Volume-sensitive taurine transport in bovine articular chondrocytes

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1. The swelling of bovine articular chondrocytes isolated from, or *in situ* within, cartilage by hypotonic shock rapidly activated the efflux or influx of radiolabelled taurine, an amino acid involved in volume regulation in a range of other cell types.

2. When chondrocytes were isolated by the use of collagenase into media of 280 or 380 mosmol l^{-1}, the activation of taurine efflux was at about the osmolarity of the isolating medium, but it was more marked for a given hypotonic shock in the cells isolated at the lower osmolarity. The volume of chondrocytes following isolation in these two osmolarities was the same, suggesting that the cells possess volume regulatory capacity.

3. In isolated chondrocytes, the induced pathway had some of the characteristics of a volume-activated channel, i.e. no transport saturation with increasing substrate concentration, and lack of *trans* acceleration. The pattern of inhibition of the volume-activated pathway by pharmacological blockers (e.g. pimozide, [(dihydro-indenyl)oxy]alkanoic acid (DIOA) and tamoxifen) differed from that described for a similar pathway in other cell types.

4. The transport of other potential osmolytes (uridine, sorbitol and inositol) was stimulated by cell swelling, independent of sodium and inhibited by pimozide with a selectivity ratio of taurine, 1·00; uridine, 0·84; sorbitol, 0·66; and inositol, 0·38. The selectivity of taurine : inositol was not altered at different cell volumes.

5. The intracellular taurine concentration of chondrocytes within cartilage was low (about 2 mmol (l cell water)$^{-1}$) showing a negligible role for taurine as an osmolyte during recovery from cell swelling. The swelling-induced loss of taurine from chondrocytes *in situ* was largely inhibited by pimozide and other drugs, showing that despite the rigid nature of cartilage, the chondrocytes were osmotically sensitive within the extracellular matrix.

The mechanical characteristics of articular cartilage are determined by the properties of the extracellular matrix, which principally comprises collagens and proteoglycans. These macromolecules are synthesized and degraded by chondrocytes, and although the cells comprise only about 5% by volume of the tissue, their activity determines the structure and composition of the matrix and hence its ability to withstand mechanical forces (Stockwell, 1991). The polyanionic proteoglycans markedly alter the ionic environment of the chondrocytes. The concentrations of free cations and anions are determined by the Gibbs–Donnan relationship – cation concentrations are high (e.g. Na^+, 250–300 mM; K^+, 8–10 mM; Ca^{2+}, 15–20 mM) whereas anion concentrations are correspondingly lower (e.g. Cl^-, 60–90 mM). The activity coefficients are the same as in free solution and the measured osmotic pressure is 350–450 mosmol l^{-1}, with the precise value at any point being determined by the local proteoglycan concentration (Maroudas, 1979; Urban & Hall, 1992).

Articular cartilage is exposed to significant mechanical forces *in vivo* and although it is known that loading is essential for cartilage health, the signals which the chondrocytes respond to are poorly understood. The effects of load on articular cartilage are complex, and difficult to separate out for independent study (see Hall, Urban & Gehl, 1991). The first event following the application of load is an increase in pressure (within milliseconds) to about 180 × atmospheric pressure (18 MPa; Hodge, Fijan, Carlson, Burgess, Harris & Mann, 1986). When cartilage loading is sustained, fluid expression occurs, the proteoglycan concentration rises, the cation concentration increases and conversely the anion concentrations fall. When load is removed, fluid is re-imbibed by the proteoglycans and the tissue swells towards normal hydration. Other changes to the tissue osmotic pressure have been reported, e.g. in osteoarthritic cartilage, increased hydration is the first detectable change before loss of matrix occurs (Stockwell, 1991). Since chondrocyte membrane water transport is very

rapid (Urban, Hall & Gehl, 1993), their volume will vary with changes in extracellular osmolarity. Chondrocytes are therefore exposed to cyclical changes in their osmotic and ionic environment and these alterations can influence matrix metabolism and ultimately the mechanical properties of the tissue (Urban *et al.* 1993).

There have been few studies on the physiology of chondrocytes (Stockwell, 1991), particularly the response of membrane transporters to anisotonic conditions. In other cell types, it is known that cells possess pathways whose permeability to osmolytes increases in response to changes in cell volume (Hoffmann & Simonsen, 1989). Thus when cells are swollen, regulatory volume decrease (RVD) is initiated whereby osmolytes are lost, resulting in cell shrinking towards normal volume. Although in most cell types K^+ and Cl^- play a major role in the RVD response, many cells utilize small organic molecules which can account for up to half the total osmolyte loss (e.g. Garcia-Romeu, Cossins & Motais, 1991). Recovery of cell volume following hypotonic shock has been observed in isolated chondrocytes (Deshayes, Hall & Urban, 1993). Conversely the process of regulatory volume increase (RVI) can be initiated in a range of cell types by which the uptake of osmolytes is stimulated resulting in cell swelling to normal volume (Hoffmann & Simonsen, 1989). Since matrix synthesis by chondrocytes, and ultimately the mechanical integrity of cartilage, is dependent on cell volume (Urban *et al.* 1993), an understanding of the processes involved in volume regulation by chondrocytes is important.

Amino acids, in particular taurine (2-amino-ethane-sulphonic acid), a non-essential sulphonic amino acid, play an important role during RVD in a range of cell types (e.g. brain cells: Pasantes-Morales & Schousboe, 1988; Law, 1991; erythrocytes: Fincham, Wolowyk & Young, 1987; Garcia-Romeu *et al.* 1991; Goldstein & Brill, 1991; MDCK cells: Sanchez-Olea, Pasantes-Morales, Lazaro & Cereijido, 1991; Roy & Malo, 1992; rabbit lymphocytes: Jesus-Garcia, Sanchez-Olea & Pasantes-Morales, 1991; hepatocytes: Ballatori & Boyer, 1992; ascites tumour cells: Lambert & Hoffmann, 1993). In view of the role of taurine in volume regulation in other cell types, as a first step towards understanding these processes in chondrocytes it was thought appropriate to determine taurine transport in anisotonic media and its sensitivity to a range of pharmacological blockers which have been shown to block a similar transporter in other cell types (e.g. Jackson & Strange, 1993). The results show that volume-activated fluxes of taurine can be measured in chondrocytes *in situ* and isolated from the matrix with the characteristics of a 'channel' with relatively low selectivity. A preliminary report on some of these findings has been presented (Hall, 1994).

METHODS

Biochemicals

Dulbecco's modified Eagle's medium (DMEM; 10 mM Hepes; pH 7·4 with NaOH; 280 mosmol l^{-1}) was used in this study. For aseptic incubations, sterile medium was used with antibiotic–antimycotic solution added to give 100 units penicillin, 100 μg streptomycin and 0·25 μg amphotericin B per millilitre of medium. Osmolarities were altered using NaCl or distilled water to shrink or swell the cells/cartilage, respectively, and osmotic pressures recorded with a Wescor vapour-pressure osmometer. The pH of all solutions was 7·4 $\pm$ 0·1 at 37 °C. Salts were Analar grade and obtained from BDH (Poole, UK) or Sigma. Collagenase (Type I) and crude papain were purchased from Sigma and radiolabelled [2-^{3}H(N)]taurine and [1,2-^{14}C]taurine were obtained from Du Pont (UK) (Stevenage, UK). The inhibitors pimozide, furosemide (frusemide), 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), tamoxifen, nitrobenzyl-thio-inosine (NBMPR), quinine and phloretin were obtained from Sigma; [(dihydro-indenyl)oxy]alkanoic acid (DIOA) was purchased from Semat (St Albans, UK); and gadolinium chloride ($GdCl_3$) was obtained from Aldrich Chemical Co. (Gillingham, UK). 4,4′-dinitro-2,2′-stilbene (DNDS) and 5-nitro-2-(3-phenylpropyl-amino benzoic acid (NPPB) were gifts from Dr K. Kirk. MK 196 (indacrinone) was a gift from Merck, Sharpe & Dohm (Rahway, NJ, USA). All stock solutions of inhibitors were prepared in dry dimethyl sulphoxide (DMSO); control conditions (i.e. in the absence of inhibitors) included identical volumes of DMSO.

Cartilage and chondrocyte isolation

The feet from young (1- to 2-year-old) steers were collected on the morning of slaughter from the abattoir, washed, skinned and the hoof removed. The unopened metacarpo-phalangeal joint was immersed in 70% (v/v) alcohol for 20 min, then opened and the articular cartilage removed under sterile conditions. Joints which were bruised, or cartilage which showed any signs of fibrillation or disease, were not used. The cartilage was cut from similar areas of the joints and placed into sterile DMEM. In some cases cartilage from several animals was pooled. The cartilage was washed with fresh medium to remove synovial fluid and placed with about 2–3 g cartilage (wet wt) per 40 ml medium. The flasks were sealed and maintained at 37 °C; media were changed daily under sterile conditions.

Chondrocytes were isolated by overnight (18–20 h) incubation of cartilage with collagenase (0·8 mg ml^{-1}). An initial incubation with pronase was not found to be necessary, and no fetal calf serum was present because it did not enhance chondrocyte yields (A. C. Hall, unpublished observations). In most experiments the osmolarity of the isolation medium was increased by the addition of 50 mM NaCl to prevent chondrocytes swelling following their release from the matrix. The pH of the solution had not changed significantly by the end of the isolation procedure. The solution containing isolated chondrocytes was passed through a fine tea strainer to remove undigested matrix and the filtrate passed by vacuum through a 25 μm pore size Nitex nylon mesh screen filter (R. Cadish & Sons, Finchley, London). The filtrate was collected and centrifuged (800 *g* for 10 min) and the supernatant removed. Fresh medium was added with a composition identical to that used for the chondrocyte isolation except that collagenase and antibiotics were not present. The cell pellet was gently resuspended and the chondrocytes washed twice in fresh medium, and then the final cell suspension prepared. A cell count

and viability assessment (using Trypan Blue; 0·5% w/v) was then performed using a haemocytometer. The viability of chondrocytes was routinely better than 95%, but the total number of cells isolated from each foot varied considerably. This was probably due to various factors, including different amounts of cartilage removed from the joint and differing potencies of various batches of collagenase. Usually about 40×10^6 cells were isolated from each bovine joint. There was variability between the membrane transport properties of different batches of chondrocytes and cartilage explants. This was probably the result of several factors, including the age of the animal used and the position on the joint from which the tissue was removed. Measurements of chondrocyte diameter were performed with a Coulter counter with channel analyser attachment, calibrated against latex beads of known volume suspended in the relevant solutions (Urban *et al.* 1993).

Measurement of radiotracer taurine influx or efflux

Centrifuge tubes with $1–3 \times 10^6$ cells ml^{-1} were prepared with various agents (pharmacological blockers, water, NaCl, etc.) as described in the figure legends. For Na^+ or Na^+-free conditions, chondrocytes were washed three times by centrifugation–aspiration in (mM): NaCl (or *N*-methyl-D-glucamine chloride (NMDG-Cl)), 190; 3-(*N*-morpholino)propanesulphonic acid (Mops), 15; glucose, 10; 380 mosmol l^{-1}; pH 7·4 with Tris base or HCl). For influx studies, an aliquot of the prewarmed cell suspension was then added to each tube, the flux was started by the addition of the radiotracer, the samples were placed at 37 ± 0·2 °C and timing begun. At the end of the flux period, extracellular tracer was quickly removed by centrifugation (10 000 *g* for 10 s), aspiration and resuspension in ice-cold DMEM (380 mosmol l^{-1}). This cycle was repeated four times over 8 min and reduced extracellular radioactivity to background levels. If the washing was performed quickly and the wash solution kept ice cold, there was no loss of tracer during this period. The cells were then lysed in Triton X-100 (0·5% v/v in water) added to the scintillation fluid and measured for radioactivity. Taurine influx was calculated as:

$$\text{Influx} = (A_t/A_S)[V_S C_S]/[C_C I_t],$$

where A_t is the intracellular count at the end of incubation period I_t (h), A_S is the count of the standard of known volume V_S (ml) and concentration C_S (mmol ml^{-1}), and C_C is the cell count ($\times 10^6$), yielding units for the influx of nanomoles per 10^6 cells per hour.

For efflux experiments, isolated chondrocytes were incubated in DMEM (about 8×10^6 cells ml^{-1}) at the required osmolarity with [^{3}H]taurine or [^{14}C]taurine (80 kBq ml^{-1}, 10 μM) for about 3 h (37 °C; pH 7·4). The cells were then washed by centrifugation (10 000 *g* for 10 s, five times) with ice-cold radiotracer-free medium of the required osmolarity, and resuspended at the appropriate cytocrit in prewarmed medium. Aliquots of the cell suspension were then placed in tubes containing the required conditions to give $1–3 \times 10^6$ cells ml^{-1}. An initial time point (A_i) was obtained by centrifuging samples and removing aliquots of supernatant for scintillation counting. Timing for the flux was then begun and samples taken and centrifuged when required. The addition of a hypotonic shock was performed using several methods (see Results). The most commonly used was the rapid addition of prewarmed distilled water to the cell suspension to give the required final osmolarity. No detectable lysis occurred under the conditions used here (see Results). Samples of the cell suspension were taken for equilibrium counts and taurine loss presented as the efflux, calculated as follows:

$$\text{Efflux} = -\ln[1 - (A_t - A_i/A_{inf} - A_i)]S/t,$$

where A_t is the counts lost at time t (h), A_{inf} the equilibrium counts, A_i the initial counts and S the intracellular concentration (nmol) in the cell water of 10^6 cells determined from separate aliquots of washed cells, yielding units for efflux of nanomoles per 10^6 cells per hour. Semilogarithmic plots established that taurine influx and efflux followed first-order kinetics over the first 10 min and therefore this was taken as the incubation time.

Determination of chondrocyte intracellular taurine concentration and taurine efflux of cartilage explants

Cartilage explants (about 200–300 μm thickness) amounting to about 500 mg condition^{-1} were placed in DMEM at the required osmolarity and including radioactive and non-radioactive taurine to give 100 μM with 40 kBq ml^{-1}. For higher taurine concentrations, the specific activity was maintained constant. For all these experiments, a parallel set of samples were prepared containing heat-treated (10 min at 100 °C) cartilage, these serving to correct for non-specific taurine binding to the matrix which, at the extracellular taurine concentration ([taurine]$_o$) of 100 μM, amounted to 10 ± 3% ($n = 12$ experiments) of specific incorporated counts. The explants were then incubated for 20 h at 37 °C. In some experiments longer incubations (up to 48 h) were performed to determine if further uptake of radiotracer taurine occurred, but no significant increase was observed. The cartilage slices were then washed in ice-cold DMEM of the required osmolarity. Usually six washes over 2 h were performed, and the radioactivity of the last wash solution was at background levels. For measurement of the intracellular taurine concentration ([taurine]$_i$), the cartilage slices were then blotted dry and their hydrated weights determined. Filtered papain solution (crude papain, 0·5% (w/v); EDTA, 5 mM; sodium acetate, 200 mM; cysteine HCl, 10 mM; pH 6·5 with NaOH) was added to the explants and they were incubated (3 h at 65 °C) until digested. Samples of the digest were then counted in scintillation fluid (Pico-Fluor 40) in a β counter.

It is difficult to determine precisely the taurine concentration of chondrocytes within the cartilage matrix, but a reasonable estimate can be obtained using the following method and making some assumptions. There are approximately $37·3 \times 10^3$ chondrocytes (mg wet wt)$^{-1}$ in bovine cartilage with a volume of approximately 550 fl cell^{-1} (Urban *et al.* 1993). If 50% of the chondrocyte comprises water (Urban *et al.* 1993) then, since the specific activity of taurine in the medium has been determined and the volume of chondrocyte water and the radioactivity of the intracellular taurine present (minus the non-specific binding) in the cartilage samples is known, the number of millimoles of taurine per litre of chondrocyte water can be calculated. For taurine efflux from cartilage explants, washed cartilage slices were blotted and placed in vials containing prewarmed medium of the required osmotic pressure. The samples were then incubated at 37 °C for 10 min with thorough mixing. An aliquot of the medium was removed and placed in a scintillation vial. The cartilage samples were then blotted and weighed, and the results expressed as shown in the figure legends. Control experiments established that in the most hypotonic solutions, the chondrocytes had lost approximately 20–30% of the taurine as a single exponential during the incubation period.

Data presentation and analysis

On a typical day, the cartilage from four to six legs was obtained and pooled. This corresponded to at least two and sometimes four animals. When required, the cartilage from different animals was maintained separately to determine variability. Results are given as means ± S.E.M. (unless otherwise stated) and are typical data of at least two experiments on different days with several feet per day, again unless indicated otherwise. Significance levels were evaluated using Student's unpaired t test; n is the number of experiments performed.

RESULTS

Activation of radiotracer taurine influx or efflux of bovine articular chondrocytes by hyposmotic shock

The increase in taurine flux following cell swelling was rapid both at normal (37 °C) and reduced (21 °C) temperature (Fig. 1) with no evidence of a lag phase at either temperature. The rate of taurine loss was significantly less at the later time points compared with those immediately following hypotonic shock. At comparable time points, the loss of taurine expressed as a percentage of total intracellular taurine at the initial time point was about two times greater at 37 °C than at the lower temperature.

In view of the rapid activation of taurine efflux following hypotonic shock it was important to determine if any lysis had occurred during the period after the change in osmolarity. To check this, two different types of experiments were performed. In the first, at the appropriate time the cells were centrifuged, the 380 mosmol l^{-1} medium was removed and replaced by medium at 280 mosmol l^{-1} (37 °C) after which the efflux was determined. The rate of activation of the taurine efflux was found to be identical to that observed in Fig. 1 at 37 °C. In the second type of experiment, chondrocytes suspended at 380 mosmol l^{-1} were treated with pimozide (50 μM), an inhibitor of the volume-activated taurine efflux (see below). At the appropriate time a hypotonic shock was delivered as described in Fig. 1, but no change in the rate of taurine efflux was observed. These experiments indicate that no significant lysis of chondrocytes occurred during delivery of the hypotonic shock and show that the activation of the pathway is, within the accuracy of the measurements, instantaneous.

Changing the osmolarity of chondrocytes isolated and suspended at 380 mosmol l^{-1} markedly altered taurine influx and efflux (Fig. 2*A* and *B*). Shrinkage of chondrocytes to osmolarities higher than those used during isolation (at 280 or 380 mosmol l^{-1}) did not further inhibit either taurine influx or efflux, indicating that the taurine flux was near to a minimal value. The volume-activated taurine flux was relatively sensitive to small increments in hyposmolarity and for efflux increased by about 2-fold for only a 5% change in medium osmolarity (from 380 to 360 mosmol l^{-1}; data not shown). Chondrocytes were also isolated at 280 mosmol l^{-1} and the effects of osmotic shock on taurine efflux measured. The

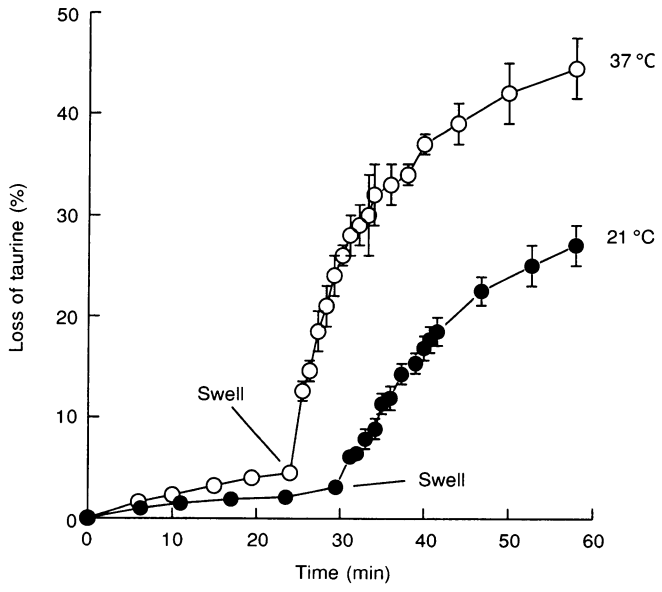

Figure 1. The rapid activation of taurine efflux from isolated bovine articular chondrocytes following hypotonic shock

Efflux was measured at 21 or 37 °C with osmolarity reduced from 380 to 280 mosmol l^{-1} at the points indicated (Swell) by the addition of water at the required temperature (see Methods). In this and subsequent figures, error bars are not shown when they are smaller than the symbols. Results are means ± S.D. from triplicate determinations made in one experiment, with similar data being obtained from two other experiments.

results (Fig. 2*B*) show that the point at which the volume-sensitive taurine flux was activated was close to the osmolarity used for chondrocyte isolation. In the most hypotonic media (120 and 180 mosmol l^{-1}), the rate of taurine efflux was not significantly different ($P > 0{\cdot}05$) between the two conditions studied. In further experiments, the osmolarity of the medium in which cells isolated at 280 or 380 mosmol l^{-1} were suspended was reduced by the same fraction (18%) and the increased taurine efflux measured. For cells isolated at 380 mosmol l^{-1} with osmolarity reduced to 312 mosmol l^{-1}, taurine efflux (in nmol $(10^6$ cells$)^{-1}$ h^{-1}) was increased from $1{\cdot}7 \pm 0{\cdot}4$ to $7{\cdot}0 \pm 1{\cdot}6$ (4·4 times); for cells isolated at 280 mosmol l^{-1} with osmolarity reduced to 230 mosmol l^{-1}, taurine efflux was increased from $2{\cdot}3 \pm 0{\cdot}6$ to $20{\cdot}2 \pm 4{\cdot}2$ (8·7 times; $n = 3$). Thus the activation of taurine efflux following the same effective hypotonic shock was more marked for cells isolated at the lower osmolarity.

In parallel experiments, chondrocyte diameters were determined following cell isolation at 280 or 380 mosmol l^{-1} as described (see Methods). Chondrocyte diameters in both osmolarities were not significantly different ($11{\cdot}44 \pm 0{\cdot}22$ and $11{\cdot}25 \pm 0{\cdot}25$ μm in 280 and 380 mosmol l^{-1}, respectively, corresponding to cell volumes of 784 ± 46 and 745 ± 52 fl; $n = 3$; $P > 0{\cdot}05$).

There might be concern that the elevated taurine influx observed under hypotonic conditions was due in part to activation of Na^+-dependent taurine influx. However, in separate experiments, there was no significant difference between the volume-sensitive taurine influx in NaCl, or Na^+-free medium (NMDG-Cl). Thus, taurine influx (at 250 μM) in Na^+-free medium was $1{\cdot}18 \pm 0{\cdot}09$ and $5{\cdot}86 \pm 0{\cdot}32$ nmol $(10^6$ cells$)^{-1}$ h^{-1} in shrunken (50 mM KCl added) and swollen (280 mosmol l^{-1}) chondrocytes, respectively, whereas in Na^+-containing medium these values were not significantly different ($1{\cdot}48 \pm 0{\cdot}12$ and $6{\cdot}28 \pm 0{\cdot}38$; $n = 3$). The magnitude of the volume-activated taurine flux ($4{\cdot}7 \pm 0{\cdot}2$ nmol $(10^6$ cells$)^{-1}$ h^{-1} in Na^+-free medium, and $4{\cdot}8 \pm 0{\cdot}3$ nmol $(10^6$ cells$)^{-1}$ h^{-1} in Na^+-containing medium) was therefore not influenced by the presence of Na^+. A Na^+-dependent component of taurine influx can, however, be observed under appropriate experimental conditions. Thus, when taurine influx was measured at low extracellular taurine concentrations (20 μM) to minimize the contribution of the non-saturable (volume-sensitive) flux and to maximize the saturable flux, in the presence of Na^+ a value of $0{\cdot}0145 \pm 0{\cdot}0008$ nmol $(10^6$ cells$)^{-1}$ h^{-1} was obtained, which was reduced significantly to $0{\cdot}0037 \pm 0{\cdot}0006$ when Na^+ was replaced by NMDG ($n = 3$; $P < 0{\cdot}001$).

Kinetic properties of the volume-activated taurine flux

By studying the concentration dependence of taurine influx as a function of the extracellular taurine

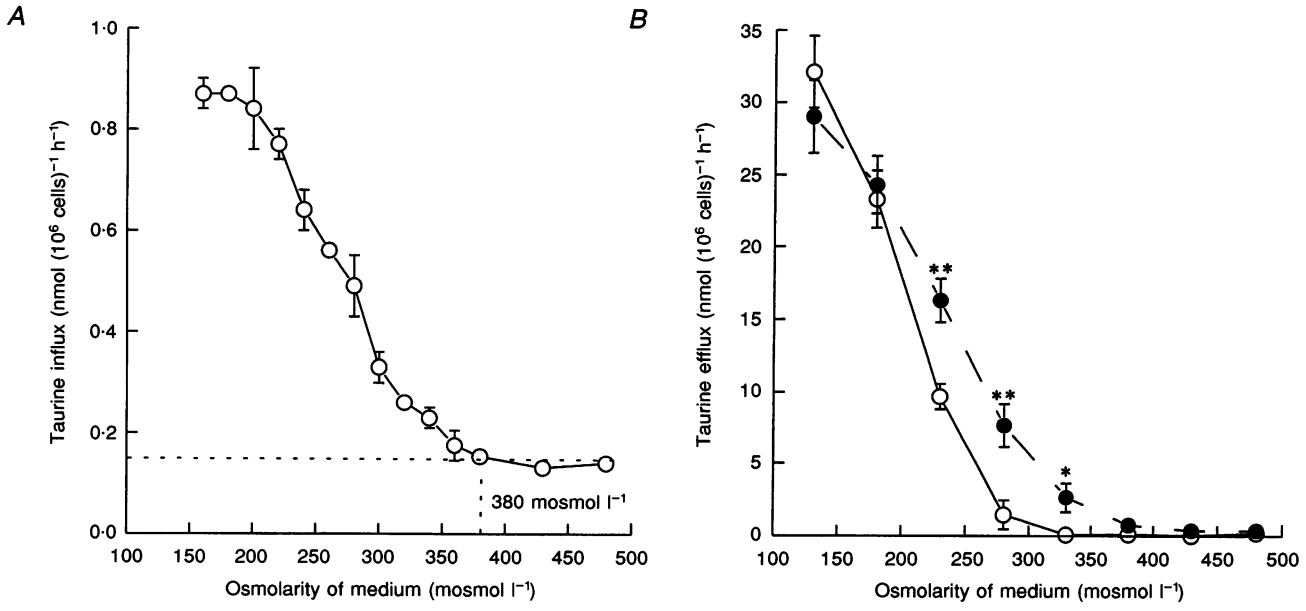

Figure 2. Increase in taurine influx or efflux from isolated bovine articular chondrocytes following changes to medium osmotic pressure

A, the [^{14}C]taurine influx and *B*, the [^{14}C]taurine efflux (both at a $[\text{taurine}]_o$ of 0·1 mM) were measured in chondrocytes isolated at 380 mosmol l^{-1} as described in Methods. For the [^{14}C]taurine efflux experiments (*B*), chondrocytes were isolated at either 280 (○) or 380 (●) mosmol l^{-1} (NaCl addition) and osmotic shocks delivered as shown on the abscissa. Data (means ± S.E.M.) are from at least 3 experiments at each osmolarity. Significant differences between the data at 380 and 280 mosmol l^{-1} are shown as $^{**}P < 0{\cdot}001$ and $^{*}P < 0{\cdot}05$.

Table 1. Inhibition of volume-sensitive taurine efflux from bovine articular chondrocytes by various pharmacological agents

Drug	Inhibition (%)	n
Pimozide (50 μM)	94·5 ± 2·5	9
Tamoxifen (10μM)	89·6 ± 8·2	3
DIOA (100 μM)	79·3 ± 5·4	3
Phloretin (100 μM)	51·3 ± 8·3	3
Niflumic acid (100 μM)	49·0 ± 5·6	3
DIDS (100 μM)	39·0 ± 8·0	8
NPPB (250 μM)	36·3 ± 2·2	3
Furosemide (100 μM)	24·7 ± 4·7	3
Quinine (100 μM)	8·9 ± 6·3	3
MK196 (100 μM)	7·1 ± 4·6	3
DNDS (100 μM)	5·3 ± 6·3	3
Gd^{3+} (10 μM)	6·4 ± 5·8	3

The activity of the pathway was determined by measuring taurine efflux from shrunken (480 mosmol l^{-1}) or swollen (280 mosmol l^{-1}) chondrocytes in the presence or absence of the drugs listed. Results are means ± S.E.M. for n independent experiments.

concentration, it is possible to obtain information about the kinetic properties of the pathway which mediates the flux in shrunken or swollen cells. The experiments (Fig. 3) show that the kinetics of taurine influx either in cells at shrunken (430 mosmol l^{-1}) or reduced (280 mosmol l^{-1}) osmolarity exhibit linear concentration dependence. This behaviour is typical for a 'channel' or 'pore' rather than for a carrier-mediated system, which would be expected to show some evidence of saturation i.e. Michaelis –Menten kinetics, over the concentration range studied.

Another test for carrier- or channel-like characteristics is the phenomenon of *trans* acceleration (Stein, 1986). When this is observed, the transport rate of substrate is increased by the presence of substrate at the other side of the membrane and is a characteristic of a variety of carrier-mediated pathways. This was tested for the volume-activated taurine flux in chondrocytes by measuring [^{14}C]taurine efflux from prelabelled chondrocytes in the presence or absence of extracellular taurine (5 mM) at reduced osmolarity (280 mosmol l^{-1}). Taurine loss was 19·7 ± 2 and 20·8 ± 1·4 in the presence

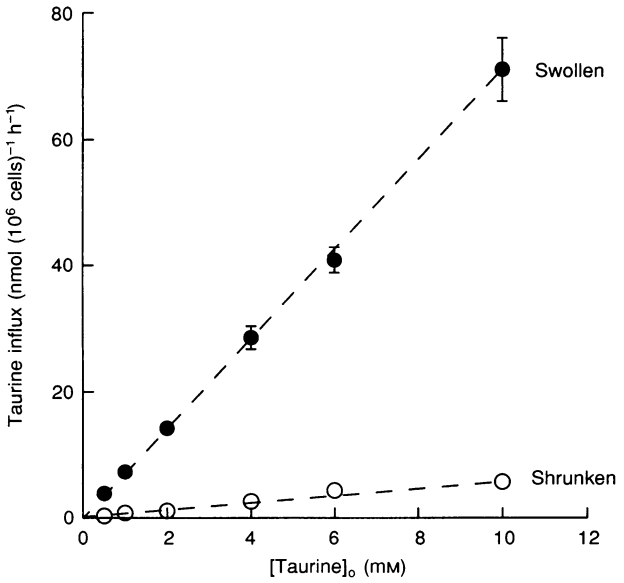

Figure 3. The kinetic properties of volume-activated taurine influx

Kinetic properties are shown as a function of extracellular taurine concentration ($[taurine]_o$) in shrunken (430 mosmol l^{-1}) or swollen (280 mosmol l^{-1}) chondrocytes isolated at 380 mosmol l^{-1}. [^{14}C]Taurine influx at varying medium taurine concentrations was measured with constant specific activity. Results (means ± S.D.) shown are from one experiment which was typical of two more.

Table 2. Selectivity of the volume-activated transporter of bovine articular chondrocytes to various osmolytes

Osmolyte	Influx (nmol $(10^6$ cells$)^{-1}$ h^{-1})		Volume-sensitive flux	Pimozide-sensitive flux	R_v	R_p
	Shrunken	Swollen				
Taurine						
Control	0·97 ± 0·10	2·89 ± 0·29	1·92	1·98	1·00	1·00
+ pimozide	0·88 ± 0·02	0·91 ± 0·03	n.s.	n.s.		
Uridine						
Control	1·09 ± 0·15	2·70 ± 0·12	1·61	1·84	0·84	0·93
+ pimozide	0·86 ± 0·12	0·86 ± 0·10	n.s.	n.s.		
Sorbitol						
Control	1·49 ± 0·08	2·76 ± 0·42	1·27	1·55	0·66	0·78
+ pimozide	1·22 ± 0·09	1·21 ± 0·10	n.s.	n.s.		
Inositol						
Control	0·70 ± 0·10	1·42 ± 0·05	0·72	0·87	0·38	0·44
+ pimozide	0·60 ± 0·04	0·55 ± 0·07	n.s.	n.s.		

Influx of radiotracer osmolytes (2·5 mM final concentration for each) was measured in Na^+-free (NMDG-Cl) media including NBMPR (50 μM) at 480 or 280 mosmol l^{-1} in the presence (+ pimozide) or absence (control) of pimozide at 100 μM). Volume-sensitive fluxes (i.e. flux in swollen − flux in shrunken cells) and pimozide-sensitive fluxes (i.e. flux in swollen cells without pimozide − flux in swollen cells with pimozide present) are shown with the ratios R_v (volume sensitive) and R_p (pimozide sensitive) relative to taurine as indicated. Results are means ± S.E.M. for 3 experiments; n.s. indicates no significant difference, $P > 0{\cdot}05$; all pairs of data, i.e. pimozide-sensitive fluxes in swollen cells, or volume-sensitive fluxes in the absence of pimozide, were significantly different, $0{\cdot}05 > P > 0{\cdot}001$).

or absence of taurine, respectively; units are the percentage loss over 10 min; $n = 3$). The finding that the rate of efflux was identical under both conditions is further support for the 'channel-like' nature of the volume-sensitive taurine pathway.

Inhibition of volume-activated taurine fluxes by pharmacological agents

A range of drugs was tested at a single concentration for their ability to inhibit volume-activated taurine efflux (Table 1). Dose–response curves of the three most potent drugs were studied in more detail, yielding $K_{½}$ values (the drug concentration required for half-maximal inhibition; μM) of: pimozide, 15 ± 5; tamoxifen, 7 ± 2; DIOA, 50 ± 10 ($n = 3$). In experiments attempting to totally inhibit the flux with tamoxifen it was found that higher concentrations (> 20 μM), rather than inhibiting, caused a marked elevation of taurine flux. Thus at 50 μM tamoxifen, taurine efflux in swollen cells was increased 3·9 ± 0·5-fold ($n = 3$) suggesting a non-specific membrane lesion.

Selectivity of the volume-sensitive taurine flux pathway

In other cell types, the transport of a range of osmolytes in addition to taurine is markedly increased following hypotonic shock (e.g. Kirk, Ellory & Young, 1992; Jackson & Strange, 1993). The selectivity of the volume-activated pathway was tested in cells suspended in Na^+-free media to reduce the contribution of endogenous pathways, and in the presence or absence of pimozide, to define the volume-sensitive component (Table 2). To abolish the contribution of the nucleoside transporter, NBMPR (50 μM) was added to all conditions. The results showed that chondrocyte swelling stimulated the influx of the osmolytes tested and pimozide abolished the induced pathway but had no effect in shrunken cells. In addition, since the extracellular concentration of these molecules is identical (2·5 mM), an estimate of the relative permeabilities of the osmolytes can be made. The order of the permeabilities (taurine > uridine > sorbitol > inositol) was the same for both volume-sensitive and pimozide-sensitive fluxes, but there were slight differences in the absolute ratios. These results support the contention that the volume-activated, pimozide-sensitive pathway of chondrocytes mediates the transport of a range of osmolytes.

The elevated flux observed with chondrocyte swelling might result either from an increase in the number of functional pathways with discrete permeability properties, or from an alteration in the dimensions of the putative channel also leading to an increased flux. One way of eliminating the first of these possibilities is by determining the selectivity of the pathway as a function of cell volume. This was tested by measuring taurine and inositol influx as described in Table 2 in chondrocytes suspended in Na^+-free media of decreasing osmolarity (480, 330, 280 and 230 mosmol l^{-1}). The ratio of volume-activated taurine : inositol flux from 480 to 330, 480 to

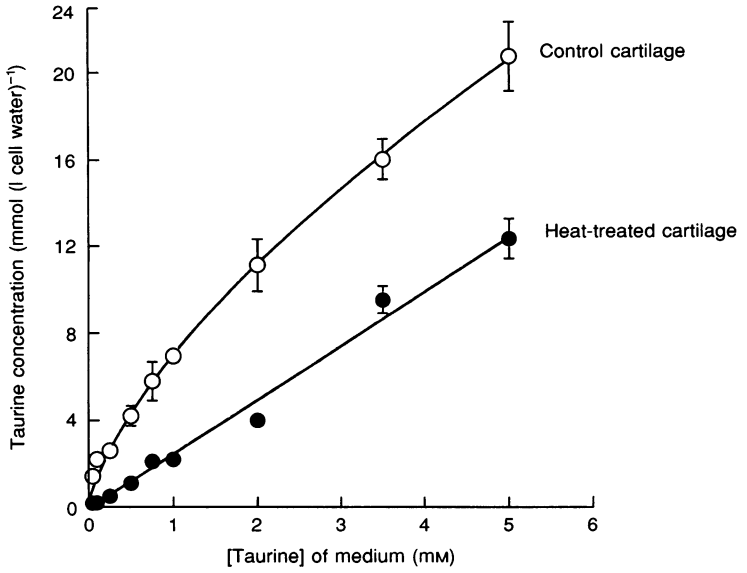

Figure 4. Concentration dependence of taurine equilibration in normal and heat-treated cartilage

The line drawn for the heat-treated cartilage is the best-fitting linear regression; the data for the control cartilage are fitted to an equation comprising saturable and non-saturable components of the form: $[Tau]_i = ([Tau]_{i,max} \times [Tau]_o)/([Tau]_{i½} + [Tau]_o) + \alpha[Tau]_o$, where $[Tau]_{i½}$ is the medium taurine concentration giving half-maximal intracellular taurine concentration, and α is the slope of the non-saturable component. The calculated $[Tau]_i$ maximum ($[Tau]_{i,max}$) is 8·9 mmol taurine (l cell water)$^{-1}$, the $[Tau]_{i1/2}$ is 0·36 mM and the slope is 2·46. Results (means ± S.E.M.) are pooled data from at least 3 separate experiments at each concentration.

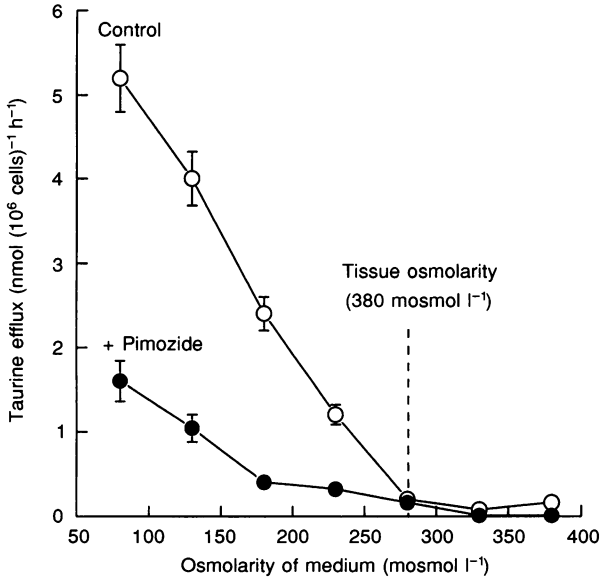

Figure 5. Stimulation of taurine efflux from bovine articular cartilage explants with changes in medium osmolarity

Explants were loaded with [^{14}C]taurine, and non-specific tracer removed by washing with cold DMEM at 280 mosmol l^{-1}. The tissue was then incubated for 30 min with pimozide (100 μM) or DMSO at 37 °C in DMEM, then the medium was aspirated off and the cartilage placed in the appropriate osmolarities in the presence or absence of pimozide. [^{14}C]Taurine efflux was then measured over 10 min as described (see Methods). Results (means ± S.E.M.) are pooled data from at least 4 separate experiments at each osmolarity.

280 and 480 to 230 mosmol l^{-1} remained constant (2·6 ± 0·2, 2·8 ± 0·2 and 2·7 ± 0·2, respectively; $n = 3$). The maintenance of this ratio shows that the permeability and hence the dimensions of the pathway remain constant with cell swelling, supporting the notion that recruitment of functional pathways accounts for the elevated flux in hypotonic solutions.

Measurement of $[taurine]_i$ and volume-activated taurine efflux from chondrocytes *in situ*

The $[taurine]_i$ of chondrocytes measured in cartilage explants obtained from the feet of eight different animals at a $[taurine]_o$ of 0·1 mM was 1·8 ± 0·09 mmol (l cell water)$^{-1}$. At this $[taurine]_o$ the non-specific binding of heat-treated cartilage was 10 ± 4% ($n = 8$) of the specific binding. Equilibrating normal and heat-treated cartilage explants at increasing $[taurine]_o$ elevated the intracellular concentration (Fig. 4). The change in taurine concentration of heat-treated tissue was linear with $[taurine]_o$ ($r = 0·9968$), whereas the relationship for living cartilage suggested saturation at high external concentrations. An estimate of the external concentration giving half-maximal internal concentration was 0·36 ± 0·08 mM (results are for cartilage from three feet).

The loss of taurine from preloaded chondrocytes within cartilage explants was a first-order process over the incubation time used (10 min; data not shown) and sensitive to medium osmolarity (Fig. 5). When the osmolarity was reduced below about 280 mosmol l^{-1} (corresponding to a tissue osmolarity of about 380 mosmol l^{-1}; Urban *et al.* 1993) there was a marked increase (25 ± 4-fold at 80 mosmol l^{-1}, $n = 3$) in taurine loss. These results parallel those of isolated chondrocytes, where the stimulation in taurine flux was observed with only small decreases in osmolarity below those to which the chondrocytes were exposed during isolation (Fig. 2). As observed for isolated chondrocytes, increasing medium osmolarity above 280 mosmol l^{-1} did not significantly decrease taurine efflux from cartilage. Pretreatment (for 30 min at 37 °C) of the cartilage explants with drugs known to reduce the volume-activated flux in isolated chondrocytes (Table 1) markedly reduced the rate of loss of taurine. With falling osmolarity, pimozide reduced, but did not abolish, taurine efflux from cartilage (Fig. 5) such that at 80 mosmol l^{-1}, pimozide had reduced taurine efflux by 68 ± 8% ($n = 3$) of the control at 80 mosmol l^{-1}. The drugs tamoxifen, DIOA and DIDS were also tested for their capacity to block swelling-activated taurine efflux from cartilage but none of them totally inhibited the process (tamoxifen, 48 ± 10; DIOA, 75 ± 8; DIDS, 15 ± 10; values are means for percentage inhibition ± S.E.M., $n = 3$). It can be seen that the inhibitory potency of these drugs does not follow the pattern observed for isolated chondrocytes (Table 1) possibly because of the partial exclusion of these drugs from the matrix.

DISCUSSION

These data show that in common with other cell types, chondrocytes possess a pathway for taurine and other osmolytes which is stimulated by an increase in cell volume (Hoffmann & Simonsen, 1989; Jackson & Strange, 1993). The lack of *trans* stimulation by extracellular taurine, the linear concentration dependence of taurine uptake (Fig. 4) and the rather poor substrate specificity (taurine, sorbitol, uridine and inositol all being transported; Table 2), suggest that this pathway has some characteristics of a 'channel' rather than those of a 'carrier' with binding sites.

The rapid rate of activation of taurine efflux in chondrocytes requires some comment when compared to similar studies performed on other cell types. A delay of about 1 min has been observed in the volume-sensitive Cl^- channel of MDCK cells (Banderali & Roy, 1992); Ehrlich ascites tumour cells (Christensen & Hoffmann, 1992); the volume-sensitive flux of organic solutes in flounder erythrocytes (Kirk *et al.* 1992); a human epithelial cell line (Kirk & Kirk, 1994); C6 glioma cells (Jackson & Strange, 1993); and KCl cotransport in rabbit erythrocytes (Jennings & Al-Rohil, 1990). In contrast, other workers have observed essentially immediate activation (trout erythrocytes: Motais, Guizouarn & Garcia-Romeu, 1991; lymphocytes: Grinstein, Rothstein, Sarkadi & Gelfand, 1984; eel erythrocytes: Fincham *et al.* 1987). It should be noted that many of these studies were performed below physiological temperatures (e.g. at room temperature for mammalian cells or cell lines), and it is possible that this would slow the biochemical processes involved in their activation. In chondrocytes there was no delay for volume-activated taurine efflux at 21 or 37 °C (Fig. 1) but taurine flux by this pathway was a temperature-sensitive process. Clearly the mechanisms of activation of different volume-sensitive transporters and ion channels vary markedly (Hoffmann & Simonsen, 1989; Pierce & Politis, 1990).

The point of activation of the taurine flux was close to the osmolarity of the medium used for cell isolation (Fig. 3). However there was no difference in volume between cells isolated at 280 or 380 mosmol l^{-1}. This is probably because when chondrocytes are released from the matrix into media of 280 mosmol l^{-1} (Urban *et al.* 1993), they regulate their volume to that of cells isolated at their *in situ* osmolarity (about 380 mosmol l^{-1}). It is obviously desirable to limit volume changes of chondrocytes during isolation from the matrix by increasing the osmolarity to within the range the cells normally experience. Although chondrocyte volume was the same at these osmolarities, the same effective hypotonic shock stimulated taurine efflux more (by 2-fold) in cells isolated at 280 compared with 380 mosmol l^{-1}. This may be related to the loss of osmolytes during RVD when the cells were isolated at

280 mosmol l^{-1}, which increases the sensitivity of the volume-sensitive transporter to subsequent hypotonic shock. There are several possible mediators of this process, including dilution of intracellular ionic strength (Motais *et al.* 1991), or membrane–cytoskeleton interactions (Hoffmann & Simonsen, 1989).

In other cell types, pathways with properties similar to those described here have been studied in detail. On the basis of kinetic, pharmacological (e.g. sensitivity to NPPB, DIDS, MK196) and electrophysiological evidence, it has been suggested that the pathway is a volume-sensitive anion channel (Banderali & Roy, 1992; Kirk *et al.* 1992; Roy & Malo, 1992; Jackson & Strange, 1993). These drugs were, however, rather weak inhibitors of volume-activated taurine efflux of chondrocytes (Table 1). In contrast, pimozide (which inhibited the pathway in chondrocytes; Table 1) blocks RVD in Ehrlich ascites tumour cells and volume-activated taurine efflux (Hoffmann & Simonsen, 1989). Other anti-calmodulin agents (e.g. trifluoperazine) block recovery from cell swelling in astrocytes (Bender, Neary, Blicharska, Norenberg & Norenberg, 1992). The drug is, however, ineffective against a similar pathway in flounder red cells (Fincham *et al.* 1987). It has been suggested that the anion exchanger band 3 plays an important role in mediating volume-sensitive taurine transport in skate erythrocytes (Goldstein & Brill, 1991). Chondrocytes however have no band 3 activity, as determined by the identical rates of recovery from acid load in the presence or absence of HCO_3^- or SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid) treatment (Wilkins & Hall, 1993). It seems unlikely therefore that in chondrocytes this transporter accounts for the marked volume-sensitive taurine flux described here, and it is more probable that, in agreement with others (Kirk *et al.* 1992), a distinct transporter is responsible.

The steroid congener tamoxifen (Wiseman, 1994) was an effective inhibitor of the swelling-induced taurine flux of chondrocytes. Tamoxifen, which has been shown to block volume-activated anion channels (Valverde, Mintenig & Sepulveda, 1993) and volume-activated taurine release in epithelial cells (Kirk & Kirk, 1994), does not inhibit cAMP- or Ca^{2+}-activated anion channels (Valverde *et al.* 1993). Its action is unlikely to be specific since it inhibits protein kinase C and Ca^{2+}–calmodulin-dependent cAMP phosphodiesterase and modulates membrane fluidity (see Wiseman, 1994). This latter observation might correlate with the increased leakiness of chondrocytes to taurine at high tamoxifen concentrations. Finally, DIOA, which was also effective, blocks anion transport in erythrocytes (Garay, Nazaret, Hannaert & Cragoe, 1988). Of the other drugs studied, furosemide and niflumic acid, which block volume-sensitive ion channels in other cell types (Cabantchik & Greger, 1992), were not very effective inhibitors (Table 1), whereas gadolinium (Gd^{3+}) at a concentration sufficient to block stretch-activated channels in other cell types (e.g. Yang & Sachs, 1989) had no effect (Table 1). It seems likely that for many of the drugs currently in use the inhibition is indirectly on the regulation of channel activity rather than solute translocation *per se*, although for some Cl^- channel blockers (e.g. NPPB) it is thought that inhibition is directly on the channel (Kirk *et al.* 1992). Since there are very different mechanisms for the activation of volume-sensitive pathways (see Hoffmann & Simonsen, 1989; Pierce & Politis, 1990) and a wide range of cell types and lines currently under study, it appears unlikely that a convenient pharmacological profile allowing comparisons between pathways of diverse cell types will be available for some time.

The low intracellular taurine concentration and the lack of a substantial sodium-dependent taurine influx pathway indicated a trivial role for this amino acid as an osmolyte in chondrocytes. This contrasts with a range of cell types (astrocytes: Pasantes-Morales, Murray, Sanchez-Olea & Moran, 1994; ascites tumour cells: Hoffmann & Lambert, 1983; myocytes: Rasmusson, Davis & Lieberman 1993; hepatocytes: Hardison & Weiner, 1980) which in addition to K^+ and Cl^-, use taurine as an osmolyte. However, results presented here (Table 2) and from various cell types (e.g. C6 glioma cells: Jackson & Strange, 1993; flounder erythrocytes: Kirk *et al.* 1992) indicate that a range of other organic osmolytes share this pathway. There is also some evidence that volume-activated Cl^- (and possibly K^+; see Starks & Hall, 1995) movements may be mediated by this system (Roy & Malo, 1992; Kirk *et al.* 1992; Jackson & Strange, 1993). The osmolytes utilized are likely to reflect several factors, including the particular metabolic properties of the cells under study and their environment. Thus, it is likely that cells exposed to high external osmotic pressures tend to employ osmolytes other than KCl for RVD because the high intracellular concentrations of salts needed to effect volume recovery can cause structural perturbations to protein structure and enzyme function (see Law, 1991). It is impossible to assess the relative contributions of the different osmolytes until their intracellular concentrations and permeability via volume-activated pathway(s) are known.

It was possible to measure the volume-activated taurine efflux from chondrocytes *in situ* (Fig. 5) because at the taurine concentration used (100 μM) only about 10% of extracellular taurine was retained within the matrix. These experiments clearly show that despite the constraints of the extracellular matrix, chondrocytes *in situ* can swell when the osmolarity of the interstitial fluid is reduced below that which the cells normally experience. The cartilage matrix, by necessity, is very tough and does not swell significantly when placed in solutions of decreasing osmolarity over the range studied here

(Maroudas, 1979). These results also imply that chondrocytes *in situ* respond to cell swelling by increased osmolyte loss; whether this results in effective RVD of chondrocytes in the matrix remains to be determined although preliminary studies using confocal microscopy suggest they possess volume-regulatory capacity (Errington & Hall, 1995).

Matrix synthesis by chondrocytes is markedly influenced by changes to osmolarity and hence cell volume and composition (Urban *et al.* 1993). Synthesis rates are optimal at the osmolarity the chondrocytes experience *in situ* (about 350–380 mosmol l^{-1}), since if the osmolarity of media suspending either cartilage explants (280 mosmol l^{-1}) or isolated articular chondrocytes (380 mosmol l^{-1}) is changed, the acute response (over about 2 h) is a reduction in matrix synthesis rates. When chondrocytes are maintained in short-term culture (about 24 h) in media of different osmolarities, the synthesis rates become optimal at the osmolarity in which the cells are incubated (Urban *et al.* 1993). Changes to the osmolarity and ionic content of chondrocytes *in situ* will occur routinely during the loading of cartilage; tissue loading causes fluid expression, a rise in proteoglycan concentration and an increase in the osmolarity of the interstitial fluid leading to a concomitant fall in chondrocyte volume. Conversely, when the tissue swells chondrocyte volume would be expected to increase. Chondrocyte metabolism is influenced by feedback from the extracellular matrix (Muir, 1981) but the mechanisms are unclear. Changes to the ionic or osmotic environment of chondrocytes can profoundly alter matrix synthesis (Urban *et al.* 1993) and alterations to cell volume and/or intracellular composition might therefore be one mechanism by which chondrocytes can 'sense' the loading pattern on the joint and produce an appropriate response in terms of changes to matrix synthesis rates. In view of the close relationship between chondrocyte volume and matrix synthesis (Urban *et al.* 1993) and the osmotic sensitivity of chondrocytes *in situ* as demonstrated in this study, the capacity of chondrocytes to regulate their volume by activation of membrane transport pathways may be important for determining matrix synthesis rates and hence the mechanical properties of healthy and diseased articular cartilage.

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