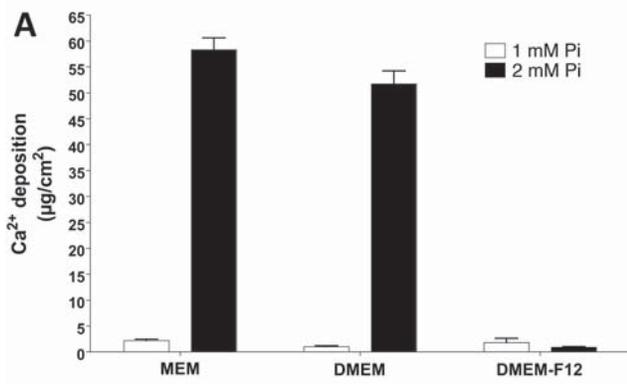


Figure 5

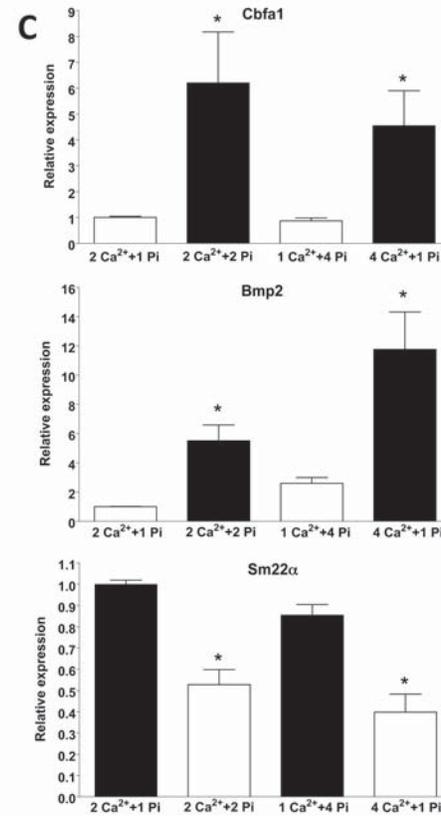
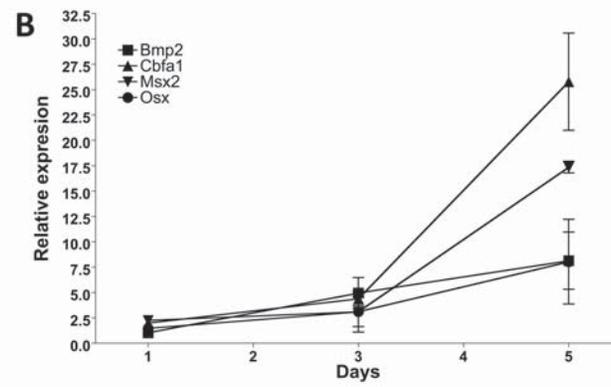
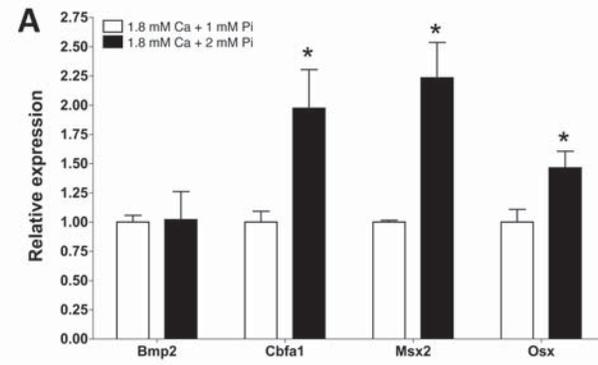


Figure 6

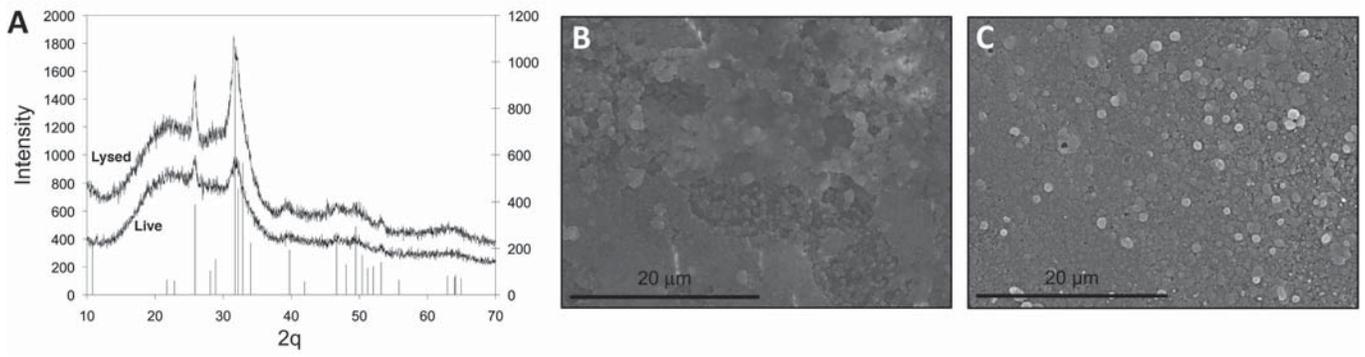


Figure 7

