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Transport of Inorganic Phosphate in Primary Cultures of Chondrocytes Isolated From the Tibial Growth Plate of Normal Adolescent Chickens

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Abstract This report describes Pi transport activity in chondrocytes isolated from the growth plate (GP) of normal adolescent chickens grown in primary cell culture. Our recent work showed that Pi transport in matrix vesicles (MV) isolated from normal GP cartilage was not strictly Na^+-dependent, whereas previously characterized Pi transport from rachitic GP cartilage MV was. This Na^+-dependent Pi transporter (NaPiT), a member of the Type III Glvr-1 gene family, is expressed only transiently during early differentiation of GP cartilage, is enhanced by Pi-deficiency, and is most active at pH 6.8. Since GP mineralization requires abundant Pi and occurs under slightly alkaline conditions, it seemed unlikely that this type of Pi transporter was solely responsible for Pi uptake during normal GP development. Therefore we asked whether the lack of strict Na^+-dependency in Pi transport seen in normal MV was also evident in normal GP chondrocytes. In fact, cellular Pi transport was found not to be strictly Na^+-dependent, except for a brief period early in the culture. Choline could equally serve as a Na^+ substitute. Activity of choline-supported Pi transport was optimum at pH 7.6–8.0. In addition, prior exposure of the cells to elevated extracellular Pi (2–3 mM) strongly enhanced subsequent Pi uptake, which appeared to depend on prior loading of the cells with mineral ions. Prevention of Pi loading by pretreatment with Pi transport inhibitors not only inhibited subsequent cellular Pi uptake, it also blocked mineral formation. Treatment with elevated extracellular Pi did not induce apoptosis in these GP chondrocytes. J. Cell. Biochem. 86: 475–489, 2002.

Key words: growth plate chondrocytes; inorganic phosphate transport; sodium ion dependency; pH dependency; mineralization; phosphonoformate; alendronate

The initiation and propagation of de novo calcium phosphate mineral formation in vertebrate bone development is a complex process. In growth plate (GP) cartilage, matrix vesicles (MV), extracellular microstructures derived from the chondrocyte plasma membrane play a key role in initiating the process of mineralization [Anderson, 1969; Ali and Evans, 1973; Wuthier et al., 1992]. The mechanisms by which calcium (Ca^{2+}) and inorganic phosphate (Pi) accumulate into MV have not been fully elucidated, however, recent studies have shown that mineralization-competent MV contain a Ca^{2+} and Pi-rich nucleational core that is essential for induction of mineralization [Wuthier et al., 1992; Wu et al., 1993, 1997; Kirsch et al., 1994]. It is currently unclear how MV form and become loaded with Ca^{2+} and Pi, but it is clear that GP chondrocytes become elevated in Pi [Wuthier, 1977; Shapiro, 1984] and Ca^{2+} [Martin and Matthews, 1970; Carson et al., 1978; Iannotti et al., 1985; Wu et al., 1997] prior to the formation of MV. Once MV form there is strong evidence that Ca^{2+} enters the vesicle lumen via a protein carrier [Genge et al., 1988]; treatment with proteases destroys the ability of MV to accumulate Ca^{2+}. It is now evident that annexin V is the mediator of Ca^{2+} influx into MV [Genge et al., 1989; Arispe et al., 1996; Kirsch et al., 1997]. While Pi transport is equally critical for mineralization, how Pi enters the MV is still an open question despite much study.

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MV isolated from rachitic chicken GP cartilage have been used to characterize this Pi transporter [Caverzasio and Bonjour, 1996], which has been reported to be strictly Na^+-dependent [Montessuit et al., 1991]. However, our recent studies with MV isolated from the normal GP cartilage indicate that Pi transport is not strictly Na^+-dependent [Wu et al., 2002]. There is clear evidence that MV originate from the plasma membrane of GP chondrocytes [Borg et al., 1978, 1981] where the Na^+-dependent Pi transporter (NaPiT) is reported to occur [Montessuit et al., 1994]. In situ hybridization studies reveal the presence of this Pi transporter in GP cartilage in vivo [Caverzasio et al., 1996; Palmer et al., 1999]. Further, transient Pi-sensitive expression of Glvr-1 mRNA, a member of the Type III NaPiT/retrovirus receptor gene family, was observed early in the differentiation of CFK2 cells, a rat chondrogenic cell line that models rat endochondral bone formation [Wang et al., 2001]. Mansfield et al. [2001] have shown that while the constitutive Glvr-1 Pi transporter and a Type II Na-Pi co-transporter are present, they are not strongly expressed in chick GP cells. Based on the timing and level of expression of these NaPiTs in relation to GP development, it is unlikely that they are solely responsible for Pi transport during chondrocyte maturation. Therefore, we searched for evidence of other Pi transporters in the normal GP chondrocytes that might be more directly responsible for the cellular accumulation of Pi that is recognized to precede mineralization [Shapiro, 1984].

Our past studies have shown that increasing the extracellular Pi concentration from the usual Dulbecco's Modified Eagle's Medium (DMEM) level of 0.9–1.9 mM, a level near to that in normal chicken GP extracellular fluid (ECF) [Wuthier, 1977], was sufficient to enable mineralization of the GP cultures, without the addition of β-glycerophosphate [Bingham and Raisz, 1974; Ishikawa and Wuthier, 1992]. However, recent studies have demonstrated that in cultured chondrocytes isolated from late embryonic (day 19) chick cartilage, elevation of Pi levels in the ECF leads to the induction of programmed cell death (apoptosis) in the treated cells [Mansfield et al., 1999, 2001]. During this process intracellular levels of Pi become elevated. Treatment with inhibitors of Pi transport, for example, phosphonoformate (PFA) and alendronate (ALN), not only blocked uptake of Pi by the cells, but also prevented induction of apoptosis [Mansfield et al., 2001]. Although NaPiTs were investigated in these studies, the authors speculated that other transport systems might be involved. Since these studies were done on chondrocytes isolated from cartilage of embryonic chicks, which depend on a defined source of available phosphate (the egg yolk), we questioned whether the same phenomena occur in chondrocytes isolated from the GP of post-natal chickens, which depend on variously available exogenous sources of Pi.

In the current study, we studied primary cultures of chondrocytes isolated from the GP of normal rapidly-growing 6-week-old broiler strain chickens. We posed the following questions: (1) Are these cells as sensitive to elevated Pi levels in the ECF as the embryonic GP chondrocytes [Mansfield et al., 1999]? (2) Since our recent studies have shown that Pi uptake by MV isolated from normal GP cartilage is not strictly Na^+-dependent [Wu et al., 2002], is Pi uptake by chondrocytes isolated from normal GP strictly dependent on Na^+? (3) Since increasing the level of extracellular Pi is essential for induction of mineral formation by GP chondrocytes [Ishikawa and Wuthier, 1992], does a step-wise increase in the level of extracellular Pi in the media of cultured GP chondrocytes cause a parallel increase in Pi uptake that is not strictly Na^+-dependent? (4) Since inhibitors of NaPiT have been shown to prevent Pi loading and apoptosis in embryonic chondrocytes [Mansfield et al., 2001], do these agents have a similar effect on GP chondrocytes isolated from adolescent chickens? For example, do inhibitors of Pi transport, that is, PFA, phosphonoacetate (PAA), and ALN, affect the growth and mineralization of GP cultures? (5) Since the expression of the Glvr-1 Type III NaPiT was only expressed very early in chondrogenesis [Wang et al., 2001], is Pi uptake by GP chondrocytes strictly Na^+-dependent at all stages of GP development?

Finally, although early studies showed that the pH of the ECF at the site of GP mineralization ($pH \sim 7.6$) is significantly more alkaline [Howell et al., 1968; Wuthier, 1977], the pH optimum of the Glvr-1 Type III NaPiT [Wang et al., 2001] is much more acidic (pH 6.8) than the pH of blood (pH 7.4). We therefore asked, what is the pH optimum for ^{32}Pi uptake by GP chondrocytes as cells mature prior to calcification of these primary cultures? If our cultures accurately model the in vivo situation, ^{32}Pi uptake should be optimal at alkaline pH.

EXPERIMENTAL PROCEDURES

Cell Culture

The epiphyseal GP were dissected from the tibia of 6-week-old hybrid broiler-strain chickens and the chondrocytes isolated as previously reported [Wu et al., 1995]. The primary cultures were grown in 35 mM dishes (2 ml per dish) or 24-well plate (0.5 ml per well) in DMEM with 10% fetal bovine serum (FBS) for the first 7–8 days. Culture media were changed every 3–4 days; a fresh supplement of ascorbic acid (25–50 μg/ml) was added, starting on day 3–4, for the duration of the experiments. Unless otherwise specified, from day 7–8 onward, the medium was changed to DATP5 mineralization medium [Wu et al., 1995] and all cultures were grown in this medium until tested for ^{32}Pi uptake. DATP5 medium was prepared from DMEM basal medium by addition of eight amino acids [Ishikawa et al., 1985], insulin-transferrin-selenite, and 5% defined FBS; it had a total Pi level of 1.9 mM (0.9 mM from DMEM + 1 mM Na_2HPO_4) [Ishikawa et al., 1998]. All culture media contained 100 IU/ml of penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B.

Treatment Protocols

For investigating the effect of Pi level, freshly isolated GP chondrocytes were grown in DMEM plus 10% FBS and ascorbic acid until day 7 or 8. The medium was then switched to "DAT5" (DATP5 minus the extra 1.0 mM Na_2HPO_4), to which incremental amounts of Pi were added (0 to 7 mM) yielding media whose Pi level ranged from 0.9–7.9 mM. The cells were then grown for the specified days prior to testing their ability to take up ^{32}Pi from separate Pi-free, sodium- or choline-containing, buffered uptake media.

To examine the effect of Pi transporter inhibitors on ^{32}Pi uptake by the GP cells, the following agents were added to the DATP5 medium. Sodium PFA was used at 0.1 μM–1 mM; sodium PAA was used at 10–100 μM; and sodium ALN (Merck, West Point, PA) was used at 0.1–100 μM. Unless otherwise stated, all chemicals were obtained from Sigma Chemical, St. Louis, MO.

Biochemical Assays

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide–thiazolyl blue) assay [McGahon et al., 1995] was used to measure chondrocyte viability. It is based on the ability of mitochondrial dehydrogenases to oxidize the thiazolyl blue tetrazolium salt to an insoluble blue formazan product. Briefly, cells were grown in 24-well plates in medium with or without the test agent. At the end of the treatment period, the medium was removed; then 0.3 ml of MTT in DMEM (120 μg/ml) was added and incubated at 37°C for 2 h. The supernatant was removed and the formazan crystals were solubilized in 0.04 M HCl in isopropanol. Optical density was read at 595 nm. To analyze other biochemical activities, cells were harvested from 35 mm dishes as previously described [Wu et al., 1995] in 1 ml TMT buffer (50 mM Tris, pH 7.5, 0.5 mM $MgCl_2$, 0.05% Triton X-100); for cells grown in 24-well plates, 0.25 ml TMT was directly put into the wells after washing the cell layer, and the entire plate was sonicated in a water bath. Aliquots were taken for alkaline phosphatase (ALP), DNA, Lowry protein, Ca^{2+} and Pi analyses as previously described [Ishikawa et al., 1998].

Chondrocyte ^{32}Pi Uptake

Assay of ^{32}Pi uptake by the GP chondrocytes was performed essentially as described by Veldman et al. [1997] and Gupta et al. [1997] using a Pi-free, buffered salt solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 15 mM HEPES, pH 7.4 (Buffer A). For studying the dependence of Pi uptake on sodium (Na^+), choline chloride was substituted for NaCl in Buffer A, producing Na^+-free "Buffer B." The cell layer from the cultured GP chondrocytes was first washed twice with either buffer, and then ^{32}Pi uptake was initiated by adding 1 ml of the buffer containing 1 μCi of carrier-free $H_3(^{32}P)O_4$, followed by incubation at room temperature for 30 min. The incubation was stopped by aspiration of the radiolabeled medium followed by three rapid washes with ice-cold unlabeled buffer. The cell layer was then solubilized by adding 1 ml of 2% SDS and the radioactivity measured by liquid scintillation counting. The effect of extracellular pH on ^{32}Pi uptake by the chondrocytes was studied using either Buffer A or B, adjusted to various pH levels (pH 6.8–pH 8.0), and incubated for 30 min as above.

Effect of Pi Transporter Inhibitors

The cultured GP chondrocytes were treated with PFA, PAA, and ALN at the specified

concentrations and times indicated in the figure legends. Then, following removal of the culture medium, uptake of ^{32}Pi by the inhibitor-pretreated and untreated control chondrocytes was monitored in Buffers A and B as described previously.

RESULTS

Effect of P-Containing Supplements

We first examined the effect of the Pi level in ECF on GP chondrocytes by varying the level of various Pi-producing supplements in the DAT5 culture medium. We measured cellular proliferation (protein), ALP activity, and mineral deposition (Ca and Pi content/dish). Table I shows that in control cultures, while the 0.9 mM level of Pi found in DMEM enabled good cell growth, it did not support significant mineral formation. As expected, supplementation with increasing levels of β-glycerophosphate (BGP) (0.5–5.0 mM) progressively increased mineralization, accompanied by a progressive decline in ALP activity [Genge et al., 1988], and a slight decline in Lowry protein. Substitution of Na_2HPO_4 as the Pi source provided nearly equivalent growth and stimulation of mineral formation. Addition of 0.5–2.5 mM Na_2HPO_4 caused progressive mineral deposition, equivalent to that seen with 0.5–5.0 mM BGP.

Two Pi transport inhibitors were then tested for their ability to inhibit mineralization of cultured chondrocytes in vitro. Figure 1A shows that PFA, added to the culture medium at concentrations between 1 nM and 100 μM, caused progressive inhibition of chondrocyte mineralization. Half-maximal inhibition (IC_{50}) of PFA for Ca^{2+} deposition was 18.0 μM, and for Pi deposition it was 13.8 μM. At 100 μM PFA, mineralization was essentially blocked. Another analog, PAA, was also tested on the cultured cells (Fig. 1B). PAA was less than one-third as potent as PFA, with IC_{50} values for Ca^{2+} deposition of 58 μM and for Pi of 53 μM. Since the effects of these drugs could be due to inhibition of either Pi transport or mineral crystal growth [Wu et al., 2002] or both, in subsequent studies we explored the ability of GP chondrocytes to acquire ^{32}Pi before mineralization ensued.

Effect of Pi Levels in the Culture Medium on ^{32}P Uptake by GP Chondrocytes

To study the effect of elevating extracellular Pi on transport of Pi into GP cells, we examined ^{32}Pi uptake by GP chondrocytes early in the culture period, well before extracellular mineral deposition became evident (typically after 21–24 days). Variation of the Pi level of the cultures in DAT5 media (pH 7.4) was started on day 8 and continued for 6 days; ^{32}Pi uptake by the chondrocytes was then examined (total culture period = 14 days). The control DAT5 media contained 0.9 mM Pi; graded levels of supplemental Pi similar to those used by Mansfield et al. [2001] were added to give final levels of 1.4, 1.9, 3.4, 5.9, and 7.9 mM. After washing the cell layers to remove extracellular Pi, either a Na^+-containing (Buffer A) or a Na^+-free (Buffer B) solution (pH 7.4) was used to measure ^{32}Pi uptake. Figure 2A depicts the stimulatory effect caused by culturing the cells for 6 days in progressively higher Pi level media. Compared to cells cultured in the low-Pi (0.9 mM) DAT5 basal medium, or those in which 0.5 mM Pi had been added (total Pi = 1.4 mM), cells cultured in media supplemented with 1 mM Pi (total

TABLE I. Effect of P-Containing Supplements on the Development of Cultured GP Chondrocytes

Phosphate supplement	Concentration (mM)	Protein (mg/dish)	ALP (mu/mg)	Ca^{2+} (μmol/dish)	Pi (μmol/dish)	Ca^{2+}/Pi (molar)
None	0	1.00 ± 0.05	1.04 ± 0.20	1.13 ± 0.26	0.88 ± 0.13	1.27 ± 0.10
Naβ-GP	0.5	0.83 ± 0.06	1.55 ± 0.30	2.52 ± 0.50	1.79 ± 0.27	1.40 ± 0.07
	1.0	0.81 ± 0.14	1.34 ± 0.60	4.66 ± 0.31	3.16 ± 0.34	1.48 ± 0.06
	2.5	0.72 ± 0.08	0.95 ± 0.46	6.53 ± 0.22	4.66 ± 0.12	1.40 ± 0.06
	5.0	0.75 ± 0.02	0.59 ± 0.09	7.38 ± 0.19	5.26 ± 0.08	1.40 ± 0.03
Na_2HPO_4	0.5	0.74 ± 0.03	1.60 ± 0.41	2.69 ± 0.74	1.88 ± 0.27	1.43 ± 0.04
	1.0	0.74 ± 0.03	0.98 ± 0.08	3.52 ± 0.19	2.41 ± 0.11	1.46 ± 0.05
	2.5	0.78 ± 0.04	0.86 ± 0.22	7.30 ± 0.53	5.20 ± 0.20	140 ± 0.05

Chicken GP chondrocytes were grown in DAT5 culture media, containing penicillin-streptomycin and vitamin C as previously described [Wu et al., 1995]. From day 7 onward, the cultures were supplemented with the additives shown below, and on 24 day the cultures were harvested and analyzed. Values are the mean ± SEM of four replicate analyses. Na β-GP, sodium β-glycerophosphate.

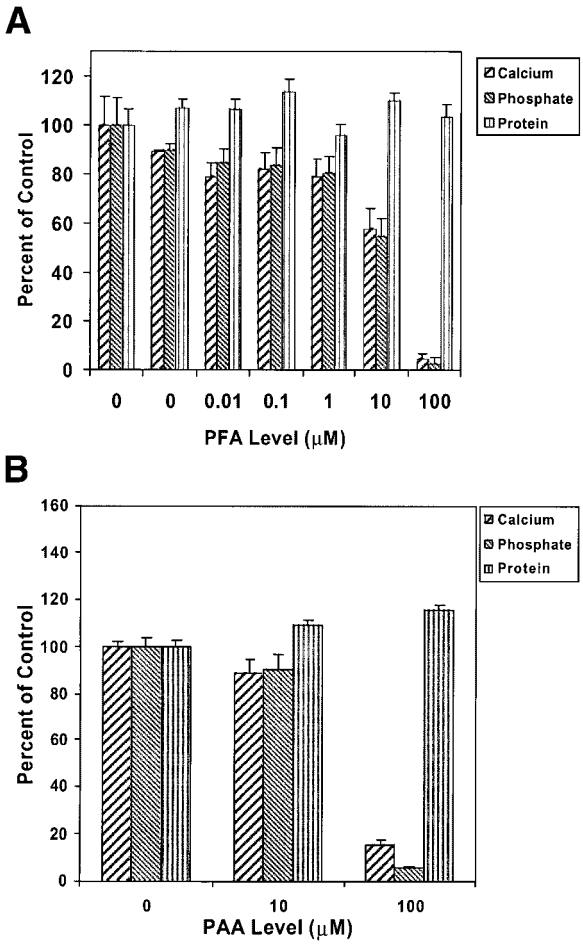


Fig. 1. Effect of phosphonoformate (PFA) and phosphonoacetate (PAA) on cell growth and mineral formation by GP chondrocytes grown in primary culture. (**A**) Various levels of PFA (0–100 μM) were added to preconfluent (day 9) cultures grown in DATP5 medium on day 9, and at each 3–4-day feeding interval thereafter until day 24 the day of harvest. (**B**) Various levels of PAA (0–100 μM) were similarly added to the preconfluent cultures grown in the same medium starting on day 10, with harvesting on day 25. Values shown are the mean ± SEM of four samples for each treatment. The calcium, phosphate, and protein content of each culture dish was analyzed.

Pi = 1.9 mM) showed markedly stimulated ability to acquire ^{32}Pi from the Pi uptake buffers. Further elevation of Pi levels (to 3.4–7.9 mM) in the culture media led to progressively higher ^{32}Pi uptake by the preconditioned chondrocytes (Fig. 2A). While the patterns of cellular ^{32}Pi uptake were generally similar, uptake from the Na^+-free, choline-containing Buffer B was significantly higher than that seen in the Na^+-containing Buffer A.

A longer supplementation of Pi in the culture media (9 days–total culture period = 17 days) led to a further increase in the capacity of the

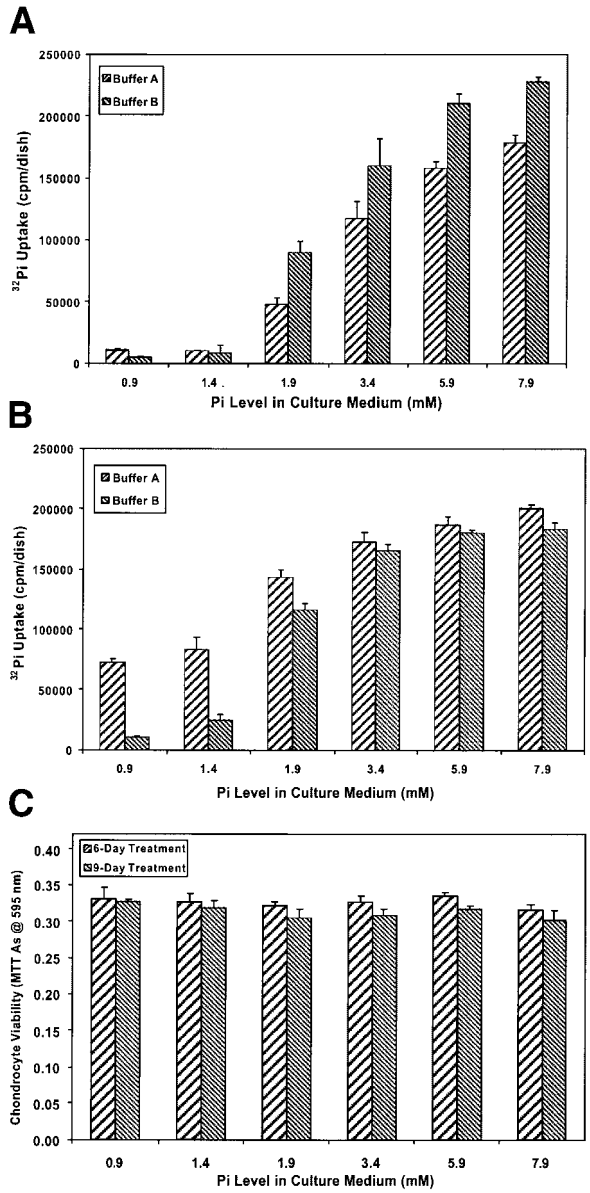


Fig. 2. Effect of Pi level in the culture medium on subsequent uptake of ^{32}Pi by GP chondrocytes. Primary cultures were grown in DMEM + 10% FBS until day 4, and then given DAT5 medium on day 8 containing incremental amounts of added Na_2HPO_4 (Pi) supplement: 0, 0.5, 1, 2.5, 5, and 7 mM. (DAT5 basal medium contains 0.9 mM Pi). The media were changed every 4 days thereafter, each with its respective Pi addition. Pi treatment was continued to day 14–6 days (**A**), or to day 17–9 days (**B**). ^{32}Pi uptake of the adherent cell monolayer was measured in either Na^+-containing (Buffer A), or Na^+-free, choline-containing (Buffer B) ^{32}Pi uptake medium. The Y-axis shows the total uptake of ^{32}Pi in cpm/dish; the X-axis shows the total Pi level in each set (0.9 mM Pi + each Pi addition). (**C**) The effect of Pi level in the culture medium on cell viability, using the MTT assay on either day 14 or day 17. Values shown are the mean ± SEM of four samples for each treatment. Note that the cells were viable even in the highest level of Pi tested (7.9 mM), which caused precipitation of extracellular mineral.

cells to accumulate ^{32}Pi (Fig. 2B). Addition of 1 mM Pi to the DAT5 medium (total Pi = 1.9 mM) again led to a marked increase in ^{32}Pi uptake, especially from the Na^+-free, choline-containing Buffer B. Contrary to what is seen with embryonic chondrocytes [Mansfield et al., 1999, 2001], viability of these GP cells indicated by the MTT assay was not affected by the addition of increasing levels of Pi to the media (Fig. 2C). These findings reveal that prior exposure of the GP chondrocytes to elevated levels of Pi enabled them to take up ^{32}Pi from the Pi transport buffers.

Effect of Exposure Time on Induction of Enhanced Cellular Pi Uptake

To gain insight into the mechanism of Pi-stimulated, not strictly Na^+-dependent ^{32}Pi uptake by the GP cells, we explored the timing of this effect. Cultures of GP chondrocytes were exposed to the various levels of Pi on day 8 of culture for 3, 6, or 9 days, after which ^{32}Pi uptake in Buffer B was measured. Figure 3 shows that the stimulatory effect of extracellular Pi was both time and concentration dependent. After 3 days of exposure, only the highest levels of Pi (3.4, 5.9, and 7.9 mM) caused significant stimulation of Pi uptake; with longer exposure (6 and 9 days) lower levels of Pi (1.9 and 1.4 mM) caused significant stimulation of cellular Pi uptake.

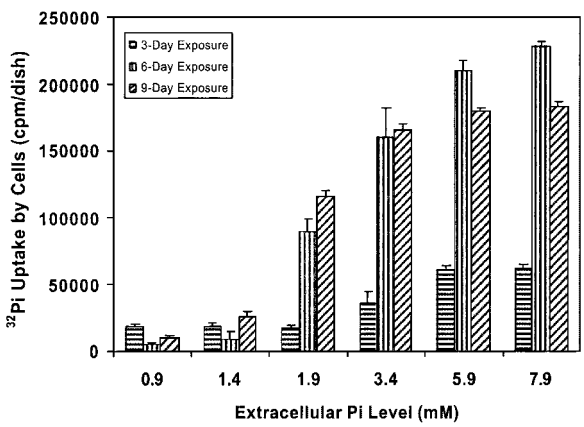


Fig. 3. Effect of length of exposure and Pi level on subsequent not-strictly-Na^+-dependent uptake of ^{32}Pi by GP chondrocytes. Primary cultures of GP chondrocytes were grown as described in Figure 2 with Pi addition on day 8 and continued for either 3, 6, or 9 days. ^{32}Pi uptake by the chondrocytes was measured in Na^+-free, choline-containing Buffer B. Values shown are the mean ± SEM of four samples for each treatment. Note that the stimulation of ^{32}Pi uptake by the chondrocytes was both dosage and time dependent: after 3 days exposure, only those cells treated with 3.4 mM and higher extracellular Pi had significant increases in ^{32}Pi uptake. With longer exposure time, lower levels of extracellular Pi exerted even greater stimulation: by day 6, 1.9 mM Pi caused over 17-fold stimulation of subsequent ^{32}Pi uptake; by day 9, even 1.4 mM Pi caused almost 2.5-fold stimulation.

Effects of Extracellular pH on ^{32}P Uptake by Chondrocytes

Because different Pi transporters have been shown to vary in pH optima as well as Na^+ ion dependency, we examined the effect of varying the pH of both the Na^+-containing (Buffer A) and Na^+-free (Buffer B) Pi uptake media on the ability of the GP chondrocytes to accumulate ^{32}Pi. Further, because there is a stage-dependent progression in the differentiation of the GP chondrocyte cultures during the induction of mineral formation in vitro, we examined Pi uptake at successive stages of the culture development. The cells were first established in DMEM + 10% FBS and then preconditioned by growing them for the indicated times in DATP5 (total Pi = 1.9 mM). Figure 4 demonstrates the effects of varying the pH of the Pi uptake medium on ^{32}Pi uptake by GP chondrocytes harvested at successive stages of culture: days 4, 8, 13, and 21. The ^{32}Pi uptake media, ranging in pH from 6.8 to 8.0, were made in either Na^+- containing Buffer A or Na^+-free (choline-containing) Buffer B, and ^{32}Pi transport into the chondrocytes was measured after 30 min incubation.

During the early culture period (e.g., days 4–5), ^{32}Pi uptake by the GP chondrocytes was highest at pH 6.8 in the Na^+-containing Buffer A, progressively decreasing at increasingly higher pH. At this early stage, Pi uptake from the Na^+-free choline-containing Buffer B was very limited and showed little pH dependency (Fig. 4A). However, as the cells began to rapidly divide and differentiate (day 8), there was a marked shift in both the Na^+ and pH dependence of ^{32}Pi uptake by the chondrocytes (Fig. 4B). There was an obvious reduction in the strictly Na^+-dependent, low pH-optimal uptake of ^{32}Pi, and a marked shift toward non-Na^+-dependent, high pH-dependent Pi uptake. Maximal ^{32}Pi uptake was seen at pH 8.0 in both Na^+-free and Na^+-containing uptake media. When the cells reached confluence (day 12–13) the switch to non-Na^+-dependent, high pH-dependent ^{32}Pi uptake was essentially complete (Fig. 4C). Later as the cultures approached calcification, there was no significant difference between uptake from the Na^+-containing and

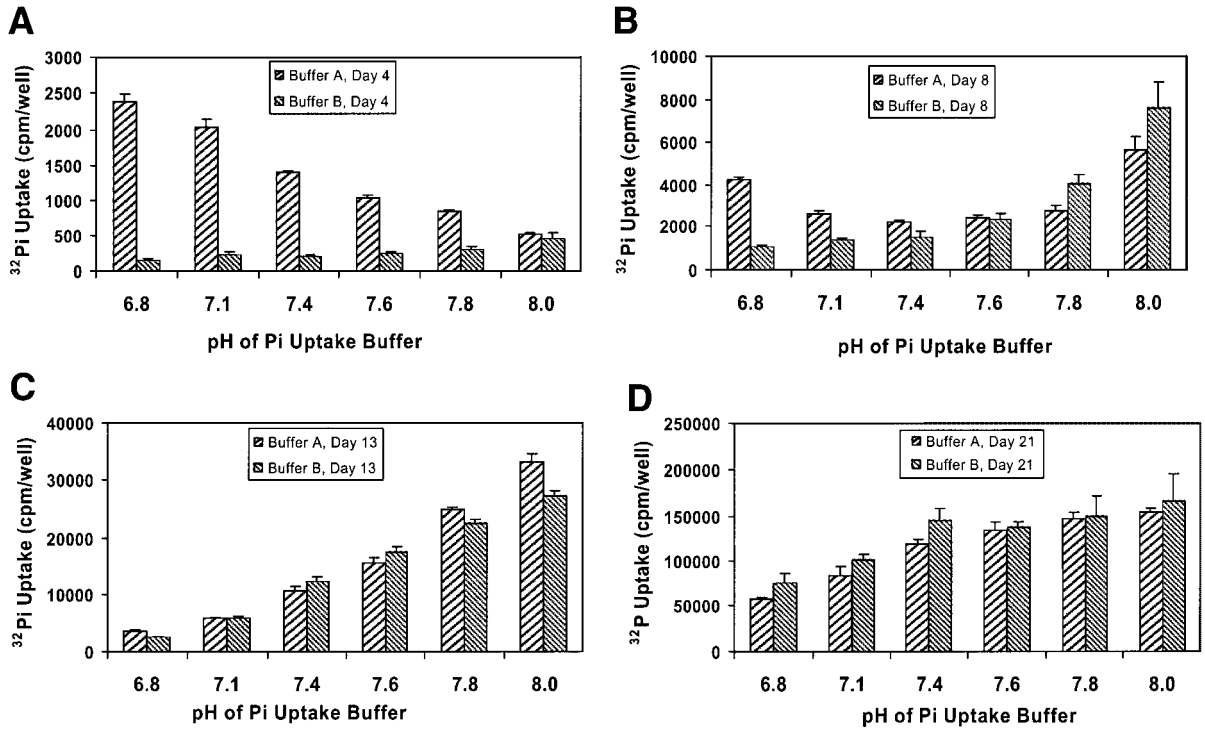


Fig. 4. Effect of culture time on pH and Na^+-dependent uptake of ^{32}Pi by GP chondrocytes. The time course of expression of Pi transport activity in primary cultures of GP chondrocytes, with respect to pH and Na^+-ion dependency, was measured. Cells were established for 8 days in DMEM + 10% FBS (total Pi = 0.9 mM) and then switched to DATP5 (total Pi = 1.9 mM) thereafter. Media were changed every 4 days. On the specified days (**A**) day 4, (**B**) day 8, (**C**) day 13, and (**D**) day 21, cellular ^{32}Pi uptake was measured at incremental increases in pH from 6.8 to 8.0 in either Na^+-containing (Buffer A) or Na^+-free, choline-containing (Buffer B). Values shown are the mean ± SEM of four samples for each treatment.

Na^+-free media, and the marked alkaline pH dependence became blunted (Fig. 4D). These findings point to the presence of two very different Pi transport systems in these differentiating GP chondrocytes: a strictly Na^+-dependent, low-pH-optimum transporter evident very early in the culture period, and a non-Na^+-dependent, high-pH-optimum transporter that becomes evident soon after the cells begin to differentiate.

Effect of Pi Transport Inhibitors on ^{32}Pi Uptake by GP Chondrocytes

To gain insight into the behavior of these two apparently distinct Pi transporters, and to explore the mechanism of the potentiation of Pi uptake of GP chondrocytes by prior exposure to elevated level of ECF Pi, we examined the effects of known inhibitors of Pi transport. PFA, a known competitive inhibitor of the strictly NaPiT in renal epithelial cells [Szczepanska-Konkel et al., 1986; Loghman-Adham et al., 1987; Szczepanska-Konkel et al., 1987], was then investigated for its ability to prevent the induction of ^{32}Pi uptake by GP chondrocytes. Treatment of preconfluent chondrocytes with PFA in DATP5 (total Pi = 1.9 mM) media was begun on day 10 and continued for 10 days; treatment of post-confluent cells was begun on day 16 and continued for 7 days. ^{32}Pi uptake by the chondrocytes in both Na^+-free and Na^+-containing media was then determined as above.

Treatment of the preconfluent GP chondrocytes with 10 μM PFA reduced ^{32}Pi uptake from Buffer A and B to 34 ± 2 and 19 ± 1% of the control, respectively; the IC_{50} (50% inhibition levels) were 3.9 and 2.7 μM PFA, respectively (Fig. 5A). PFA also reduced ^{32}Pi uptake by post-confluent chondrocytes, but required significantly higher levels (IC_{50} levels for Buffer A and Buffer B were 68 and 36 μM PFA, respectively) (Fig. 5B). The MTT assay shows that PFA treatment did not cause significant cell death (Fig. 5C).

Because pretreatment of the chondrocytes with the lowest level of PFA tested (10 μM) already caused marked inhibition of subsequent

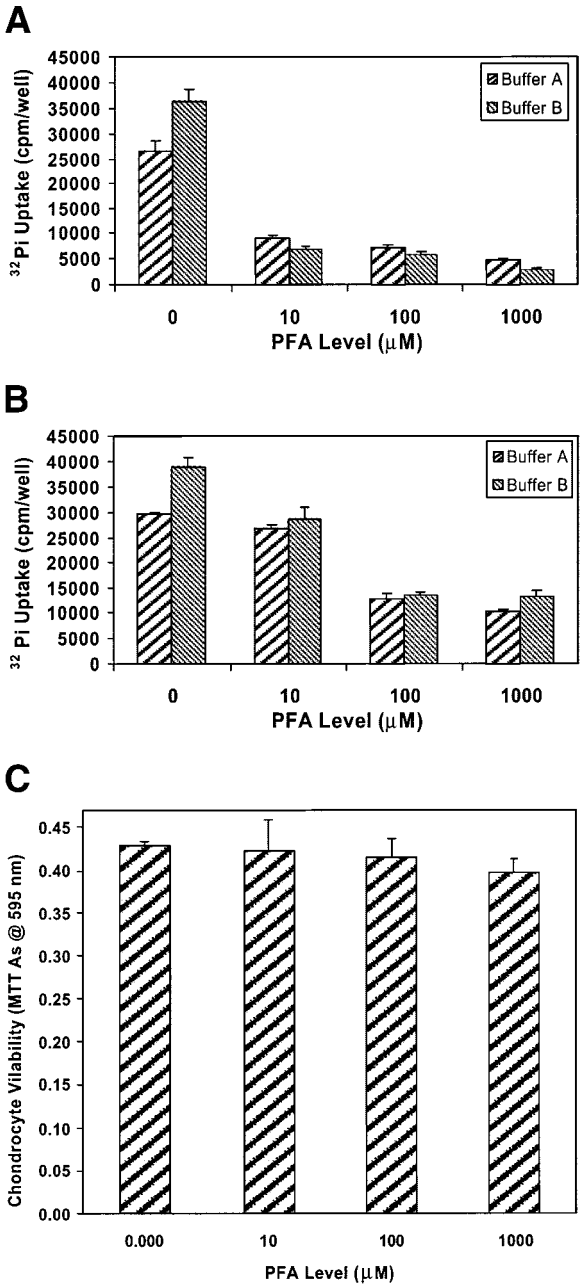


Fig. 5. Effect of pretreatment of pre- and post-confluent GP chondrocytes with the Pi transport inhibitor, phosphonoformate (PFA), on subsequent cellular uptake of ^{32}Pi. Chondrocyte cultures were grown in DATP5 (1.9 mM total Pi) as described in Figure 4. (**A**) PFA was added to the culture medium of preconfluent cells on day 10 and treatment continued to day 20. (**B**) PFA was also added to postconfluent cells on day 16 and treatment continued to day 23. ^{32}Pi uptake by the chondrocytes was then measured in either Na^+-containing (Buffer A) or Na^+-free, choline-containing (Buffer B). (**C**) Using the MTT assay on day 20, cell viability was measured on day 10-treated cultures set up in parallel to those shown in (A). Values shown are the mean ± SEM of four samples for each treatment. Note the marked inhibition of subsequent ^{32}Pi uptake by the PFA-treated cells, which was not caused by an affect on cell viability.

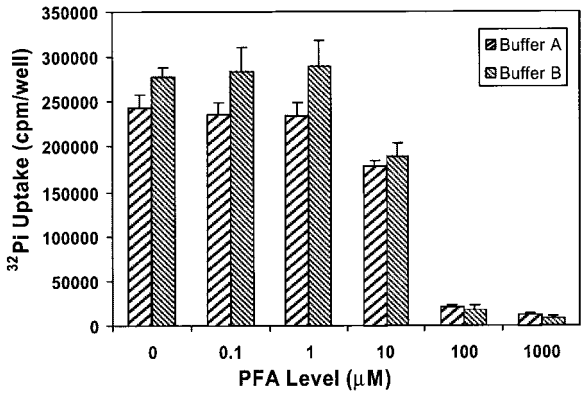


Fig. 6. Effect of low levels of PFA treatment on subsequent cellular ^{32}Pi uptake by GP chondrocytes. Cultures were grown as in Figure 4. Graded levels of PFA (0.1–1000 μM) were added to preconfluent cultures on day 10 and continued for 10 days. Cellular ^{32}Pi uptake was then measured in either Na^+-containing (Buffer A) or Na^+-free, choline-containing (Buffer B). Values shown are the mean ± SEM of four samples for each treatment.

^{32}Pi uptake, we tested the effect of a larger range of PFA level (0.1–1000 μM), studying the effect on preconfluent cells. Figure 6 shows that after 10 days exposure, PFA caused a dosage-dependent inhibition of the subsequent ability of the chondrocytes to take up ^{32}Pi from either Na^+-containing or Na^+-free media. This effect of PFA again was not the result of deterioration of the cells; there was no difference in MTT levels between the control and PFA-treated cells (data not shown).

The effects of ALN pretreatment on chondrocyte viability and subsequent cellular uptake of ^{32}Pi are shown in Figure 7. Treatment of the GP cells with ALN was begun on day 12 and continued for either 4 or 8 days. Effects of ALN on cell viability were assessed at the end of each treatment period (Fig. 7A,B). In contrast to PFA, ALN caused significant cell death after only 4 days exposure to the cells (Fig. 7C). The IC_{50} of ALN for cell viability was 6.7 μM after 4 days exposure and 4.8 μM after 8 days exposure. Subsequent ^{32}Pi uptake by the treated cells showed that low levels of ALN (0.5–1.0 μM) caused significant inhibition of ^{32}Pi uptake (Fig. 7A,B), but the highest level (20 μM), which caused major loss of cell viability, showed a reversal of ^{32}Pi uptake inhibition, presumably because of the high level of cell death. Half maximal inhibition of ^{32}Pi uptake by the cells occurred at ~6 μM for both the 4- and 8-day ALN-treated cells.

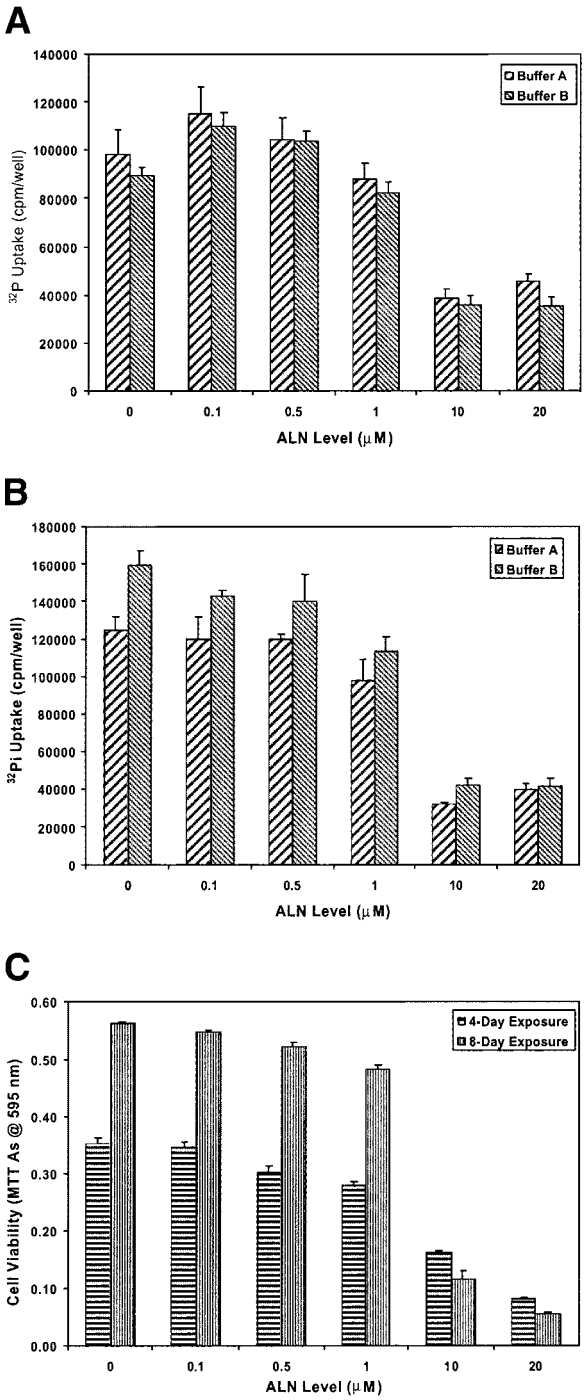


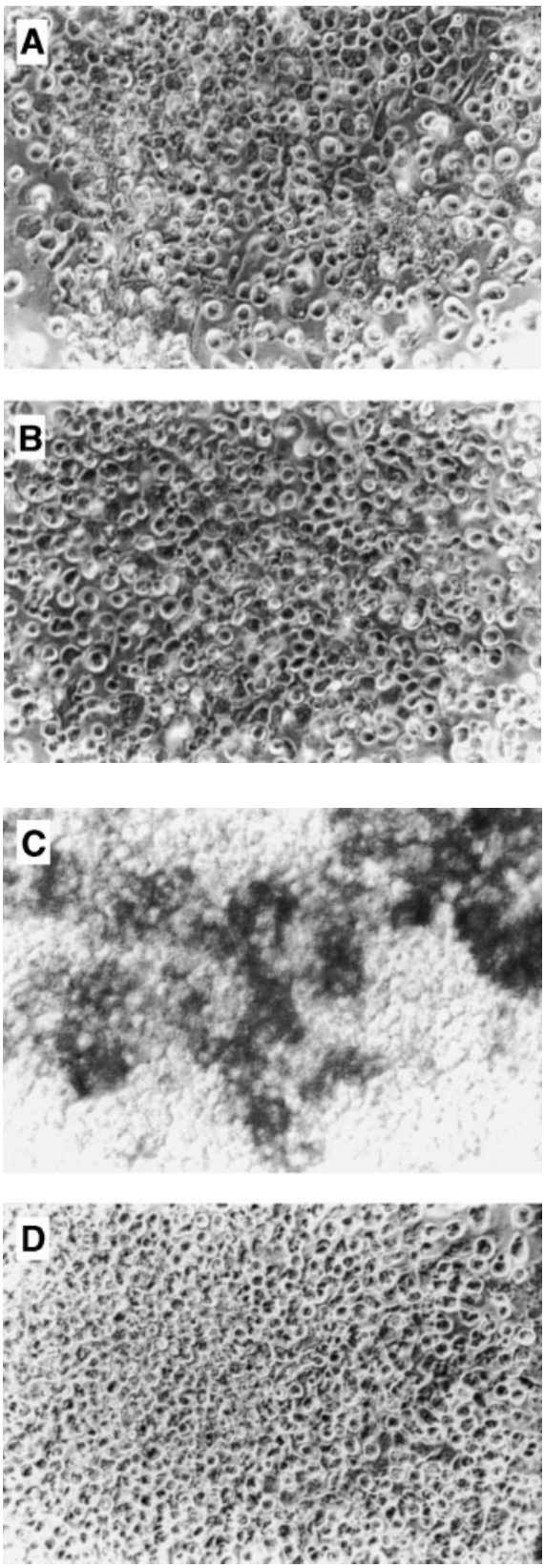
Fig. 7. Effect Alendronate (ALN) treatment on subsequent cellular ^{32}Pi uptake by GP chondrocytes. Cultures were grown in double sets under conditions described in Figure 4. ALN was added on day 12 of culture and treatment continued for either 4 days (**A**) or 8 days (**B**). ^{32}Pi uptake by the treated cells was measured in Buffer A or B as before. MTT assays of cell viability were also performed (**C**). Note in C that in contrast to all other previous treatments, ALN caused a significant time and dose-dependent loss in cell viability. Values shown are the mean ± SEM of four samples for each treatment.

Morphology of Chondrocytes After Treatment

Treatment with graded levels of PFA did not cause changes in the morphology of chondrocytes (Fig. 8A–D). ALN treatment caused the cells to become smaller, accompanied by apparent apoptotic cell death evidenced by the release of cellular granules into the matrix (Fig. 9A–C). Regarding the effect of Pi supplementation to the cultures, cells grown in the DAT5 medium (0.9 mM Pi, basal medium) became round and hypertrophic after 8 days (Fig. 10A). There was no apparent change when total Pi in the medium was increased to 1.4 mM (Fig. 10B). Increasing the Pi level to 1.9 mM caused the cells to form vesicular structures on the cell surface, but otherwise they appeared unchanged (Fig. 10C). Further increase of Pi in the medium caused substantial mineral formation in the extracellular space (Fig. 10D–F). While formation of cell surface vesicles was enhanced, the cells did not appear to become apoptotic. This was confirmed by the MTT assays (Fig. 2C).

DISCUSSION

The studies now reported describe the characterization of Pi transport in GP chondrocytes isolated from the proximal tibia of rapidly growing normal adolescent chickens. These cells were grown as primary cultures under conditions that enable the cells to undergo normal differentiation and mineral formation [Ishikawa and Wuthier, 1992; Wu et al., 1995]. We observed significant changes in ^{32}Pi uptake by these cells at progressive time periods in culture in terms of Na^+- and pH-dependency, as well as their response to prior incubation in media containing elevated Pi and various Pi transport inhibitors. Based on these findings we conclude that there must be at least two distinct types of transporters expressed during the culture of these GP chondrocytes. One type of Pi transporter, evident very early in the cultures (day 4–8), showed Na^+- and pH-dependency essentially identical to that previously described by others in GP chondrocytes [Montessuit et al., 1994; Caverzasio and Bonjour, 1996; Wang et al., 2001]. This activity was strictly Na^+-dependent, and had a pH optimum of 6.8. However, it became evident that another higher capacity Pi transporter became expressed shortly thereafter, and remained dominant for the rest of the culture period leading up to mineralization. We discovered this transporter activity when



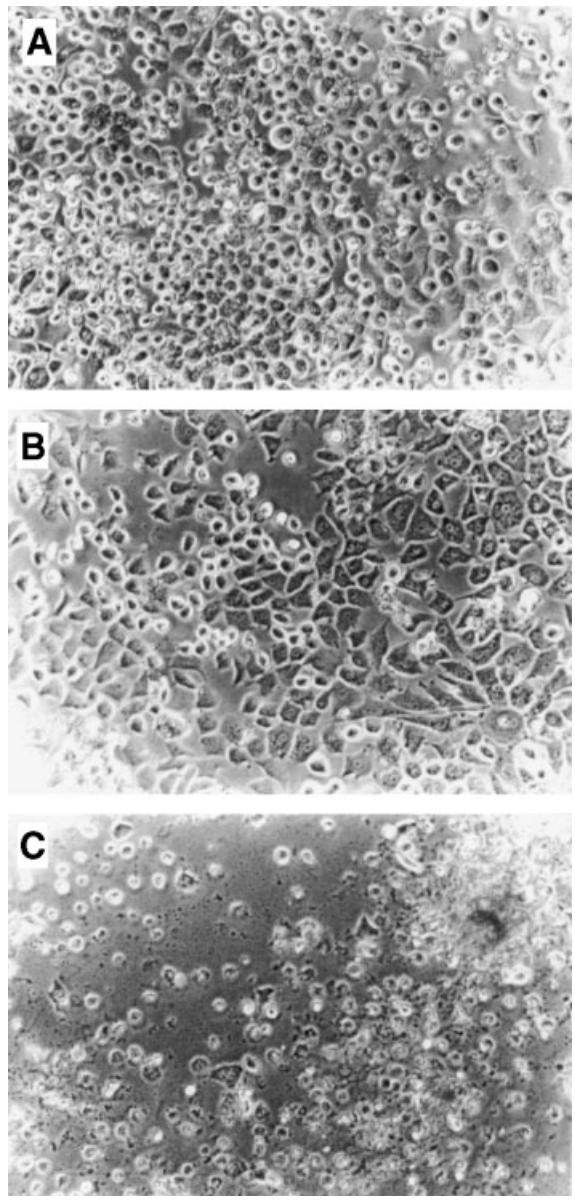


Fig. 9. Phase-contrast photomicrographs of primary cultures of GP chondrocytes grown in DATP5 medium and starting day 12 treated with ALN at either 1 μM (**B**) or 20 μM (**C**) until day 16. (**A**) is the untreated control. Note the marked effect of even 1 μM ALN on the morphology of the cells. At 20 μM, ALN caused massive cell death.

Fig. 8. Phase-contrast photomicrographs of primary cultures of GP chondrocytes grown in DATP5 medium (1.9 mM total Pi) in the absence (**A,C**) or presence (**B,D**) of PFA (100 μM) starting on day 10. Photographs were taken on days 15 (**A**,**B**) and 24 (**C**,**D**) of culture. Note the absence of mineral formation in the PFA-treated cultures, whereas the control cultures had begun to form extracellular mineral on day 15, and formed abundant mineral deposits on day 24.

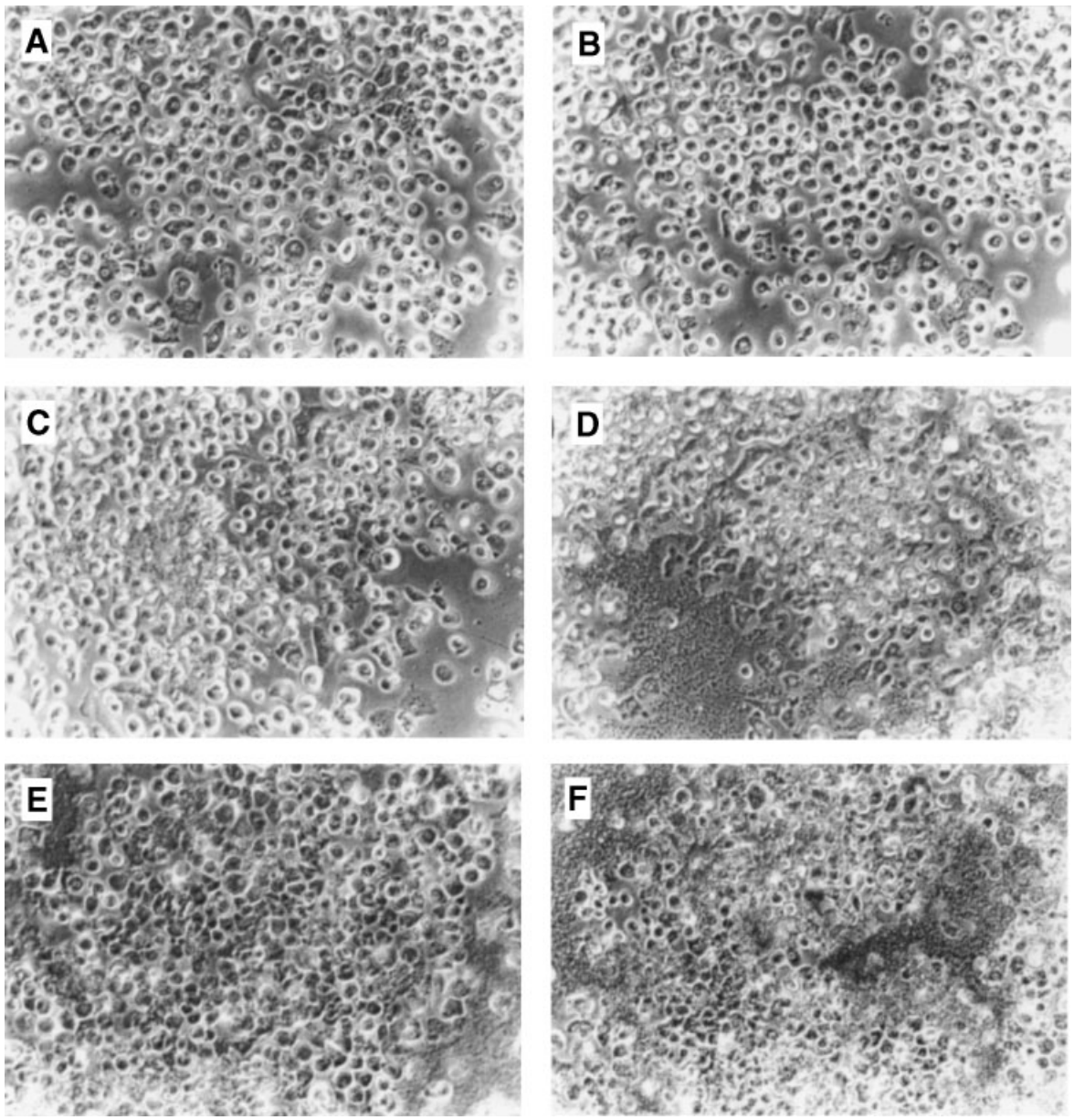


Fig. 10. Phase-contrast photomicrographs of primary cultures of GP chondrocytes grown in DAT5 medium containing graded levels of additional Na_2HPO_4 (Pi). Treatment with Pi was begun on day 8 and continued until day 14 when the photomicrographs were taken. (**A**) DAT5 control (0.9 mM total Pi), (**B**) DAT5 + 0.5 mM (1.4 mM total Pi), (**C**) DAT5 + 1.0 mM (1.9 mM total Pi), (**D**) DAT5 + 2.5 mM (3.4 mM total Pi), (**E**) DAT5 + 5.0 mM (5.9 mM total Pi), and (**F**) DAT5 + 7.0 mM (7.9 mM total Pi). Note that despite the presence of mineral precipitates at levels of 3.4 mM Pi and higher, the cells remained round and refractive with no evidence of apoptosis. This was confirmed by the MTT assays (Fig. 2C).

we examined the Na^+-dependency of ^{32}Pi uptake and found that after 8 days of culture, significant Pi uptake by the cells occurred in Na^+-free Buffer B, especially when the pH was increased progressively to pH 8. The activity of this high-capacity Pi transport system, which appears to be essentially identical to that recently described in normal MV [Wu et al., 2002], became dominant by the time the cells had almost reached confluence on day 13 of the culture.

When these chondrocytes were grown in DMEM + 10% FBS for 8 days, and then grown in DAT5 culture media supplemented with additional Pi for several (3–10) days, the ability of the cells to acquire ^{32}Pi from either Na^+-containing or Na^+-free uptake buffers was markedly enhanced in a dosage-dependent manner.

This finding demonstrates that the level of extracellular Pi regulates the activity of this not-strictly-NaPiT. Uptake of Pi by these cells was not due to the induction of programmed cell death (apoptosis). MTT assays (Fig. 2C) show that there was no evidence of cell death in these high-Pi, serum-containing media. As we have noted earlier [Ishikawa and Wuthier, 1992; Wu et al., 1995], the presence of a higher Pi level in the culture medium (Table I) also enhances the induction of mineralization. While the importance of extracellular Pi for mineralization has long been recognized [Howell et al., 1968], there is a growing recognition that the levels of Pi in particular are of key importance to GP mineralization [Kakuta et al., 1985; Wang et al., 2001]. Our current findings help elucidate the reasons for this effect.

Recently Mansfield et al. [1999, 2001], using 19-day chick embryo tibial chondrocyte cultures, showed that when the Pi level of the culture medium was raised above 3 mM, programmed cell death occurred. Specifically, they showed that Pi induced apoptosis in terminally differentiated hypertrophic chondrocytes. The apparent discrepancy between these and our current findings could result from several differences in experimental approach. In their studies, the chondrocytes were first conditioned by exposure for 1 day to serum-free medium, whereas our cells were maintained in serum-containing media until ^{32}Pi uptake was measured. It is possible that factors present in serum are protective to the cells. Second, they used nonadherent chondrocytes, whereas in our studies the cells we left in situ. It is thus possible that detached the cells were left in situ. Third, there was a difference in the tissue source of the cells. Their chondrocytes were isolated from the tibia of 19-day chick embryos, whereas the cells studied in this report were from tibia of 6–8-week-old adolescent chickens. During the embryonic period in ovo when the egg yolk constitutes a defined readily available source of Pi, it is probable that variations in extracellular Pi do not occur; thus, the cells would be vulnerable to any significant deviation from the normal state. In contrast, in the post-natal state there needs to be an ability to adapt to availability of dietary phosphate. In fact, it is well known that in young growing animals a phosphate-deficient diet produces rickets, typically accompanied by an arrest in growth [Parsons et al., 1970; Harrison et al., 1980; Lapatsanis et al., 1981]. Finally, the cells utilized in the Mansfield studies were terminal hypertrophic chondrocytes, which are particularly susceptible to induction of apoptosis. In the current studies, we focused on events occurring early during the culture period to avoid confusion that would occur if the cells were already involved in the induction of mineral formation.

The importance of "preloading" of phosphate by GP chondrocytes for subsequent mineralization was elucidated by the use of Pi transport inhibitors that in effect prevent the cells from accumulating phosphate. Studies using PFA pretreatment of the cells prior to analysis of ^{32}Pi uptake by chondrocytes reveal that this Pi analog significantly impaired the ability of the cells to acquire ^{32}Pi in subsequent assays of Pi transport activity, without causing any significant effect on cell growth or survival. In contrast, although ALN pretreatment caused similar inhibition of cellular Pi transport, this agent caused a significant decrease in viability of the GP chondrocytes; the IC_{50} for the apoptotic effect of ALN was 5–7 μM.

The sensitivity of primary cultures of GP chondrocytes to ALN was unexpected because in short-term assays, Mansfield et al. [2001] had previously shown that this drug protected chondrocytes exposed to high extracellular Pi concentration from apoptosis. ALN is an amino bisphosphonate that binds to hydroxyapatite in bone and has been reported to specifically inhibit the activity of osteoclasts [Sato et al., 1991]. In fact, Reszka et al. [1999] showed that several bisphosphonates, including ALN, could induce osteoclast apoptosis. However, in their investigation the level of ALN used was in the mM range, 1000 times the concentration utilized in this study.

Several studies have reported that a Type III NaPiT is active in chondrocytes and in other osteogenic cells [Montessuit et al., 1994; Montessuit et al., 1995; Palmer et al., 1999]. It is most active at acidic (6.8) pH, with decreased activity at alkaline pH. This strictly NaPiT is induced by phosphate deprivation and is upregulated in the rachitic animals [Montessuit et al., 1991]. Its activity is stage-specific [Wang et al., 2001] and under humoral control [Montessuit et al., 1994]. Treatment with IGF-1 stimulated the activity of this transporter in a time and dose-dependent manner, with maximal increase (~110%) being seen at ~8 h at a level of ~10 nM [Montessuit et al., 1994]. PTH

also stimulated the activity of this NaPiT in a biphasic manner. Wang et al. [2001], using the CFK2 chondrogenic cell line, also noted Pi-dependent regulation of this Glvr-1 NaPiT by PTHrP-(1–34).

Mansfield et al. [2001] have also demonstrated the presence of a Type II NaPiT in both adolescent and embryonic chick chondrocytes by immunohistochemical methods. Using an anti-Type II NaPiT antibody, these authors show that proliferative chondrocytes of the chicken GP are minimally stained, whereas the plasma membranes of the hypertrophic chondrocytes are more heavily stained.

While the exact timing and locus depends on the system studied, the Glvr-1 Type III NaPiT appears to be expressed early in the GP, although this may depend on whether the animals are embryonic or postnatal. For example, in day 17–18 mouse embryos using in situ hybridization, this transporter was found to be expressed (weakly) in both early hypertrophic cells expressing Type X collagen and in slightly more mature cells [Palmer et al., 1999]. On the other hand, studies using chondrogenic CFK2 cells that closely model GP development, expression of Glvr-1 mRNA was restricted to early stages well before expression of Type X collagen; more fully differentiated cells no longer produced it [Wang et al., 2001]. The transient period of Glvr-1 mRNA expression appears to correspond to a stage before GP chondrocytes become differentiated. Thus, the Glvr-1 NaPiT may be involved in the commitment of chondrocytes to GP differentiation. Our finding of the early expression (day 4) of acidic pH- and strictly Na^+-dependent Pi transport activity in the cultured GP chondrocytes is consistent with these earlier studies. This is further supported by our unpublished in situ hybridization studies that reveal the Type III Glvr NaPiT is predominantly expressed in the proliferative zone of the GP of adolescent chickens. The not-strictly-Na^+-dependent and alkaline pH-specific Pi transport activity we report here is clearly of a different type.

We are now investigating the molecular identity of this transporter. It is apparent that this Pi transporter becomes active in GP chondrocytes when extracellular Pi is plentiful. Conversely, it seems to be inactive when Pi levels are limited. Such a Pi transporter would be of benefit to growing animals because it would enable mineralization to occur rapidly when nutritional supplies of Pi are abundant, but would shut down when Pi was only marginally available. Under conditions of Pi deprivation, usage of Pi would be prioritized to prevent "squandering" of phosphate for bone formation when it was more critically needed for cellular energy metabolism or for proliferation of cells needed for survival. Whether this Pi transporter is upregulated during terminal differentiation and mineralization remains to be clarified.

Why would so many Pi transporters be needed to regulate GP chondrocyte Pi metabolism? Perhaps NaPiT, such as Type II and Type III, are essential for the maintenance of sufficient cellular Pi for cellular metabolism. Pi is required for many metabolic functions including oxidative phosphorylation, glycolysis, and general nucleotide synthesis to meet the demands of rapidly growing cells. In the proliferative zone of GP cartilage, the cells are well removed from an immediate blood supply and perhaps need to have a high-affinity Pi transporter upregulated under conditions of limited extracellular Pi supply. The site of expression of the Type II and III transporters is consistent with this interpretation.

On the other hand the newly described Pi transporter activity, which is upregulated in response to high Pi, would be ideal for loading the extra Pi required for mineralization. The cells would thus be able to produce phosphate- and calcium-enriched MV primed to rapidly induce mineralization. It is worth noting that a number of studies indicate that GP chondrocytes load elevated amounts of Pi prior to the onset of mineralization [Howell et al., 1968; Wuthier, 1977; Kakuta et al., 1985]. This suggests that normal GP cells have a Pi-sensing system that responds to increased Pi levels in the ECF, enabling them to accumulate Pi needed for induction of mineralization.

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