Characterisation of Inorganic Phosphate Transport in Bovine Articular Chondrocytes

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Key Words

Articular cartilage • Mineralisation • Osteoarthritis • Phosphate • NaPi

Abstract

In mineralising tissues such as growth plate cartilage extracellular organelles derived from the chondrocyte membrane are present. These matrix vesicles (MV) possess membrane transporters that accumulate Ca^{2+} and inorganic phosphate (P_i), and initiate the formation of hydroxyapatite crystals. MV are also present in articular cartilage, and hydroxyapatite crystals are believed to promote cartilage degradation in osteoarthritic joints. In the present study, P_i transport pathways in isolated bovine articular chondrocytes have been characterised. P_i uptake was temperature-sensitive and could be resolved into Na^+ -dependent and Na^+ -independent components. The Na^+ -dependent component saturated at high concentrations of extracellular P_i , with a K_m for P_i of 0.17 mM. In solutions lacking Na^+ , uptake did not fully saturate, implying that under these conditions carrier-mediated uptake is supplemented by a diffusive pathway. Both Na^+ -dependent and Na^+ -independent components were sensitive to the P_i transport

inhibitors phosphonoacetate and arsenate, although a fraction of Na^+ -independent P_i uptake was resistant to these anions. Total P_i uptake was optimal at pH 7.4, and reduced as pH was made more acidic or more alkaline, an effect that represented reduced Na^+ -dependent influx. RT-PCR analysis confirmed that two members of the NaPi III family, Pit-1 and Pit-2, are expressed, but that NaPi II transporters are not.

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Introduction

Chondrocytes are the only cell type normally resident in articular cartilage and, by the secretion of matrix macromolecules and enzymes that degrade them, are solely responsible for the upkeep of this tissue. An imbalance between anabolic and catabolic processes results in cartilage disorders such as osteoarthritis [1].

The presence of basic calcium phosphate (hydroxyapatite) crystals is a common feature of osteoarthritic joints [2, 3]. It is not clear how these crystals are associated with osteoarthritis, although they could initiate an inflammatory response [4], induce proteinase release from chondrocytes [5, 6] or act as mechanical

irritants within the joint [7].

Although largely performed in avian tissue, studies of mineralisation in growth plate cartilage provide a starting point for understanding crystal deposition in articular cartilage. There is evidence to believe that matrix vesicles (MV), extracellular organelles of up to several hundred nm in diameter that are derived from the chondrocyte membrane, are critical in the early stages of crystal formation [8, 9]. Studies of growth plate cartilage have shown that hydroxyapatite crystals form secondary to concentration of Ca^{2+} and P_i within MV, where the internal environment is specialised for the deposition of mineral complexes [10-12]. As Ca^{2+} and P_i concentrations are elevated within MV, their solubility product is exceeded leading to deposition of BCP at the inner surface of the MV membrane and the growth of needle-like hydroxyapatite crystals [13]. These act as a template for further mineral formation, until such time that the crystals grow to a certain size and break through the MV membrane [8]. Once released into the surrounding matrix, there is sufficient Ca^{2+} and P_i to support continued formation of hydroxyapatite crystals and tissue mineralisation.

Transport of Ca^{2+} and P_i across the MV membrane is, therefore, an important component of the calcification process. Growth plate MV possess annexins II, V and VI, which act as membrane channels through which Ca^{2+} can be taken up [14, 15] and, like the cells from which they are derived [16], they possess at least two transport pathways - one Na^+ dependent and the other Na^+ -independent - to accumulate P_i .

Although MV are present in articular cartilage, little is known concerning P_i transport in MV, or in the chondrocytes from which they are derived. In other cells, three distinct families of Na^+ -dependent transporters have been cloned and characterised [17-21]. NaPi-I most likely functions as an organic anion transporter [22], while NaPi-II transporters are expressed primarily in epithelia [21, 23, 24]. NaPi-III expression is more widespread, and this transporter performs a P_i 'housekeeping' role [25]. NaPi III has been demonstrated in cultured chondrogenic cells [26], and its upregulation by pro-inflammatory cytokines shown in cultured articular chondrocytes [27]. Although a stilbene-sensitive Na^+ -independent P_i -anion exchanger has also been described [28], these two studies excluded a role for Na^+ -independent P_i transport systems [26, 27].

In the present study, the membrane transport pathways mediating P_i uptake into isolated bovine articular chondrocytes (and potentially into MV derived from

them) have been determined. We report the functional presence at least two transport mechanisms, Na^+ -dependent and independent, and provide details of their kinetics and inhibitor sensitivity. We discuss their significance in the context of chondrocyte physiology.

Materials and Methods

Solutions

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Co. Ltd., Poole, U.K. In preliminary experiments, solutions containing 2mM Ca^{2+} were used and some precipitation of $[\text{}^{33}\text{P}]\text{-P}_i$ occurred, distorting values for subsequent P_i uptake measurement (data not shown) [29]. All uptake solutions were therefore prepared without Ca^{2+} or Mg^{2+} to prevent unwanted precipitation of insoluble phosphate compounds. Previous studies have reported that, while the absolute rate of P_i uptake is altered in Ca^{2+} -free solutions, the affinity of transporters for their substrate is unaffected [30]. HEPES-buffered saline (HBS; 300mOsm), for use in P_i uptake experiments and following chondrocyte isolation comprised (in mM): NaCl (145), KCl (5), D-glucose (10), ethylenediaminetetraacetic acid (EDTA; 0.1), and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES; 10), with pH adjusted to 7.4 (or, for certain experiments, to a different value) using concentrated NaOH. Ice cold KCl stop solution was used to halt P_i influx and comprised: KCl (150) and HEPES (10), with pH adjusted to 7.4 using concentrated NaOH. Na^+ -free solution was identical in composition to HBS except that *N*-methyl-D-glucamine (NMDG) chloride was substituted for sodium chloride and pH was adjusted using concentrated HCl. For experiments in which extracellular Na^+ concentration was varied, osmolarity was preserved by addition of Na^+ -free solution of equivalent pH in appropriate quantities. For all measurements of P_i transport chondrocytes were centrifuged through oil to stop P_i uptake. For this, a corn oil/dibutylphthalate (DBP) mixture was prepared with a final relative density 1.010 x water.

Stock solutions of sodium arsenate and phosphonoacetic acid were made up in distilled water. A stock solution of 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid sodium salt (SITS) was prepared in dimethylsulphoxide (DMSO). In all cases the concentration of the stock inhibitor was at least 100 times greater than the final concentration in the flux solution. Parallel control fluxes were measured in the presence of vehicle alone. Trichloroacetic acid (TCA; 5%) and Triton X-100 (0.1%) were prepared in distilled water.

Radioactive phosphate ($[\text{}^{33}\text{P}]\text{HPO}_4^{2-}$), used to measure P_i uptake, was obtained from Perkin-Elmer, Beaconsfield, U.K., with an initial activity of approximately 40 MBq ml^{-1} . Prior to each experiment, stock solutions of $[\text{}^{33}\text{P}]\text{HPO}_4^{2-}$ were made up in the relevant flux solution (approximately 2 $\mu\text{Ci ml}^{-1}$) supplemented with P_i (initial concentration 0.02 to 4 mM). Influx measurements were started by adding an equivalent volume of cell suspension to radioactive phosphate stock (below).

Chondrocyte isolation

Articular chondrocytes were isolated using a method modified from one previously described [31, 32]. Feet from steers were obtained immediately after slaughter at an abattoir and stored at 4°C until required. All feet used were less than 96 hours old. Prior to chondrocyte isolation, feet were cleaned, skinned and the hooves were removed, and the metacarpophalangeal joints were opened under near aseptic conditions in a sterile hood, using standard dissecting equipment washed in 70% ethanol. Cartilage shavings (approximately 1 mm thick) were removed from the articular surfaces and placed immediately in a culture flask containing 50 ml DMEM supplemented with collagenase (type I; 1 mg ml⁻¹), and incubated overnight (15–19 h, 37°C, 5% CO₂).

At the end of this period, undigested cartilage was removed by filtration, first using a cell strainer (pore size approximately 500 µm), followed by Nitex filter cloth (pore size 20 µm). The resulting cell suspension was centrifuged (800 x g, 10 min), the supernatant was discarded and the pellet resuspended in fresh HBS. This procedure was repeated twice before suspending chondrocytes in approximately 20 ml fresh HBS.

Chondrocytes were counted and their viability was assessed by the trypan blue exclusion technique in conjunction with light microscopy. A small sample of washed cells (50–100 µl) was diluted threefold with trypan blue dye (0.5% w:v in HBS), and transferred to a Neubauer haemocytometer, for examination by light microscopy. Cell preparations were discarded when the total proportion of viable cells was less than 95%. Viable cell suspensions were diluted with fresh HBS, such that the final concentration of cells was 1.0 x 10⁶ cells ml⁻¹, and incubated in a water bath at 37°C.

Measurement of P_i influx

To initiate measurements of P_i uptake, a known volume of chondrocyte suspension in a flux solution (37°C) was transferred into an Eppendorf tube which contained an equivalent volume of flux solution supplemented with a known concentration of sodium phosphate and approximately 2 µCi ml⁻¹ of [³³P]HPO₄²⁻. For certain experiments, the uptake solution was also supplemented with putative inhibitors of P_i uptake, at doses previously shown to be effective in other studies [33, 34]. Immediately after addition of the chondrocyte suspension (at time 0) the tubes were mixed for approximately 2 s prior to incubation in a water bath at 37°C or, for certain experiments, on ice (4°C).

After the required time, the uptake reaction was stopped by transferring 0.15 ml of influx solution into an Eppendorf tube containing 1 ml ice-cold KCl stop-solution layered above 0.3 ml DBP/corn oil mixture. The cells were immediately pelleted by microcentrifugation from the aqueous phase through the oil (10,000 x g, 20 s). The aqueous phase was aspirated and the tube washed four times. Cells were lysed by the addition of Triton X-100 (0.5%, 0.5 ml) and proteins were precipitated and pelleted by the addition of TCA (5%, 0.5 ml) and centrifugation (10,000 x g, 10 min). The resulting 1 ml supernatant was transferred to scintillation vials and supplemented with 4 ml scintillation fluid prior to counting. Counts recorded at t=0 min

were subtracted from values measured at all subsequent timepoints. Standard tubes containing known amounts of [³³P]HPO₄²⁻ from each separate influx solution were prepared and subjected to scintillation counting. The concentration of P_i taken up into chondrocytes was determined from an estimate of cell volume (assuming chondrocytes are spherical with a diameter of 10 µm).

RT-PCR

Total RNA was extracted from freshly isolated bovine chondrocytes, rat liver and rat kidney, according to the manufacturer's protocol of a commercially available extraction kit (QuickPrep Total RNA Extraction Kit, Pharmacia Biotech). Approximately 2 x 10⁶ cells or 0.5 g of rat tissue were used for each RNA preparation. RNA quantities were measured spectrophotometrically and 5 µg of total RNA was reverse transcribed using Superscript II and oligo-d(T)_{12–18} in a 20 µl reaction (Invitrogen). An aliquot of 1 µl of this reaction was used as a template in the subsequent PCR reactions. The primers were designed to the reported sequences of the *Bos taurus* transporter isoforms as detailed below, and custom synthesised by Sigma-Genosys. Sequences were as follows:

SLC34A1 (NaPi IIa) predicted from genomic sequence, accession number XM_883312.1: sense 5' ATC CTC CTT GTC AAG ATG CTC AAC TCT CTG CT 3' (bases 1150–1181), antisense 5' GAA GAA GAA GTG GCA GAG GGC AAT CTG 3' (1491–1465);

SLC34A2 (NaPi IIb) accession number NM_174661.2 [35]: sense 5' CTA CCT GGC CAT CCT TGT GGG G 3' (bases 1349–1370), antisense 5' CCA CGC CCA CCA GCA CTG 3' (1776–1759);

SLC20A1 (NaPi III/Pit 1) predicted from genomic sequence, accession number XM_874960.1: sense: 5' GCAATG ACG TCA GCA ATG CCA T 3' (bases 2905–2936), antisense: 5' CTG TGA CGA ACC AGG CCA TAA AAA TG 3' (bases 3282–3257);

SLC20A2 (NaPi III/Pit 2) predicted from genomic sequence, accession number XM_597108.2: sense: 5' GCA CCT GCT CTT CCA CTT CCT GC 3' (bases 1452–1474), antisense: 5' ATG AGG AGA GCC ATG ATA GCA GCA CTG 3' (1937–1911).

PCR was performed using *Taq* polymerase (Gibco) with a 57.5°C annealing temperature in a Hybaid Omnigene thermocycler for 35–40 PCR cycles. Products were separated on a 1% agarose gel and visualised by ethidium bromide fluorescence with a 100 base pair (bp) ladder (New England Biolabs Inc) as size markers. Sequencing was performed by the DNA Sequencing Service, Department of Biochemistry, University of Oxford.

Statistical analysis

Results are presented as mean ± S.E.M., where n is the number of independent determinations on cells isolated from joints of different animals. Experiments on cells from each isolation batch were performed in triplicate. Significant differences were determined using Student's *t* test for unpaired samples. Analysis was performed using Prism 3.0 (GraphPad Software).

Fig. 1. Effect of temperature on uptake of P_i into isolated bovine articular chondrocytes. Transport of radiolabelled P_i was determined over 90 min for chondrocytes suspended in HBS at 37°C (●) and at 4°C (○). HBS (pH 7.4) contained 145 mM Na^+ and 0.1 mM P_i . Results are presented as mean $\pm$ SEM, $n=6$ (37°C), $n=3$ (4°C).

Results

Time course of P_i uptake

Uptake of P_i into isolated articular chondrocytes was measured over 90 min using standard radioisotopic flux techniques. In line with studies in growth plate chondrocytes, unless otherwise stated, extracellular P_i concentration ($[P_i]_o$) was 0.1 mM. At 37°C, P_i uptake was linear for approximately 10 min and slowed, although did not plateau, over the subsequent 80 min (Figure 1). In contrast, for cells incubated at 4°C, P_i uptake was linear for 90 min, albeit at a considerably reduced rate (Figure 1). After 90 min the total uptake of P_i was approximately five-fold less at 4°C than at 37°C.

Effects of varying P_i and Na^+ concentration

Experiments were conducted to determine the effect of varying $[P_i]_o$ on P_i uptake into chondrocytes. Initial rates for influx were derived from uptake values measured during the initial 10 min. For cells suspended in HBS, P_i uptake rate increased as $[P_i]_o$ increased. Although the rate did not exhibit such a steep dependence on $[P_i]_o$ at the higher concentrations tested, it did not plateau (Figure 2a), indicating that - over the concentration range of $[P_i]_o$ tested here - uptake was not saturable. For chondrocytes incubated in Na^+ -free solution, P_i uptake at any given $[P_i]_o$ was significantly reduced. In-

Fig. 2. (a) Dependence of P_i transport on $[P_i]_o$. P_i uptake into articular chondrocytes was determined for 10 min in HBS (●) and Na^+ -free solution (○) at 37°C (pH 7.4). Uptake solutions were supplemented with varying amounts of P_i to achieve $[P_i]_o$ of 0.01 to 2 mM. Results are presented as mean $\pm$ SEM, $n=17$ (HBS), $n=6$ (Na^+ -free solution). Na^+ -dependent uptake (dashed line) was calculated by subtracting the Na^+ -independent component (in Na^+ -free solution) from P_i uptake measurements determined in HBS. Results are presented as mean $\pm$ SEM, $n=18$ (HBS), $n=6$ (Na^+ -free solution). (b) Separation of total Na^+ -independent uptake (●) into a saturable (○) fraction and a linear fraction (dotted line). Influx in Na^+ -free solution was fitted using the equation $P_i \text{ uptake} = \{((V_{max} [P_i]) / (K_m + [P_i])) + ([P_i] K_c)\}$, where K_c is a constant.

creased $[P_i]_o$ continued to elicit an increase in the rate of P_i uptake, although again saturation was not observed (Figure 2a). The Na^+ -dependent component of uptake exhibited saturation kinetics, with a K_m for P_i of 0.17 ± 0.03 mM (determined using Prism, GraphPad software). Uptake in the absence of Na^+ could be fitted to a two component model (Prism, GraphPad software) with satu-

Fig. 3. Na^+ -dependent P_i transport: additional P_i uptake induced in the presence of varying $[\text{Na}^+]_o$. P_i uptake into articular chondrocytes was determined for 10 min in mixtures of HBS and Na^+ -free solution (final $[\text{Na}^+]_o$ 0 to 145mM), supplemented with 0.1 mM P_i at 37°C (pH 7.4). P_i uptake recorded in Na^+ -free solution was subtracted from all other values. Results are presented as mean $\pm$ SEM, $n = 11$. Variable slope curve fitting (Prism, GraphPad software).

able and linear fractions (Figure 2b). The saturable fraction exhibited a K_m of $0.22 \pm 0.07\text{mM}$.

Experiments were next performed to determine the effect of varying extracellular Na^+ concentration ($[\text{Na}^+]_o$) on the rate of P_i uptake. Figure 3 shows the Na^+ -dependent component of P_i influx as a functional of $[\text{Na}^+]_o$. As $[\text{Na}^+]_o$ was increased from 0 to 50 mM, P_i uptake increased in parallel. However, at $[\text{Na}^+]_o$ concentrations of 75mM or higher, little further increase in rate of P_i uptake was apparent. Variable slope curve fitting (Prism, GraphPad software) yielded a K_m for Na^+ of 53.6 ± 12.4 mM.

Inhibitor studies

In subsequent experiments, the effects on P_i uptake rate of the anionic P_i analogues phosphonoacetate (PA) and arsenate (AsO_4^{3-}), were tested. PA concentrations of 2mM or higher significantly inhibited P_i uptake ($P < 0.05$), with approximately 80% inhibition achieved by 5mM (Figure 4a). Similarly, AsO_4^{3-} had a significant inhibitory effect ($P < 0.05$) at concentrations of 0.1 mM and greater, reaching a maximal inhibition of approximately 70% of the control uptake at a concentration of 10 mM (Figure 4b).

We further tested the effects of these inhibitors in Na^+ -free solutions. From these data, an inhibitory con-

Component	K_i ($\pm$ SEM, mM)	
	Phosphonoacetate	Arsenate
Na^+ -dependent	1.81 ± 1.4	5.03 ± 3.6
Na^+ -independent	0.88 ± 0.76	0.27 ± 0.06

Table 1. K_i values for inhibition of P_i uptake into bovine articular chondrocytes by phosphonoacetate and arsenate determined from dose-response curves of cells suspended in HBS and Na^+ -free solution at 37°C containing 0.1mM $[\text{P}_i]_o$ (pH 7.4). Na^+ -independent values are from cells in Na^+ -free solution. Na^+ -dependent values are calculated by subtracting Na^+ -independent values from uptake measured in HBS. Measured IC_{50} values were corrected for the effects of P_i binding using the equation $K_i = \text{IC}_{50} / (1 + [\text{P}_i] / K_m(\text{P}_i))$ where K_m for P_i is 0.17 and 0.22mM for Na^+ -dependent and Na^+ -independent uptake respectively.

stant (K_i) could be calculated for each of the inhibitors on the Na^+ -dependent and Na^+ -independent components of P_i uptake (Table 1). Both inhibitors were able to effectively abolish the Na^+ -dependent component of influx. In contrast, a fraction of Na^+ -independent influx was resistant to PA and AsO_4^{3-} , with little further inhibition achieved by doses of 1mM (Figure 4c and 4d). For the fractions that could be inhibited, estimates of K_i values showed that PAA and arsenate exhibit low affinities for the Na^+ -dependent component, but markedly higher affinities for the Na^+ -independent component.

Finally, treatment with SITS (0.2mM) reduced P_i uptake to $81.1 \pm 7.9\%$ of control values, although this action did not reach the level of statistical significance ($P > 0.05$).

Dependence on pH_o

P_i transport in other cells (including growth plate chondrocytes) has been shown to be sensitive to changes in pH. This may be an important property, given the acidic environment inhabited by the chondrocyte. To confirm whether P_i transporters in articular chondrocytes exhibit similar pH-dependence, we characterised the pH sensitivity of P_i uptake by these cells. Here, cells suspended in HBS demonstrated maximal uptake of P_i at pH_o 7.4, with P_i uptake especially reduced at more acidic pH values (Figure 4). For cells suspended in HBS at pH_o 5.5, P_i uptake was less than one third of that pH_o 7.4. In contrast, P_i uptake into cells suspended in Na^+ -free solution was insensitive to alterations in pH_o (Figure 5).

Fig. 4. Sensitivity of total P_i uptake and of Na^+ -dependent and -independent components of P_i uptake to (a, b) phosphonoacetate (0-10mM) and (c, d) arsenate (0-10mM). P_i uptake was measured in HBS supplemented with 0.1 mM P_i at 37°C (pH 7.4), in the presence or absence of the relevant inhibitor. Na^+ -dependent uptake (dashed line) was calculated by subtracting the Na^+ -independent component (in Na^+ -free solution; $\bigcirc$) from P_i uptake measurements determined in HBS. Data are presented as mean $\pm$ SEM, $n=6$, * $P<0.05$, ** $P<0.005$, (compared to rate in the absence of inhibitor).

Molecular identification of Na^+ -dependent P_i transporters

cDNA was prepared from freshly isolated bovine chondrocytes and RT-PCR performed using similar methodology to that previously described [36]. PCR primers were selected to detect potentially expressed phosphate membrane transport systems in articular chondrocytes: SLC34A1 (NaPi IIa), SLC34A2 (NaPi type IIb), SLC20A1 (Pit-1) and SLC20A2 (Pit-2). Rat cDNA (from kidney for NaPi IIa [37], from liver for NaPi IIb, Pit-1 and Pit-2 [38]) constituted positive PCR controls. Aliquots of PCR reactions were run on 1% agarose-TBE gels and the DNA was visualised under UV illumination

by staining with ethidium bromide. Where a band was obtained from chondrocyte cDNA samples it was sequenced (Department of Biochemistry, University of Oxford) to confirm its identity. Figure 5 illustrates the PCR products obtained. Using primers specific for SLC34A1 and SLC34A2, amplification yielded PCR products of the predicted size (341bp and 427bp respectively) in the positive control tissues but not in bovine chondrocytes. In contrast, when using primers specific for SLC20A1 and SLC20A2, amplification yielded PCR products of the predicted size (377bp and 485bp respectively) in both control tissues and bovine articular chondrocytes. The products amplified from bovine cDNA

Fig. 5. Effect of extracellular pH on P_i uptake by articular chondrocytes. Uptake was measured for 10 min in HBS (●) or in Na^+ -free solution (○) supplemented with 0.1mM P_i at 37°C. Na^+ -dependent P_i uptake (dotted line) was calculated by subtracting the Na^+ -independent component (in Na^+ free solution) from P_i uptake measurements determined in HBS at any given pH_o . Data are presented as mean $\pm$ SEM, $n=3$. * $p<0.05$ (compared to rate at pH 7.5).

were extracted, purified and sequenced. The sequences of these products were identical to the predicted isoforms in *Bos taurus*.

Discussion

This study has characterised the uptake of P_i by freshly isolated bovine articular chondrocytes. P_i is a vital cellular nutrient, and is particularly important to macromolecular synthesis and cellular energy production. In articular cartilage there is evidence that the inappropriate formation of BCP contributes to the onset and progression of osteoarthritis. Evidence from other tissue types, for example growth plate cartilage and bone, suggests that phosphate transporting mechanisms are essential for the formation of BCP. Furthermore, previous reports have indicated the significance of type-III Na^+ -dependent phosphate transporters to chondrocyte differentiation and the onset of osteoarthritis [26, 27]. However, we contend that the significance of these findings is not clear until a detailed kinetic study of all articular chondrocyte phosphate transporters is made. Furthermore, understanding the phosphate transporters present in the chondrocyte membrane is a necessary

Fig. 6. (a) Detection of SLC34A1 and SLC34A2. M: 100 base pair ladder; Lane 1: SLC34A1 in bovine chondrocytes; Lane 2: SLC34A1 in rat kidney; Lane 3: SLC34A2 in bovine chondrocytes; Lane 4: SLC34A2 in rat liver. (b) Detection of SLC20A1 and SLC20A2. M: 100 base pair ladder; Lane 1: SLC20A1 in bovine chondrocytes; Lane 2: SLC20A1 in rat liver; Lane 3: SLC20A2 in bovine chondrocytes; Lane 4: SLC20A2 in rat liver.

prerequisite to studies of MV physiology.

In preliminary experiments, the uptake of radiolabelled phosphate at 37°C was measured over a 90 min timecourse. Uptake was linear for 10 min, after which it slowed, although equilibrium was not achieved within 90 min. In contrast, P_i uptake measured at 4°C (when membrane transport mechanisms are predicted to be largely inoperative [39]) was significantly attenuated over the 90 min timecourse, a finding indicative of the operation of one or more membrane transporters for P_i uptake in articular chondrocytes. Subsequent experiments characterised the concentration dependence of P_i uptake during the initial 10 min period, when influx was a linear function of time. While a clear dependence of P_i uptake on $[P_i]_o$ was apparent, P_i uptake did not saturate over the concentration range tested here. However, the component of uptake that was dependent upon extracellular Na^+ did exhibit saturation kinetics. The $[P_i]$ of uncalcified cartilage matrix is around 0.7mM [40], so the K_m value derived here suggests that *in situ* this system will exhibit considerable activity and will be responsive to changes in prevailing P_i levels. In contrast, kinetic analysis of the Na^+ -dependence of this system shows that it will be saturated for Na^+ in the cation-rich environment of the extracellular matrix. In the absence of Na^+ ions,

significant P_i uptake persisted, which failed to saturate over the range of $[P_i]_o$ tested. Although previous studies of phosphate transport in articular chondrocytes have described only Na^+ -dependent transport, mediated by a Pit (NaPi III) system [26, 27]; it appears that Na^+ -independent processes are contributing to the P_i uptake that we have measured here.

At the current time, little information exists concerning the nature of Na^+ -independent P_i transport in mammalian cell membranes, and the identity of this system in these cells is unclear. Na^+ -independent influx does, however, account for a significant proportion of total P_i uptake into articular chondrocytes and its role is, therefore, likely to be important. In particular, Na^+ -independent transport is likely to play the dominant role in P_i uptake by MV derived from chondrocytes, since these structures lack the metabolic machinery to maintain Na^+ gradients across their membranes.

Anion exchange (AE) has been proposed to mediate Na^+ -independent P_i transport in epithelial cells [34]. It did not prove possible to determine whether a fraction of the Na^+ -independent flux in chondrocytes is mediated by this carrier. Our findings using the AE inhibitor SITS - which consistently reduced P_i uptake without this effect reaching the level of statistical significance - were somewhat inconclusive, perhaps because in Na^+ -free solution our data could be resolved into saturable and linear components.

Further support for the belief that the Na^+ -independent component comprises both carrier-mediated and diffusive pathways comes from the observation that a distinct uptake of P_i was recorded at $4^\circ C$, and from the studies we performed using the inhibitors PAA and AsO_4^{3-} . Although PAA and AsO_4^{3-} are frequently described as inhibitors of Na^+ -dependent P_i transport, they have previously been shown also to exert effects against Na^+ -independent systems [33]. In the present study, these competitive compounds were able to inhibit effectively the Na^+ -dependent component - albeit with low affinity - whereas a sizeable fraction of the Na^+ -independent component was resistant. PAA and AsO_4^{3-} nevertheless exhibited high affinity for that fraction of the Na^+ -independent component that they did inhibit.

In contrast to Na^+ -independent P_i transport, Na^+ -dependent P_i uptake was sensitive to alterations in

extracellular pH. P_i uptake was maximal at pH 7.4 and was markedly inhibited at more acidic values. Given the pK_a values for P_i of 2.1 and 6.8, the predominant species at pH 7.4 will be HPO_4^{2-} (although the amount of the $H_2PO_4^-$ species will not be insignificant), suggesting that this species is preferentially transported by the Na^+ -dependent system. Inhibition of P_i uptake in acidic solutions is noteworthy, given the acidic environment that the chondrocyte inhabits [41].

Although Na^+ -independent P_i transporters have yet to be identified, several Na^+ -dependent transport proteins have been cloned. The RT-PCR experiments performed in the present study detected mRNA for two type III NaPi cotransporters, Pit-1 and Pit-2, but failed to detect mRNA for the NaPi type IIa and IIb isoforms. Recent findings indicate that human Pit-1 and Pit-2 (NaPi III isoforms) expressed in *Xenopus* oocytes exhibit K_m values for P_i of 0.32mM and 0.16mM, respectively [30], values similar to the K_m for Na^+ -dependent uptake that we have obtained. Furthermore, both transporters are affected by pH changes in a similar fashion to that reported here [42]. It therefore seems probable that at least one member of the NaPi type III family is responsible for Na^+ -dependent P_i uptake in articular chondrocytes, a view that is in keeping with the near-ubiquitous expression in non-epithelial cells of NaPi III variants that perform a 'housekeeping' role in P_i uptake. Interestingly, Pit-1 expression in human knee articular chondrocytes is upregulated in OA cartilage [27].

Taken together, these findings suggest that at least two different systems transport P_i across the plasma membrane of articular chondrocytes. The identification of these P_i transport systems could have future therapeutic implications with regards to the treatment of degenerative joint disorders such as osteoarthritis. This disorder is frequently characterised by BCP crystal formation [43], secondary to the transmembrane accumulation of Ca^{2+} and P_i . Hence, the pathways identified here constitute a potential target for pharmacological intervention to prevent crystal formation and osteoarthritis.

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