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The presence of PHOSPHO1 in matrix vesicles and its developmental expression prior to skeletal mineralization.

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Abstract

PHOSPHO1 is a phosphoethanolamine/phosphocholine phosphatase that has previously been implicated in generating inorganic phosphate (P_i) for matrix mineralization. In this study we have investigated PHOSPHO1 mRNA expression during embryonic development in the chick. Whole-mount *in situ* hybridization indicated that PHOSPHO1 expression occurred prior to E6.5 and was initially restricted to the bone collar within the mid-shaft of the diaphysis of long bones but by E11.5 expression was observed over the entire length of the diaphysis. Alcian blue/alizarin red staining revealed that PHOSPHO1 expression seen in the primary regions of ossification preceded the deposition of mineral suggesting that it is involved in the initial events of mineral formation. We isolated MVs from growth plate chondrocytes and confirmed the presence of high levels of PHOSPHO1 by immunoblotting. Expression of PHOSPHO1, like TNAP activity was found to be up-regulated in MVs isolated from chondrocytes induced to differentiate by the addition of ascorbic acid. This suggests that both enzymes may be regulated by similar mechanisms. These studies provide for the first time direct evidence that PHOSPHO1 is present in MVs and is its developmental expression pattern is consistent with a role in the early stages of matrix mineralization.

Introduction

Matrix mineralization is a biphasic process that occurs in the matrix surrounding terminally differentiating chondrocytes, osteoblasts and ondontoblasts and is the mechanism by which growth plate cartilage, bone and tooth formation occurs by each of these cell types, respectively [1,2]. The initial phase concerns the formation of Ca^{2+} ions and inorganic phosphate (P_i) within matrix vesicles (MVs) [3,4]. The accumulation of Ca^{2+} is controlled by calcium binding molecules such annexins, phosphatidylserine and bone sialoprotein [5-7], however, the source of P_i is less clear. It has long been speculated that the generation of P_i results from the action of phosphatases, the most abundant being tissue non-specific alkaline phosphatase (TNAP), an isozyme of alkaline phosphatase expressed in bone, liver and kidney [4]. Once sufficient levels of Ca^{2+} and P_i have accumulated within the MVs, calcium phosphate precipitates, before the formation of insoluble hydroxyapatite [8]. The second phase involves the breakdown of the MV membranes, which expose the preformed hydroxyapatite to the extracellular fluid.

Generation of P_i for mineralization does not appear to be entirely due to the activity of TNAP as in newborn TNAP knockout mice, bone development and mineralization appear normal. Hypomineralization and other abnormalities do subsequently appear [9-11] but have been shown primarily to be due to a build up of inorganic pyrophosphate (PP_i), a known substrate of TNAP [12] and a potent inhibitor of hydroxyapatite crystal formation [13]. However, hypomineralization is greatly reduced in TNAP/PC-1 double-knockout mice and only observed in the long bones and not the vertebrae or cranium [11,14]. PC-1 encodes the enzyme, phosphodiesterase I, which generates PP_i from nucleotide triphosphates [15]. Additional studies have shown that TNAP can be removed from MVs without

reducing their potential to mineralize [16], whilst specific inhibitory studies on TNAP provide further evidence that other phosphatases are required for P_i generation [17].

PHOSPHO1 is a phosphatase with up-regulated expression in avian growth plate chondrocytes [18] and is localized to mineralizing regions of chick bone [19]. Since its identification in the chicken, PHOSPHO1 orthologues have also been identified in a number of other species including, humans, mice and zebrafish [20,21]. Analysis of PHOSPHO1 sequences using the web-based program, SignalP v1.1 [22] predicts the absence of a signal peptide. This suggests PHOSPHO1 to be a soluble cytoplasmic phosphatase. We have recently shown that human PHOSPHO1 exhibits high specific activity toward the phospholipid metabolites, phosphoethanolamine and phosphocholine [23]. It has been demonstrated that phosphoethanolamine and phosphocholine are the two most abundant phosphomonoesters in cartilage [24]. In addition, the proportions of membrane phospholipids containing these groups decrease in MVs during mineralization, whilst 1,2-diacyl glycerol has been shown to accumulate, indicative of phospholipase C activity [25]. This gives rise to the possibility of a novel mechanism whereby phosphate locked within the plasma membrane may be unleashed through the action of PHOSPHO1 and phospholipase C to contribute to the P_i concentration inside the MV.

Despite the localization of PHOSPHO1 to mineralizing regions of bone in chicks, little is known about its expression in relation to the onset of mineralization during embryonic development or in adult bone. More importantly, it has also yet to be demonstrated whether PHOSPHO1 is present within MVs. In the present work, we investigate PHOSPHO1 expression from the initial stages of skeletal formation in chick embryos to adult and assay for the presence of PHOSPHO1 in MVs isolated from the growth plate and primary chondrocyte cultures.

Materials and Methods

Whole-Mount In Situ Hybridization

A 750 bp DNA fragment corresponding to the third exon of the chicken PHOSPHO1 gene was obtained via PCR amplification of cDNA derived from RNA isolated from chick growth plate chondrocytes using primers F; ATGGCAGCTCCCGGC and R; GCAGTTCTTGAGGAGCTCCG, and cloned into a pGEM-T Easy vector (Promega, Southampton, UK). Digoxigenin (DIG)-labeled sense and anti-sense RNA probes were synthesized by *in vitro* transcription of the DNA template in the presence of ribonucleotides using the DIG-labeling mix (Roche, Lewes, UK) with SP6 and T7 RNA polymerases (both from Roche), respectively. Embryos (E6.5, E9.5 and E11.5) were fixed in 4% paraformaldehyde/phosphate buffered saline (PBS) overnight and dehydrated in methanol. One ml of 50% methanol/50% DMSO was added on ice then 0.25 ml of 10% Triton X-100 was added and incubated at room temperature for 30 min. Embryos were then washed three times in PBT (PBS plus 0.1% Tween 20), pre-hybridized for 1 hour (67˚C) then hybridized at 67˚C for 4 days with 5.0 μg/ml of each respective probe. Following hybridization the embryos were washed twice in 2 × SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0) at 70˚C, three times in 2 × SSC/0.1% CHAPS (3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) at 70˚C, three times in 0.2 × SSC/0.1% CHAPS at 70˚C and twice with KTBT (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM KCl, 1% Triton X-100) at room temperature. Embryos were blocked in 20% heat-inactivated fetal calf serum/KTBT for 3 hours. Alkaline phosphatase-conjugated mouse anti-DIG antibody (Roche) was added (1:1000 dilution) and allowed to react overnight at 4˚C. The next day the embryos were washed five times with KTBT and incubated overnight at 4˚C. The embryos were washed twice in NTMT (100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50

mM MgCl, 0.1% Triton X-100) then a solution containing 3.5 µl/ml *p*-nitrotetrazolium blue, 3.5 µl/ml 5-bromo-4-chloro-3-indoyl-phosphate in NTMT was added for color detection. This reaction was carried out in the dark and was stopped by addition of 4% formal saline.

Alcian Blue/Alizarin Red Staining

The embryos (E9.5 and E11.5) were fixed in 90% ethanol and prior to staining, skin and viscera were removed. Alcian blue solution (0.01% alcian blue, 3% acetic acid) was added to the embryos and left for 3 days. The embryos were rehydrated in increasing dilutions of ethanol. Embryos were then placed in 1% KOH for 2 days to clear and then in alizarin red solution (0.001% alizarin red, 1% KOH) for 3 days. Embryos were washed with 1% KOH and stored in 100% glycerol.

Immunohistochemistry and Calcein Labeling

Tibiae were removed from female chickens aged 1 day, 3 weeks and 2 years and E6.5 and E.9.5 embryonic chickens. The mineralized tissue was briefly immersed in 5% polyvinyl alcohol (Sigma, Poole, UK) and chilled in an n-hexane freezing bath at –70˚C [26]. In some cases the tibiae were decalcified in 10% EDTA at 4˚C for up to 14 days before. Sections were cut (10 µm thickness), fixed in ice-cold acetone and stored at –80˚C until required. Immunolocalization of PHOSPHO1 was performed using a standard immunoperoxidase procedure which included an overnight incubation at 4˚C with affinity purified antiserum diluted to 4 µg/ml of IgG in PBS containing 5% fetal bovine serum (FBS) or 4 µg/ml of normal sheep IgG as control (Sigma). In some cases DAB peroxidase substrate tablets containing cobalt chloride (Sigma) were used. All sections were mounted without counterstaining. The

antiserum to PHOSPHO1 was raised in sheep against recombinant chicken PHOSPHO1 as previously described and shows specificity with avian tissues only [19]. For calcein labeling, 3 and 24 week-old female chickens were injected with 10 mg/kg calcein and culled by cervical dislocation 3 days after injection. The presence of calcein stain in sections of tibiae was visualized by fluorescence microscopy. Some sections were stained with haematoxylin and eosin or von Kossa using standard protocols to identify mineralizing regions.

Matrix Vesicle Isolation

Three week-old chickens were culled by cervical dislocation and the tibias removed. The growth plate was dissected from the overlying and underlying tissue and diced into small pieces (1 mm^3). Cartilage pieces were incubated at 37˚C in the presence of 0.1% trypsin type II (Sigma) in HBSS (Hank's buffered salt solution) for 30min. The tissue was washed in HBSS and incubated in 0.07% collagenase at 37˚C for 3 hours. The cell suspension was passed through a sieve (45 μm) to remove remaining tissue fragments and spun (1,000 g for 5 min) to pellet the cells. Supernatants were subjected to differential centrifugation. Briefly, the supernatant from the chondrocytes was spun at 30,000 g for 20 min to remove sub-cellular debris and finally at 100,000 g for 1 hour to pellet MVs. To obtain primary chondrocyte cultures, cartilage pieces were digested by collagenase and hyluronidase as previously described [27]. Chondrocytes were seeded in T25 culture flasks at a density of $1x10^6$ cells/cm^2 in Dulbecco's modified eagles medium containing 10% FBS, 2mM L-Glutamine. Following cellular attachment the cells were treated with or without ascorbic acid (100 μg/ml) and cultured for up to 12 days (37˚C, 5% CO_2). When harvesting the cells, the monolayer was washed with HBSS and incubated in 0.07%

collagenase type II solution at 37˚C for 90 minutes. The mixture was then subjected to differential centrifugation as described above.

TNAP activity was assayed using the ALP bio-assay kit (Thermo Electron, Melbourne, Australia). MVs activity was determined by measuring the cleavage of *p*-nitrophenyl phosphate (pNPP) at 405 nm, pH 10.7. Total TNAP activity was expressed as nmol pNPP hydrolyzed/min/mg of total protein.

Immunoblotting

The resultant MVs were analysed for the presence of PHOSPHO1 by immunoblotting. LDS sample buffer was added to the MV lysate extracted from the primary chondrocyte culture, whilst MVs extracted directly from chick cartilage were lysed in lithium dodecyl sulfate (LDS) sample buffer prior to electrophoresis. Samples corresponding to 10 μg total protein were incubated at 70˚C for 10 minutes before loading. Samples were run on a 10% Bis-Tris NuPAGE gel and electroblotted to nitrocellulose, which were then blocked in 5% non-fat milk in Tris buffered saline with 0.1% Tween 20 (TBST). The membranes were then probed with 2 μg/ml sheep-anti-PHOSPHO1 antibody in blocking solution [19] and washed three times with TBST. Blots were then incubated with mouse anti sheep/goat IgG-peroxidase (DAKO, Cambridgeshire, UK) diluted 1:2,000 in blocking solution followed by three washes in TBST. The immune complexes were then visualized by enhanced chemiluminescence. Finally the proteins on the blot were stained with India ink after alkali pre-treatment, as described by Sutherland and Skerrit (28). Briefly, the membranes were washed in TBST before incubation with 0.2M NaOH for 5 minutes. The membrane was then submerged in 10% India ink solution for 120 minutes and finally washed repeatedly in TBST until only the protein bands were visible.

Results

Embryonic Localization of PHOSPHO1

PHOSPHO1 mRNA expression during embryonic development was investigated in the chick using whole-mount *in situ* hybridization, staining was seen in embryos incubated with the antisense oligonucleotides corresponding to PHOSPHO1 gene expression at the ossification centres of the long bones. No hybridization was observed in any other tissues or in embryos incubated with the sense oligonucleotides (Fig. 1A). PHOSPHO1 expression began prior to E6.5 around the mid-shaft of metatarsi and by E11.5 had spread to the ends of the diaphysis (Fig. 1B-D). The unmineralized cartilaginous ends of the bones remain unstained.

The temporal expression of PHOSPHO1 in relation to deposition of mineral was also investigated in the chick embryos. Figures 1E and F show PHOSPHO1 staining of the front limb bones (E9.5) and hind phalange (E11.5), respectively. The entire diaphysis of the front limb bones appeared to be stained whereas staining had a more restricted location within the diaphysis of the hind phalanges of the older mice. This developmental feature was confirmed by alcian blue and alizarin red staining. Between E9.5 to E11.5 these bones consisted primarily of a cartilaginous matrix with alizarin red staining of mineral restricted only to a small region around the centre of the bones in the front limb by E9.5 (Fig. 1G) whereas the phalanges at E11.5 show a complete lack of mineralization (Fig. 1H). Immunohistochemical staining of frozen sections of tibia from embryonic chicks (E6.5 and E9.5) mirrored that of the *in situ* hybridization and revealed that the PHOSPHO1 staining was localised to the osteoid (bone collar) and associated periosteal osteoblasts within the mid-diaphyseal region (Figs 2A-C). There were also indications that some chondrocytes within the rudiment

also stained positively (Fig. 2C). At this developmental stage the PHOSPHO1 stained osteoid and the matrix of the differentiating chondrocytes were not mineralized as assessed by von Kossa staining (data not shown). Control sections in which the primary antibody was substituted with normal sheep IgG showed no positive staining (Fig. 2D). By developmental stage E9.5 cortical bone formation had began and had a trabecularized appearance (Fig. 2E and F), which was distinct from osteonal bone present in post-hatch birds (Fig. 2J-2L). PHOSPHO1 staining was limited to the bone forming surfaces within cortical bone (Fig. 2G and H) and not on pre-existing mineralised surfaces as visualized by von Kossa staining (Fig. 2I).

PHOSPHO1 in Growing and Adult Bone

Strong immunoreactivity was observed at the periosteum and also the bone forming surfaces of the primary osteons situated within the cortical bone (Fig. 2J and K) of the one day-old and 3 week-old chicks. All osteons within the cortical bone of the 1 day-old chick were immunoreactive whereas only those within the periosteal area of the 3 week-old bone were positive. Staining within the periosteum of the 1 day-old and 3 week-old chicks was limited to the osteoid layer and mirrored that of the calcein fluorescence (Fig. 2M), with no staining present in the fibroblastic or osteogenic layers. In the 3 week-old chicks, closed osteons were negative and staining was also absent from the endosteal surface and the cells within the marrow cavity. PHOSPHO1 immunostaining was also present on medullary bone surfaces (Fig. 2L) but not within the periosteum or osteons of a 2 year-old adult chicken. At this age, bone apposition has ceased and no mineralization within osteons or at the periosteal surface takes place. Mineralization in medullary bone of adult female birds was observed by calcein fluorescence (Fig. 2N).

PHOSPHO1 in Isolated Matrix Vesicles

The presence of PHOSPHO1 was examined in MVs isolated from chick growth plate cartilage. Two forms of PHOSPHO1 (30.4 and 28.6 kD) were detected in MVs isolated directly from the tibial growth plates of 3 week-old chicks (Fig. 3A) and from primary cultured chondrocyte cells (Fig. 3B)**.** PHOSPHO1 expression in the MVs isolated from the primary cells was found to increase with time in culture and appeared to be much greater in the presence ascorbic acid after 12 days in culture. Equal loading of the samples was confirmed by India ink staining of the membrane (Fig. 3C). No overt signs of matrix mineralization were noted in these cultures. TNAP activity in the presence of ascorbic acid was found to double after 5 days in culture and was 4-times that of the control after 12 days but was not found to alter significantly with time in the control cultures (Fig. 3D).

Discussion

In the embryo, bones begin as condensations of mesenchymal cells that act as models (anlages) for further development. In the developing long bone, capillary invasion of the perichondrium coincides with a switch in perichondrial cell differentiation to the osteoblast lineage. These cells secrete a primary bone collar, which is a thin layer of mineralized bone, lying around the outside of the mid-section of the bone. The bone collar maintains the structural integrity of the bone that is weakened by osteoblasts eroding the internal calcified cartilage scaffold. The ossification process begins at the mid-diaphyseal region and progresses towards the bone extremities [29, 30].

PHOSPHO1 has previously been shown to be localized to mineralizing regions of chick bone [19] and has therefore been implicated in the generation P_i for

mineralization. If this hypothesis is true then developmental expression of PHOSPHO1 should precede the deposition of mineral and follow the same pattern. Whole-mount *in situ* hybridization indicated that PHOSPHO1 expression occurred prior to E6.5 and was present within the bone collar within the mid-shaft of the diaphysis of the long bones. This was confirmed by immunohistochemistry where both the non-mineralized bone collar and associated periosteal osteoblasts, which first appear at E6.5 -7 [31], stained strongly. The matrix staining of PHOSPHO1 on bone forming surfaces may reflect the presence of the enzyme in osteoblast derived MVs, which are deposited within newly formed osteoid [32]. The expression of PHOSPHO1 preceded the mineralization of the osteoid which is known not to occur until E7.5 in the chick [29]. Also the appearance of PHOSPHO1 in the cartilage rudiment at E6.5 is coincident with chondrocyte hypertrophy in the mid-diaphysis [33] and agrees with our previous observations that PHOSPHO1 is expressed by hypertrophic chondrocytes prior to them mineralizing their extracellular matrix [19].

By E11.5 expression was observed over the entire length of the diaphysis. In addition, mRNA expression preceded the deposition of mineral as revealed by alcian blue/alizarin red staining**.** The absence of PHOSPHO1 immunoreactivity on the surface of existing mineralized cortical bone is in accord with previous observations by us where PHOSPHO1 immunoreactivity was absent from terminally differentiated chondrocytes situated deep in the mineralized zone [19]. This suggests that PHOSPHO1 is required for the *de novo* formation of the inorganic phase but not for the continued crystal growth of hydroxyapatite. It is therefore likely that PHOSPHO1 has a pivotal role in first phase of the mineralization process during embryonic development.

By the time of hatching, the chick skeleton is essentially mineralized but continues to grow by periosteal apposition until adulthood. Expression of PHOSPHO1 was observed in all osteons within the cortical bone of the 1 day-old chick but only those situated in the periosteal region of the 3 week-old chick. This difference in PHOSPHO1 distribution reflects the vastly different bone apposition rates between the two ages of birds and is similar to TNAP protein localisation, which has previously been shown to be present at the surface of growing osteons but absent in closed osteons where mineralization is complete [34]. Further compelling evidence that PHOSPHO1 plays a role in mineralization comes from the observation that it is present in medullary bone but not cortical bone of adult chicks. In adults, bone apposition has ceased and no mineralization at the periosteum takes place. However, medullary bone in adult female birds constantly undergoes remodelling [35] as illustrated by calcein fluorescence.

Previous studies by us have shown that 30.4 and 28.6 kD forms of PHOSPHO1 exist in growth plate chondrocytes, corresponding to transcripts derived from alternative start sites in the PHOSPHO1 gene [19]. The amino acid sequences of both putative transcripts contain all three of the catalytic motifs found within the HAD enzyme superfamily [36] and so both forms are likely to be catalytically active. It is unknown whether each form has a distinct physiological significance. Both forms of PHOSPHO1 were found to be present in MVs providing strong evidence that PHOSPHO1 is involved in the first phase of the mineralization. Primary chondrocytes were cultured in the presence and absence of ascorbic acid. The addition of ascorbic acid was found to increase both PHOSPHO1 levels and TNAP activity in isolated MVs. Ascorbic acid is a known stimulant for osteogenesis and mineralization *in vitro* [37,38]. This suggests that PHOSPHO1 expression and TNAP activity are both

influenced by the state of differentiation and may be controlled by similar mechanisms within growth plate chondrocytes.

Ca^{2+} and P_i are known to be present at high levels in MVs even before induction of mineral formation [39]. The action of PHOSPHO1 in MVs will contribute to the P_i generated, although the extent this contribution is unknown. As well as its generation inside MVs it is also clear that some P_i is imported from the extracellular matrix with at least two types of P_i transporter known to be present in growth plate chondrocytes [40-43]. The ambient concentration of P_i in the extracellular fluid is close to 2 mM [44]. This may be due to TNAP, which is GPI-anchored to the outer MV membrane [45] as it's proposed role in the bone matrix is to generate the Pi needed for hydroxyapatite crystallization [46-48]. However, TNAP has also been hypothesized to hydrolyze the mineralization inhibitor PP_i [13] to facilitate mineral precipitation and growth [12, 49, 50]. It is therefore doubtful that the amount of P_i released from MV lipids would be sufficient to increase the overall level of extracellular P_i. However, the local effect of PHOSPHO1 within MVs may be sufficient to facilitate mineral formation. A schematic representation of this process is shown in Fig. 4.

In conclusion, PHOSPHO1 is expressed at the primary regions of ossification in developing embryonic bone prior to deposition of mineral and is thus likely to play a role in the initial events leading up to mineralization. We also show for the first time that PHOSPHO1 is enriched in MVs. This study provides further evidence that PHOSPHO1 expression is associated with skeletal mineralization and indirectly supports our hypothesis that PHOSPHO1 is involved in generating P_i within MVs for mineralization.

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FIGURE LEGENDS

Figure 1. Whole mount *in situ* hybridization showing PHOSPHO1 mRNA expression in the long bones. (**A**) Metatarsi from chicken embryos (E9.5) incubated with antisense (above) and sense (below) PHOSPHO1 oligonucleotides. The staining can be seen only around the ossification centre (or bone collar) of the bone from the embryo incubated with the antisense probe. (**B-D**) PHOSPHO1 expression in metatarsi from embryos (E6.5, E9.5 and E11.5, respectively). The region of PHOSPHO1 expression extends from the mid-shaft region to the tips between these stages. (**E** and **F**) PHOSPHO1 expression pattern in front limb (E9.5) and hind limb phalange (11.5), respectively. (**G** and **H**) Alcian blue/alizarin red staining showing the presence of cartilaginous matrix/mineral in front limb (E9.5) and hind limb phalange (11.5), respectively. Magnification bars represent 1 mm.

Figure 2. (**A**) Immunohistochemical staining of the bone collar (arrows) and chondrocytes with affinity purified antibodies to PHOSPHO1 in a cross-section of a tibia from E6.5 chick embryo. (**B**) Immunohistochemical staining of longitudinal tibial section from E6.5 embryo showing positive staining is present in the bone collar of the mid section of the diaphysis (arrows). The area delineated by the box is shown at higher magnification in (**C**) where the staining is present in the bone collar (arrows), periosteal osteoblasts (arrow heads) and chondrocytes (*). (**D**) Control longitudinal section of E6.5 chick incubated with normal sheep IgG. The bone collar (arrows) and chondrocytes (*) are unstained. (**E** and **F**) Haematoxylin and eosin stained sections showing trabecularized morphology of the developing cortical bone in longitudinal (**E**) and cross section (**F**) of E9.5 chick tibia. (**G** and **H**) Immunohistochemical staining of longitudinal tibial section from E9.5 embryo. (**H**)

Shows the box in (**G**) at higher magnification. Staining is limited to the bone forming surfaces (arrows) and is not present on all mineralized bone surfaces. (**I**) Von Kossa staining of mineral in longitudinal section of E9.5 chick tibia. Immunohistochemical staining of PHOSPHO1 in sections of tibia from (**J**) 1 day-old, (**K**) 3 week-old and (**L**) 2 year-old chickens and calcein fluorescence showing (**M**) mineralization within the periosteum and primary osteons of cortical bone in 3 week old chick and (**N**) mineralizing surface of medullary bone in 24 week old chickens. MB, medullary bone; CB, cortical bone. Staining within the cortical bone (**J** and **K**) was limited to the osteoid layer of the periosteum and the bone forming surfaces of the primary osteons (arrows) and mirrored that of the calcein fluorescence (**M**), with no staining present in the fibroblastic or osteogenic layers. No immunostaining was observed within the cortical bone of the adult (**L**). DAB peroxidase substrate tablets containing cobalt chloride giving a grey/black end product were used in B, C, G and H. Magnification bars represent 0.1 mm in **A**-**I** and 0.2 mm in **J**-**N**.

Figure 3. (**A**) Immunoblot showing PHOSPHO1 expression in MVs isolated from the tibial growth plates of 3 week old chicks. (**B**) Immunoblots examining PHOSPHO1 expression in MVs isolated from primary chondrocytes cultured in the presence or absence of 100 μg/ml ascorbic acid. (**C**) India Ink Staining of MV Immunoblot demonstrating equal loading in each lane. (**D**) TNAP activity of MVs isolated from primary chondrocyte cultures.

Figure 4. Schematic representation of Ca^{2+} and P_i accumulation in MVs. BSP, bone sialoprotein; PCho, phosphocholine; PEA, phosphoethanolamine; PLC, phospholipases C; A-C, annexin complex; Pi-T, phosphate transporter.