Histol Histopathol (1999) 14: 1023-1031

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Sodium transport systems in human chondrocytes II. Expression of ENaC, $Na^+/K^+/2Cl^-$ cotransporter and Na^+/H^+ exchangers in healthy and arthritic chondrocytes

E. Trujillo[1,2], D. Alvarez de la Rosa[1], A. Mobasheri[3], T. González[2], C.M. Canessa[4] and P. Martín-Vasallo[1]

[1]Laboratory of Developmental Biology, Department of Biochemistry and Molecular Biology, University of La Laguna, La Laguna, Tenerife, Spain, [2]Rheumatology Service, Universitary Hospital of Canarias, La Cuesta, Tenerife, Spain, [3]Department of Biomedical Sciences, School of Biosciences, University of Westminster, London, United Kingdom and [4]Department of Molecular and Cellular Physiology, Yale University School of Medicine, New Haven, USA

Summary. In this article, the second of two, we continue our studies of sodium-dependent transport systems in human cartilage from healthy individuals and with osteoarthritis (OA) and rheumatoid arthritis (RA). We demonstrate the presence of the epithelial sodium channel (ENaC), previously undescribed in chondrocytes. This system is composed of three subunits, α, ß and γ. We have shown that the human chondrocytes express at least the α and the ß subunit of ENaC. The expression of these subunits is altered in arthritic chondrocytes. In RA samples the quantity of α and ß is significantly higher than in control samples. On the other hand, ENaC α and ß subunits are absent in the chondrocytes of OA cartilage. Human chondrocytes also possess three isoforms of the Na^+/H^+ exchanger (NHE), NHE1, NHE2 and NHE3. The NHE system is composed of a single protein and is believed to participate in intracellular pH regulation. Furthermore, our studies indicate that at least one isoform of the electroneutral $Na^+/K^+/2Cl^-$ cotransporter (NKCC) is present in human chondrocytes. There are no obvious variations in the relative expression of NHE isoforms or NKCC between healthy and arthritic cartilage. Our data suggests that chondrocytes from arthritic cartilage may adapt to changes in their environmental sodium concentration through variations in ENaC protein levels. ENaC is also likely to serve as a major sodium entry mechanism, a process that, along with cytoskeletal proteins, may be part of mechanotransduction in cartilage.

Key words: Epithelial sodium channel, Na^+/H^+ exchanger, $Na^+,K^+,2Cl^-$ cotransporter, Osteoarthritis, Rheumatoid arthritis

Offprint requests to: Pablo Martín-Vasallo, MD, PhD, Laboratorio de Biología del Desarrollo (LBD), Departamento de Bioquímica y Biología Molecular, Universidad de La Laguna, 38206 La Laguna, Tenerife, Spain. Fax: 34.922.318354. e-mail: pmartin@ull.es

Introduction

This article is the second part of a study reporting the sodium transporters of the human chondrocyte from healthy individuals and patients afflicted by OA and RA. The concentration of free cations in the extracellular environment of the cartilage is unusually high (350-450mM Na^+, 8-15mM K^+) (Maroudas, 1979). The intracellular sodium concentration ($[Na^+]$) will depend on chondrocyte permeability and the concentration of free extracellular $[Na^+]$; although the permeability of cations such as Na^+ and K^+ across the chondrocyte plasma membrane is low, they tend to move down their electrochemical gradient. The ion channels, cotransporters and exchangers previously shown to be involved in Na^+ transport are summarized in Figure 1 (adapted from Mobasheri et al., 1998). The Na^+,K^+-ATPase which has been the subject of part I of this study, is the principal regulator of intracellular $[Na^+]$ and $[K^+]$. The density of the Na^+,K^+-ATPase in isolated animal chondrocytes has been reported to be relatively high (Mobasheri et al., 1997a) and its isoforms constituents have been identified (Mobasheri et al., 1997b). The Na^+,K^+-ATPase has also been shown to be responsive to changes in extracellular ionic and osmotic parameters (Mobasheri et al., 1997b, 1998). In contrast to the Na^+,K^+-ATPase, little is known about other Na^+ dependent transport systems and their role in ion homeostasis and matrix synthesis in chondrocytes. Cartilage matrix is very acidic; the fixed negative charge of cartilage extracellular matrix attracts protons in addition to cations resulting in an unusually acidic extracellular environment (pH 6.9-7.1). The accumulating lactate produced by anaerobic metabolism of chondrocytes further exacerbates the low pH in cartilage (Stockwell, 1991). Chondrocytes have a resting intracellular pH (pH_i) of approximately 7.1 units and changes to pH_i have been shown to modify the synthesis of matrix macromolecules in bovine articular chondrocytes

(Wilkins and Hall, 1992). It has also been demonstrated that extracellular pH regulates the synthesis of matrix macromolecules (Wilkins and Hall, 1995). Extremes of extracellular acidity result in a reduction in the pH_i of isolated articular chondrocytes and physiological manipulations resulting in intracellular acidosis inhibits matrix synthesis in cartilage (Wilkins and Hall, 1995). Therefore, pH regulation is important to the health and turnover of cartilaginous tissues. Amiloride sensitive Na^+/H^+ exchangers and proton-extruding ATPases are normally involved in the process of acid extrusion.

The Na^+/H^+ exchanger or cation exchanger is one of the better characterized pH_i regulators (for a recent review see Orlowski and Grinstein, 1997). The Na^+/H^+ exchanger is involved in multiple cellular functions in addition to its primary role, the regulation of pH_i. These include the control of cell volume and trans-epithelial ion transport. It is involved in the exchange of Na^+ and H^+; Na^+ ions move down their steep concentration gradient into the cell via NHE proteins and are exchanged for protons within the cytoplasm. The mode of exchange is electroneutral with a stoichiometry of 1:1. These transporters are regulated by a remarkably wide variety of stimuli that can modulate their expression level and activity (Bianchini and Pouyssegur, 1994). To date, six NHE isoforms have been described (NHE1-6). These isoforms share the same overall structure but exhibit differences with respect to amiloride sensitivity, cellular localization, kinetic variables, regulation by various stimuli and plasma membrane targeting in polarized epithelial cells. Isolated bovine articular chondrocytes possess two distinct isoforms, NHE1 and NHE3 (Wilkins et al., 1996), the regulation of which are significantly different. Although there is no information on NHEs in human cartilage the finding that NHE3 is expressed in bovine chondrocytes confirms that NHE1 is not the only Na^+/H^+ exchanger in these cells. Overall, chondrocytes exploit the steep inward Na^+ gradients that exist across the plasma membrane by expressing the amiloride sensitive acid extruding Na^+/H^+ exchangers to regulate pH_i.

One form of the bumetanide sensitive $Na^+/K^+/2Cl^-$ co-transporter is expressed in bovine chondrocytes (Errington and Hall, 1995; Errington et al., 1997). This transporter is involved in the process of regulatory volume increase as incubation of chondrocytes with bumetanide (a specific inhibitor of the $Na^+/K^+/2Cl^-$ co-transporter) inhibits this volume regulation. The $Na^+/K^+/2Cl^-$ co-transporter acts as a major Na^+ entry mechanism in chondrocytes (along with K^+ and Cl^-) and particularly following the hypertonic shock effect of increasing extracellular $[Na^+]$.

The fundamental role of the epithelial sodium channel (ENaC) is the sodium reabsortion following the electrochemical gradient generated by Na^+,K^+-ATPase. It is involved in the maintenance of sodium homeostasis. The sodium transport through ENaC is not coupled to any other ion. It is a multimeric system that consists of three subunits, α, ß and γ (Canessa et al., 1993, 1994). The ENaC shows distinct tissue- and cell type-dependent expression and differential sensitivity to inhibition by the diuretic amiloride and its analogs (Fyfe et al., 1998). It has been shown in a big variety of tissues, however, until now, the ENaC has never been studied in cartilage.

In this article we show the expression of ENaC, $Na^+/K^+/2Cl^-$ cotransporter and Na^+/H^+ exchanger in the human healthy and arthritic cartilage.

Materials and methods

Tissue sampling and immunohistochemistry

Pathological samples of human hip (17 samples, 11 OA and 6 RA) and knee (7 samples, 4 OA and 3 RA) joints were obtained following orthopedic replacements, hip (4 samples) and knee (2 samples) from traumatic incidents and necropsies (2 samples). Refer to part I of this study for information about immunohistochemical procedures. For immunofluorescence double labeling we applied consecutively the polyclonal primary antibody, the anti-rabbit-FITC secondary antibody, the monoclonal primary antibody and finally the anti-mouse-rhodamine. All solutions, incubation times and reagents were as in part I of this study.

Antibodies used

Information and references about the specific

Table 1. Antibodies used in this study.

ANTIBODY	ISOFORM	WORKING DILUTION	SOURCE	REFERENCE
Epithelial Sodium Channel (ENaC)				
Anti αEnaC	α	1:400	C. Canessa	Duc et al., 1994
Anti ßEnaC	ß	1:200	C. Canessa	Duc et al., 1994
Na^+/H^+-exchanger				
Anti NHE1	NHE1	1:1000	B. Bianchini	Tse et al., 1991
Anti NHE2	NHE2	1:1000	M. Donowitz	Tse et al., 1991
Anti NHE3	NHE3	1:1000	M. Donowitz	Tse et al., 1991
$Na^+/K^+/2Cl^-$ cotransporter				
T4	NKCC1	1:100	DSHB	Lytle et al., 1995

DSHB: Developmental Studies Hybridoma Bank, National Institute of Child Health and Development (NIHCD), maintained by The University of Iowa.

antibodies used in this study to detect subunits and isoforms of the transporters are shown in Table 1. Polyclonal antibodies against αENaC and ßENaC were generated and characterized as described (Duc et al., 1994). Dr. Bianchini (INSERM, U244, FRANCE) provided isoform specific polyclonal antibodies against NHE1. Dr. Donowitz (Johns Hopkins University, Baltimore) kindly provided antibodies against NHE2 and NHE3. A monoclonal antibody against the $Na^+/K^+/2Cl^-$ cotransporter developed by Dr. C. Lytle (University of California, Riverside) and Dr. B. Forbush III (Yale University School of Medicine, New Haven) (Lytle et al., 1995) was obtained from the Developmental Studies Hybridoma Bank maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA52242, under contract NO1-HD-7-3263 from the National Institute of Child Health and Development (NIHCD, USA). This antibody which recognizes the NKCC1 and NKCC2 isoforms of the $Na^+/K^+/2Cl^-$ cotransporter was developed against a fusion protein encompassing the carboxy-terminal 310 amino acids of the human colonic crypt NKCC (between MET-902 to SER-1212). This antibody has a remarkably wide species specificity, which includes mammalian, avian, and elasmobranch species (Lytle et al., 1995).

Results

We have investigated the expression of α and ß

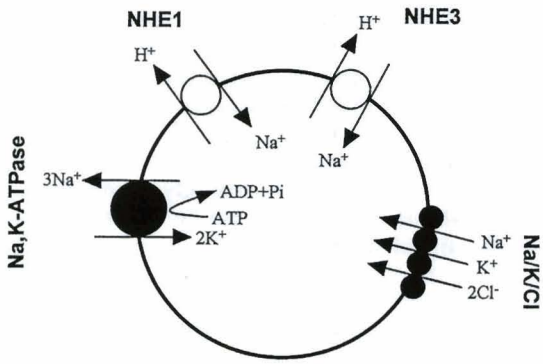

Fig. 1. This figure illustrates and summarizes the ion transporters known to be involved in Na^+ regulation in chondrocytes (studies in animal models).

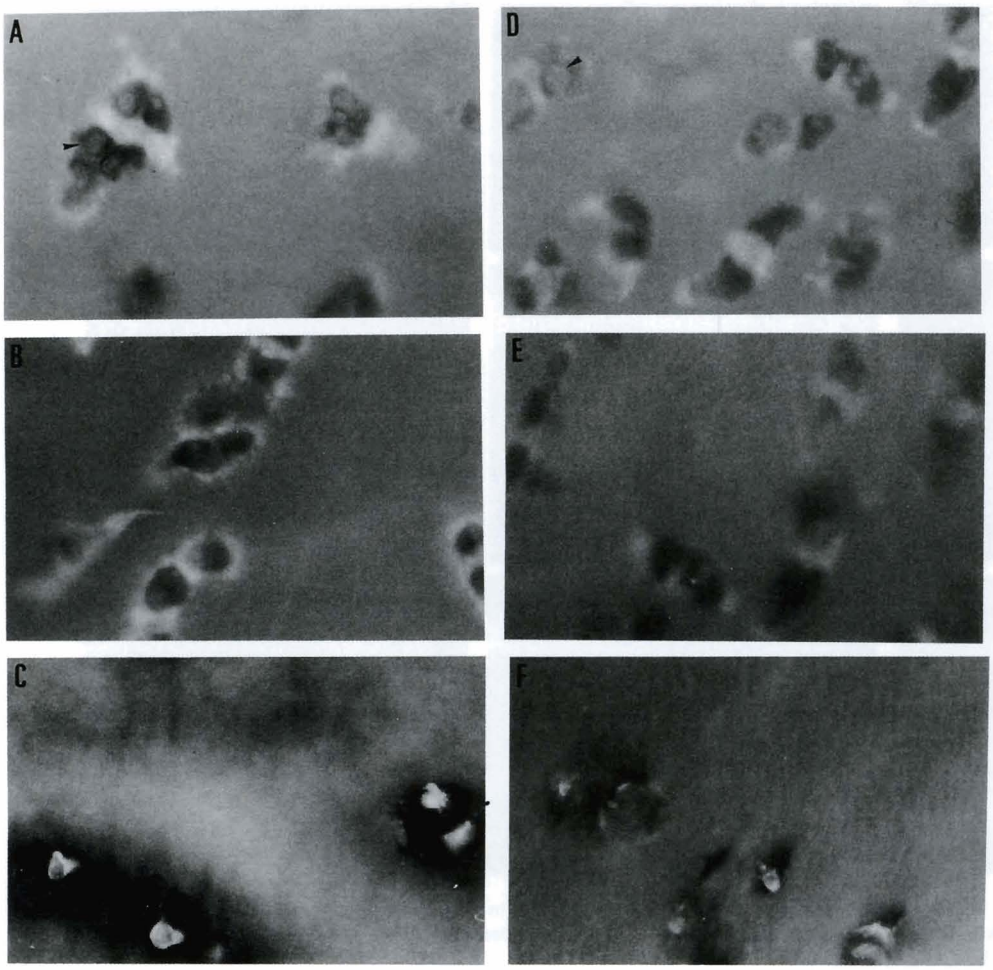

Fig. 2. Localization by indirect immunofluorescence of the α and the ß subunits of ENaC in 5 μm cryostat sections of the adult cartilage. **A and D.** Positive immunoreactivity of the α and the ß subunits in the adult normal cartilage. **B and E.** Absence of signal for the α and the ß subunits in cartilage of a patient affected of gonarthrosis (lateral femoral condyle). **C and F.** Localization of the α and the ß subunits in cartilage of the femoral head of a female affected of RA. Arrowheads pointing inside the chondrons show plasma membrane specific staining. x 570

subunits of the epithelial sodium channel, three isoforms of the Na^+/H^+ exchanger (NHE1, NHE2 and NHE3) and the $Na^+/K^+/2Cl^-$ cotransporter in the chondrocyte of the human adult cartilage. To this end we have performed immunohistochemistry using antibodies as specific probes. These studies were performed in cartilage from healthy adults and from patients afflicted by OA or RA.

Figure 2 shows the immunolocalization of the α and ß subunits of the ENaC in human normal and pathologic adult cartilage. Normal chondrocytes show positive immunoreactivity for both subunits (Fig. 2A,D). Fluorescent staining for ENaC α subunit shows a brighter signal than the staining for the ß subunit. This is probably due to differences in antibody affinities and no conclusions about α:ß stoichiometry can be assessed. The specific signal for both ENaC subunits completely disappears in the chondrocytes of patients affected of OA (Fig. 2B and E show representative pictures), although the characteristic unspecific staining remains apparent (see part I of this study for discussion). Two samples of OA cartilage showed a faint staining in a group of superficial cells (data not shown).

To eliminate the possibility of an artifact we repeated this experiments with another well-characterized detection method, the avidin/biotin/peroxidase system (Vector Laboratories, Burlingame, CA), using 3,3'-diaminobenzidine as the chromogen. The expression pattern was consistent with the immunofluorescence data in all the samples (data not shown). To test the integrity of the OA chondrocytes during the procedure, a double label experiment was done. The same sample was stained with the polyclonal anti αENaC antibody and with the monoclonal McK1 antibody, specific for Na^+,K^+-ATPase α1 isoform, which has been previously shown to give a positive signal in this cells (see part I of this study). The chondrocytes remained negative for αENaC, but a bright positive signal was detected for Na,K-ATPase α1 isoform (data not shown).

Chondrocytes in RA cartilage show an intense immunoreactivity for both α and ßENaC (Fig. 2C,F), significantly higher than healthy controls. This pattern

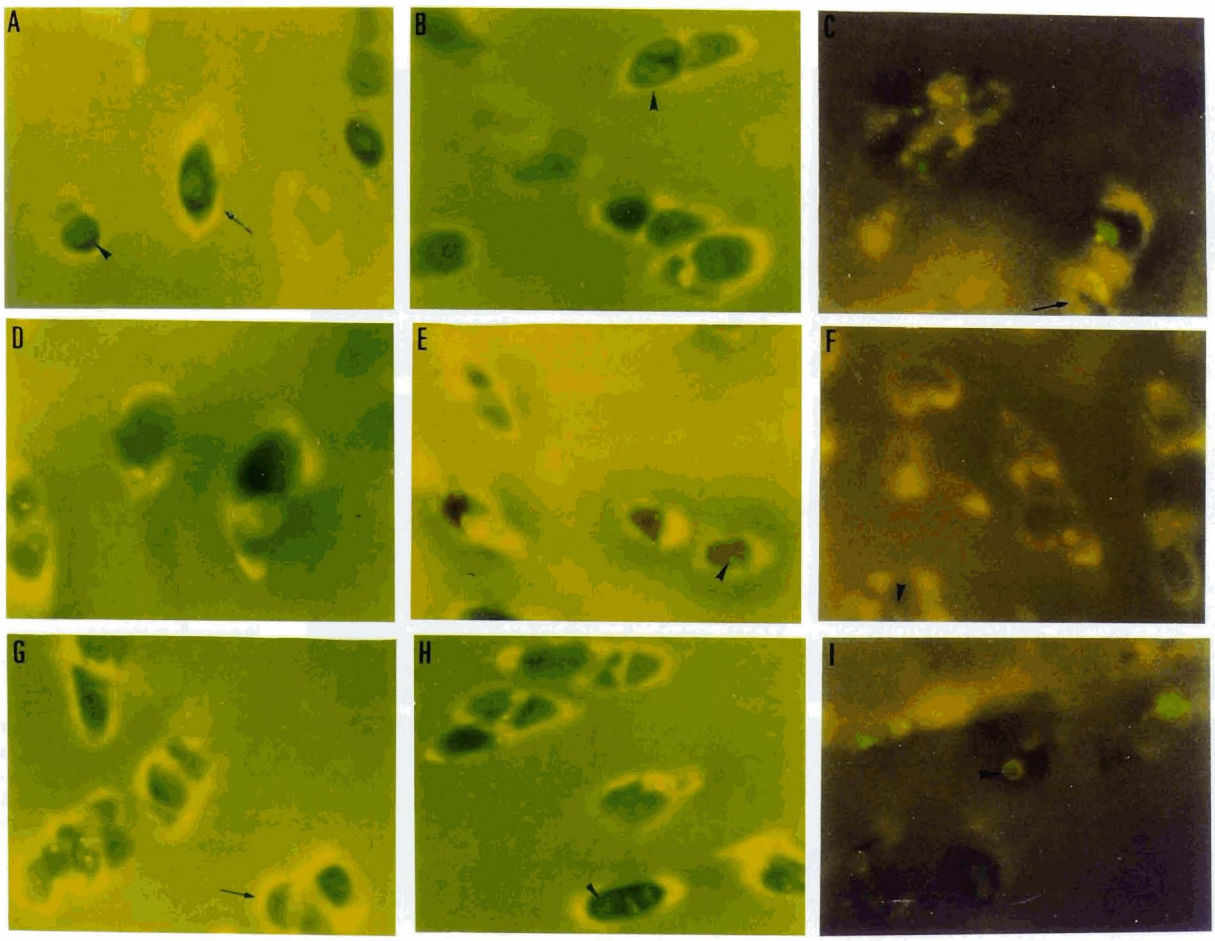

Fig. 3. Immunolocalization of NHE1, NHE2 and NHE3 isoforms of $Na^+/,H^+$ exchanger in human adult articular cartilage. **A-C** show NHE1 immunoreactivity in normal, OA and RA cartilage respectively. **D-F.** Faint label for NHE2 in normal, OA and RA cartilage respectively. **G-I.** Positive reactivity for NHE3 in parallel sections. x 570

was constant in all the samples tested and also when the avidin/biotin staining method was used (data not shown).

Figure 3 show the immunolocalization of NHE1, NHE2 and NHE3 isoforms of the Na^+/H^+ exchanger in adult human cartilage. The anti-NHE1 antibody gives an intense signal in both healthy and arthritic chondrocytes (Fig. 3A-C), with no apparent changes in fluorescence intensity between them. The NHE2 isoform is detectable, but the staining is very faint (Fig. 3D). This signal showed a similar level in OA and RA chondrocytes (Fig. 3E,F). The NHE3 isoform is also positive in human chondrocytes, with no changes in the immunofluorescence intensity between healthy and pathologic samples (Fig. 3G-I).

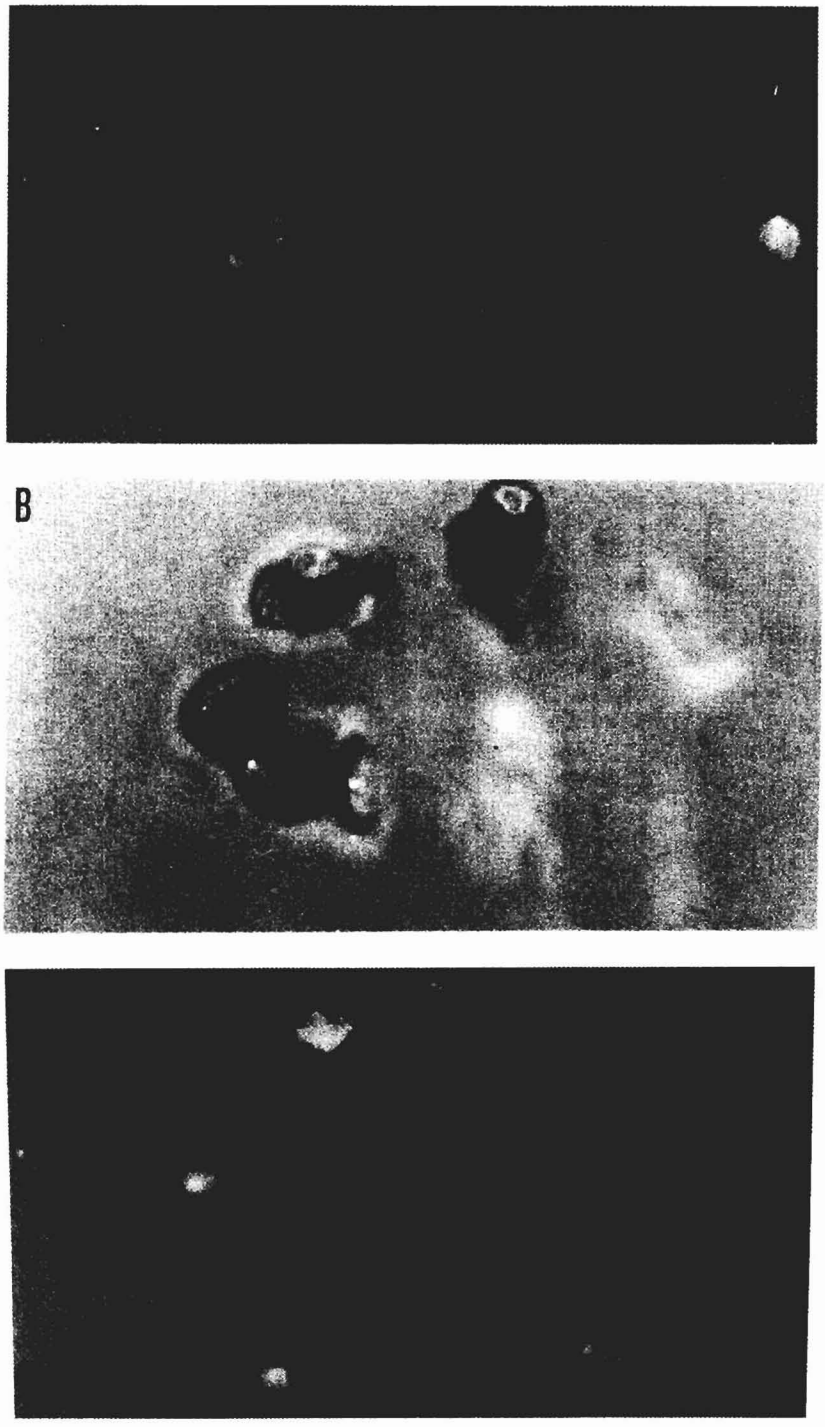

Fig. 4. Immunolocalization of the $Na^+/K^+/2Cl^-$ cotransporter in human adult articular cartilage. **A.** Normal cartilage. **B.** OA cartilage. **C.** RA cartilage. Arrowheads show specific staining. x 570

Figure 4 shows the bright positive signal for the anti-NKCC antibody in human cartilage. Both healthy and arthritic chondrocytes appears highly reactive, with no apparent changes in fluorescence intensity between samples, as shown in figure 4A-C.

Discussion

The information currently available about fundamental aspects of cartilage ion transport is limited compared to other tissues. More importantly, the majority of investigators working on ion transport systems in chondrocytes have focused on animal models (mainly bovine, porcine, equine and ovine cartilage and corresponding isolated chondrocyte cultures) and, to our knowledge, there are no reports of transport systems in human articular cartilage. In this article, three of the principal sodium transport pathways of human articular chondrocytes have been characterized in an attempt to understand how the chondrocyte adapts to its challenging ionic environment and how the altered expression of ion transport systems may be important in pathologies of human cartilage.

Significance of ENaC Expression

We have demonstrated the presence of at least two subunits of ENaC, namely the α and ß subunits, in human cartilage. The presence of ENaC γ subunit could not be tested because the γ subunit antibody, to our knowledge the only antibody currently available, gives high background in immunohistochemistry. It would be interesting to know the precise subunit composition of ENaC in chondrocytes. There are examples of tissues such as lung that exhibit differential expression of subunits (Farman et al., 1997). The different composition of subunits form channels with variations in their functional properties (McNicholas and Canessa, 1997). Although there is a previous report of ENaC α subunit mRNA expression in cartilage (Killick and Richardson, 1997), this was in cartilage from chicken. Preliminary reports suggest that bovine articular cartilage expresses relatively low levels of ENaC. Also, high levels of the cystic fibrosis transmembrane conductance regulator (CFTR) are expressed in bovine chondrocytes (Mobasheri, unpublished observations). A recent report (Briel et al., 1998) correlates the high level of CFTR expression with the inhibition of ENaC activity in *Xenopus* oocytes which could explain the low abundance of ENaC in bovine chondrocytes, as the dominant presence of CFTR would inhibit its activity. There is no information available about CFTR expression in human chondrocytes. This is the first report of ENaC at the translational level in chondrocytes. The presence of ENaC in chondrocytes is surprising because most cells display low Na^+ permeability unless they are involved in vectorial transport of Na^+. Chondrocytes are not polarized epithelial cells involved in transcellular ion transport. However, the ionic environment of chondrocytes is highly unusual and the high extracellular [Na^+] surrounding chondrocytes may be one explanation for the presence of ENaC. It is possible that these cells need to increase the internal [Na^+] to become hypertonic as a way of avoiding shrinkage following periods of excessive load. Indeed, intracellular [Na^+] in these cells has been reported to be relatively high (up to 50 mM) (Urban and Hall, 1992). Recently it has been demonstrated that αENaC acts as a stretch activated non-selective cation channel in osteoblasts (Kizer et al., 1997). The authors postulated a general role for αENaC as cation channel involved in the process of mechanotransduction. In terms of developmental origin, chondrocytes are highly related to osteoblasts and express many common phenotypic markers (Grigoriadis et al., 1988). Thus evidence of ENaC expression in osteoblasts indirectly supports our findings in human chondrocytes and the recent findings of Killick and Richardson (1997) in chicken cartilage.

Static and dynamic loadings are continuously applied to skeletal tissues such as bone and cartilage. Load applied to cartilage results in deformation of chondrocytes (Guilak, 1995) and consequently leads to

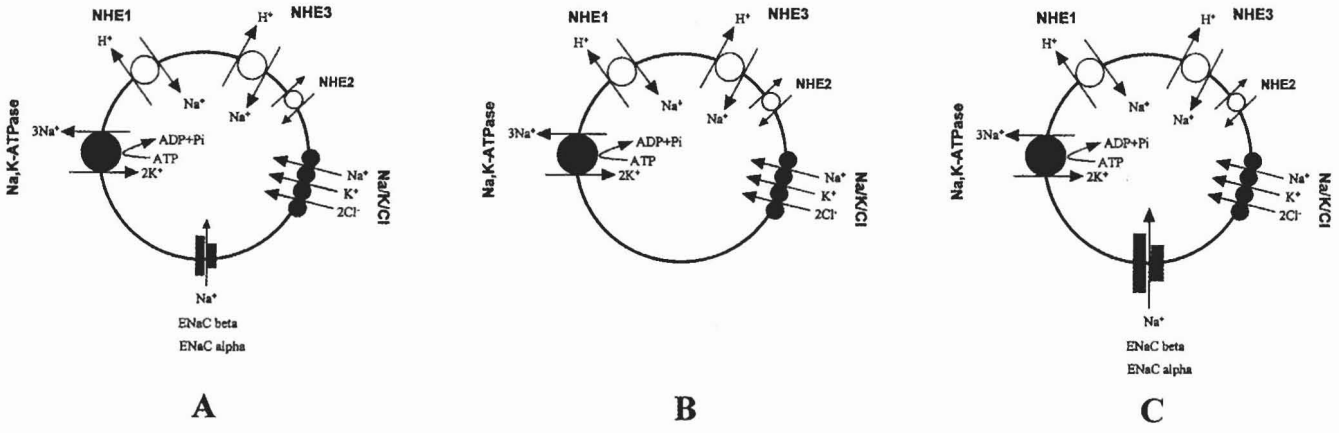

Fig. 5. Na^+ transport model in human chondrocytes. **A.** Healthy human cartilage. **B.** Cartilage affected of OA. **C.** Cartilage affected of RA.

changes in matrix synthesis (Wong et al., 1997). Load results in elevation of pressure (up to 200 atm) and perturbations in the external environment of chondrocytes (increased tissue osmolarity and ionic strength). The cells shrink and volume regulatory ion transport systems are activated (Hall et al., 1996; Mobasheri et al., 1998). Mechanosensing processes enable chondrocytes to respond to extrinsic loadings via separate processes of mechanoreception and mechanotransduction. The former transmits extracellular physical stimuli into chondrocytes and the latter transduces or transforms the energetic and informational content into an intracellular signal that results in a physiological response. Very little is known about these processes in chondrocytes. It is well established that chondrocytes are capable of physiological and adaptive responses to long-term changes in their environment (Urban et al., 1993) but nothing is known about the acute responses to mechanical load. In strained bone tissue, stretch activated ion channels have been demonstrated in bone cells. These ion channels permit passage of Na^+, K^+ and Ca^{2+}. Such transmembrane ionic activity may, in turn, initiate intracellular electrical events, for example, modulation of membrane potential and intracellular [Ca^{2+}]. Activation of ion channels and rapid changes in intracellular Na^+, K^+ or Ca^{2+} levels may constitute initial events in mechanosensory processes. Subsequent changes in membrane potential and intracellular [Ca^{2+}] may constitute key events in intracellular cascades that result in mechanotransduction. The presence of ENaC in human cartilage implies that ENaC may be a functional Na^+ channel involved in the process of mechanotransduction.

The most relevant finding of this study is the absence of α and ßENaC in cartilage affected of OA. As it has been described in the result section, this is not a consequence of artifacts in the technical procedure. Only two samples remained partially positive for α and ß ENaC. We can propose two explanations for this result; the existence of two different populations of OA patients or, alternatively, the two samples correspond to cartilage zones in an early evolutionary stage of pathology. The disappearance of ENaC subunits can be correlated with the change in [Na^+] in OA cartilage. ENaC is the Na^+ entry pathway more easily modulated by the chondrocytes because the sodium transport through this channel is not coupled to any other ion. Since isolated chondrocytes respond adaptively to rapid changes in their ionic environment (Urban et al., 1993), ENaC may be a molecular participant in the transduction of mechanical load by inducing rapid changes in intracellular [Na^+] which would subsequently be restored to previous levels by the plasma membrane Na^+, K^+-ATPase.

In RA cartilage samples we have observed a remarkable increase in α and ßENaC staining in all the samples tested and with two different detection methods. The lack of information about changes in ionic composition in RA cartilage makes it impossible to establish a correlation between ENaC subunit up-regulation and intracellular [Na^+]. It may be possible that in rheumatoid joints where the articular cartilage matrix has been drastically compromised (Buckwalter and Mankin, 1997), the upregulation of ENaC is a marker for the chondrocyte's altered mechanosensing machinery that has adapted to the altered ionic and osmotic properties of the extracellular matrix. This may be part of the cellular adaptation to new physical environment.

NHE Isoforms

There are at least three isoforms of Na^+/H^+ exchanger expressed in human chondrocytes, NHE1, NHE2 and NHE3. There are three other isoforms described, the poorly studied NHE4 and NHE5, and the putative mitochondrial isoform, NHE6 (Orlowski and Grinstein, 1997). These proteins were not included in our study due to the lack of specific antibodies.

Bovine chondrocytes have been shown to express two isoforms of the Na^+/H^+ exchanger, NHE-1 and NHE-3 (Wilkins et al., 1996), although our results suggest that NHE-2 is also found in low quantities in human chondrocytes from normal and degenerate cartilage. Regulation of pH_i in chondrocytes relies predominantly on the Na^+/H^+ exchanger NHE-1, which is ubiquitously expressed (Noel and Pouyssegur, 1995; Wilkins et al., 1996). The presence of multiple NHE isoforms in cartilage could be explained as a tissue specific adaptation to the unusually acidic extracellular matrix that surrounds chondrocytes, the characteristic anaerobic metabolism of these cells (Wilkins et al., 1996) and the continuous changes in local pH as a consequence of joint load. The expression of several NHE isoforms in chondrocytes raises the possibility of independent regulatory mechanisms. The NHE isoforms are differentially regulated by a number of factors as activators of tyrosine kinases, agonist of Ser/Thr kinases, cytosolic [Ca^{2+}] and changes in cell volume (Orlowski and Grinstein, 1997).

We have found no variations in the relative expression levels of these isoforms in OA and RA chondrocytes. At the present time there are no data available about pH regulation in OA and RA cartilage, but there is an increase in the matrix abundance of catabolic cytokines and growth factors, which could in turn alter pH_i, and hence matrix synthesis. It has been demonstrated that changes to pH_i of bovine articular chondrocytes can modify synthesis of matrix macromolecules (Wilkins and Hall, 1995) and changes in extracellular matrix composition (resulting from altered patterns of synthesis) may underlie pathological states (Kuettner et al., 1992).

Our observations suggest that in OA and RA cartilage the density of NHE proteins is unaffected and thus it is unlikely that growth factors alter NHE expression in pathologies of cartilage, although changes in the differential regulation of the isoforms due to the change in matrix permeability cannot be

discarded.

Na/K/2Cl cotransport System

The third major Na^+ entry pathway studied, the Na/K/2Cl cotransporter (NKCC), is found in high levels in human chondrocytes. To our knowledge, this is the first time that the NKCC isoforms have been described in chondrocytes using immunofluorescence techniques. Studies that have simulated physiological joint loading *in vitro* have shown that load produces continuous cell volume changes (Errington et al., 1997) and the presence of high levels of NKCC, a major cell volume regulator, is not surprising. Although several different isoforms of NKCC have been described to date (Gamba et al., 1994; Xu et al., 1994; Payne et al., 1995) we are unable to differentiate between them due to the antibody employed, which recognizes both NKCC1 and NKCC2 isoforms. The OA and RA cartilage express the same relative levels of NKCC, without any apparent changes when compared with healthy tissue. This may be an indication that the volume regulatory function of the Na/K/2Cl cotransporter is unaffected by alterations to the extracellular matrix. This would indicate that activation and regulation of the bumetanide sensitive Na/K/2Cl cotransporter is not significantly different in normal and degenerate cartilage where this transport system is involved in volume regulation following load-induced osmotic challenge (Hall et al., 1996). The apparent lack of difference in Na/K/2Cl cotransporter expression levels in normal and degenerate cartilage would also indicate that chondrocytes in degenerate joints affected by OA or RA are probably capable of maintaining normal cell volume without altering Na/K/2Cl cotransporter expression.

Based on our data and previously published material, we propose a model (Fig. 5) summarizing the major sodium transport systems involved in ion homeostasis. Our models summarize the Na^+ transport mechanisms involved in healthy and arthritic human chondrocytes.

The three Na^+ entry pathways studied in this article are receptors of diuretic drugs commonly used in clinical practice. ENaC and NHE are receptors for amiloride and its analogues. NKCC is the receptor for furosemide-bumetanide. The interaction of these diuretics with ion transport in chondrocytes could be important in the pathophysiology of cartilage, provided that this tissue is permeable to these drugs. Although normal cartilage matrix is relatively impermeable to large molecules, degenerate cartilage from OA and RA patients is significantly more permeable due to changes in the three dimensional structure of compromised matrix. Furthermore, the gradual changes in matrix permeability in OA or RA cartilage could have an effect on the regulation of these transporters because a number of growth factors and hormones entry in the tissue. The information thus gained may contribute to the basic understanding of cartilage pathology and enhance the approach for pharmacological intervention.

Acknowledgements. We thank Dr. Bianchini (INSERM, U244, France) for the kind gift of antibodies against NHE1 and Dr. Mark Donowitz (Johns Hopkins University) for the NHE2 and NHE3 antisera. This work was supported by grants 93/831 and 96/0453 from FIS, Spain, to P. M.-V. and the Department of Biomedical Sciences, School of Biosciences, University of Westminster (grant number V001173) to A.M. Elisa Trujillo and Diego Alvarez de la Rosa contributed equally to this paper, and thus both are first authors. E.T. is recipient of a fellowship from Unidad de Investigación of Hospital Universitario de Canarias.

References

Bianchini L. and Pouyssegur J. (1994). Molecular structure and regulation of vertebrate Na^+/H^+ exchangers. J. Exp. Biol. 196, 337-345.

Briel M., Greger R. and Kunzelmann P. (1998). Cl^- transport by cystic fibrosis transmenbrane conductance regulator (CFTR) contributes to the inhibition of epithelial Na^+ channels (ENaCs) in xenopus oocytes co-expressing CFTR and ENaC. J. Physiol. 508, 825-836.

Buckwalter J.A. and Mankin H.J. (1997). Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation. J. Bone Joint Surg. Am. 47, 487-504.

Canessa C.M., Horisberger J.D. and Rossier B.C. (1993). Epithelial sodium channel related to proteins involved in neurodegeneration. Nature 361, 467-470.

Canessa C.M., Schild L., Buell G., Thorens B., Gautschi I., Horisberger J.D. and Rossier B.C. (1994). Amiloride-sensitive epithelial Na^+ channel is made of three homologous subunits. Nature 367, 463-467.

Duc C., Farman N., Canessa C.M., Bonvalet J.P. and Rossier B.C. (1994). Cell-specific expression of epithelial sodium channel alpha, beta, and gamma subunits in aldosterone-responsive epithelia from the rat: localization by in situ hybridization and immunocyto-chemistry. J. Cell Biol. 127, 1907-1921.

Errington R.J. and Hall A.C. (1995). Volume regulatory properties of porcine articular chondrocytes measured in situ using confocal microscopy. J. Physiol. 482, 12-13.

Errington R.J., Fricker M.D., Wood J.L., Hall A.C. and White N.S. (1997). Four-dimensional imaging of living chondrocytes in cartilage using confocal microscopy: a pragmatic approach. Am. J. Physiol. 41, C1040-C1051.

Farman N., Talbot C.R., Boucher R., Canessa C.M., Rossier B.C. and Bonvalet J.P. (1997). Non-coordinated expression of α, β and γ subunit mRNAs of the epithelial sodium channel along the rat respiratory tract. Am. J. Physiol. 38, C511-C518.

Fyfe G.K., Quinn A. and Canessa C.M. (1998) Structure and function of the Mec-ENaC family of ion channels. Semin. Nephrol. 18, 138-151.

Gamba G., Miyanoshita A., Lombardi M., Lytton J., Lee W.S., Hediger M.A. and Hebert S.C. (1994). Molecular cloning, primary structure, and characterization of two members of the mammalian electroneutral sodium-(potassium)-chloride cotransporter family expressed in kidney. J. Biol. Chem. 269, 17713-17722.

Grigoriadis A.E., Heersche J.N. and Aubin J.E. (1988). Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. J. Cell Biol. 106, 2139-2151.

Guilak F. (1995). Compression-induced changes in the shape and

volume of the chondrocyte nucleus. J. Biomech. 28, 1529-1541.

Hall A.C., Starks I., Shoults C.L. and Rashidbigi S. (1996). Pathways for K^+ transport across the bovine articular chondrocyte membrane and their sensitivity to cell volume. Am. J. Physiol. 270, C1300-1310.

Killick R. and Richardson G. (1997). Isolation of chicken alpha ENaC splice variants from a cochlear cDNA library. Biochim. Biophys. Acta 1350, 33-37.

Kizer N., Guo X.L. and Hruska K. (1997). Reconstitution of stretch-activated cation channels by expression of the alpha-subunit of the epithelial sodium channel cloned from osteoblasts. Proc. Natl. Acad. Sci. USA 94, 1013-1018.

Kuettner K.E., Schleyerbach R., Peyron J.G. and Hascall V.C. (1992). Articular cartilage and osteoarthritis. Raven Press. New York.

Lytle C., Xu J.-C., Biemesderfer D. and Forbush III B. (1995). Distribution and diversity of $Na^+/K^+/Cl^-$ cotransport proteins: a study with monoclonal antibodies. Am. J. Physiol. 269, C1496-C1505.

Maroudas A. (1979). Physico-chemical properties of articular cartilage. In: Adult articular cartilage. Freeman M.A. (ed). Pitman Medical, Skeletal Research. Vol. 2. Academic Press. New York. pp 215-290.

McNicholas C. and Canessa C.M. (1997). Diversity of channels generated by different combinations of epithelial sodium channel subunits. J. Gen. Physiol. 109, 681-692.

Mobasheri A., Hall A.C., Urban J.P.G., France S.J. and Smith A.L. (1997a). Immunologic and autoradiographic localization of the Na,K-ATPase in articular cartilage: upregulation in response to changes in extracellular Na concentration. Int. J. Biochem. Cell. Biol. 29, 649-657.

Mobasheri A., Errington R.J., Golding S., Hall A.C. and Urban J.P.G. (1997b). Characterization of the Na, K-ATPase in isolated bovine articular chondrocytes; molecular evidence for multiple α and ß isoforms. Cell Biol. Int. 21, 201-212.

Mobasheri A., Mobasheri R., Francis M.J.O., Trujillo E., Alvarez de la Rosa D. and Martin-Vasallo P. (1998). Ion transport in chondrocytes: membrane transporters involved in intracellular ion homeostasis and the regulation of cell volume, free (Ca^{2+}) and pH. Histol. Histopathol. 13, 893-910.

Noel J. and Pouyssegur J. (1995). Hormonal regulation, pharmacology, and membrane sorting of vertebrate Na^+/H^+ exchanger isoforms. Am. J. Physiol. 268, C283-C296.

Orlowski J. and Grinstein S. (1997). Na^+/H^+ exchangers of mammalian cells. J. Biol. Chem. 272, 22373-22376.

Payne J.A., Xu J.C., Haas M., Lytle C.Y., Ward D. and Forbush B III. (1995). Primary structure, functional expression, and chromosomal localization of the bumetanide-sensitive Na-K-Cl cotransporter in human colon. J. Biol. Chem. 270, 17977-17985.

Stockwell R.A. (1991). Cartilage failure in osteoarthritis: relevance of normal structure and function. A review. Clin. Anat. 4, 161-191.

Tse C.-M., Yang V.W., Watson A.J.M., Potter J., Sardet C., Pouyssegur J. and Donowitz M. (1991). Molecular cloning of cDNA encoding the rabbit ileal villu cell basolateral membrane Na^+/H^+ exchanger. EMBO J. 10, 1957-1967.

Urban J.P.G. and Hall A.C. (1992). Physical modifiers of chondrocyte metabolism. In: Articular cartilage and osteoarthritis. Keuttner K., Hascall V.C., Schleyerbach R. and Peyron R. (eds). Raven Press Ltd. New York. pp 393-406.

Urban J.P.G., Hall A.C. and Gehl K.A. (1993). Regulation of matrix synthesis by the ionic and osmotic environment of articular chondrocytes. J. Cell Physiol. 154, 262-270.

Wilkins R.J. and Hall A.C. (1992). Measurement of intracellular pH in isolated bovine articular chondrocytes. Exp. Physiol. 77, 521-524.

Wilkins R.J. and Hall A.C. (1995). Control of matrix synthesis in isolated bovine chondrocytes by extracellular and intracellular pH. J. Cell Physiol. 164, 474-481.

Wilkins R.J., Mobasheri A. and Hall A.C. (1996). Characterization of NaxH exchange in isolated bovine articular chondrocytes. FASEB J. 10, 3843P (Abstract).

Wong M., Wuethrich P., Buschmann M.D., Eggli P. and Hunziker E. (1997). Chondrocyte biosynthesis correlates with local tissue strain in statically compressed adult articular cartilage. J. Orthop. Res. 15, 189-196.

Xu J.C., Lytle C., Zhu T.T., Payne J.A., Benz E. Jr. and Forbush III B. (1994). Molecular cloning and functional expression of the bumetanide-sensitive Na-K-Cl cotransporter. Proc. Natl. Acad. Sci. USA 91, 2201-2205.

Accepted February 10, 1999