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Acid-sensing ion channel 3 expressed in type B synoviocytes and chondrocytes modulates hyaluronan expression and release

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Abstract

Background—Rheumatoid arthritis is an inflammatory disease marked by intra-articular decreases in pH, aberrant hyaluronan regulation and destruction of bone and cartilage. Acid-sensing ion channels (ASICs) are the primary acid sensors in the nervous system, particularly in sensory neurons and are important in nociception. ASIC3 was recently discovered in synoviocytes, non-neuronal joint cells critical to the inflammatory process.

Objectives—To investigate the role of ASIC3 in joint tissue, specifically the relationship between ASIC3 and hyaluronan and the response to decreased pH.

Methods—Histochemical methods were used to compare morphology, hyaluronan expression and ASIC3 expression in *ASIC3*^{+/+} and *ASIC3*^{−/−} mouse knee joints. Isolated fibroblast-like synoviocytes (FLS) were used to examine hyaluronan release and intracellular calcium in response to decreases in pH.

Results—In tissue sections from *ASIC3*^{+/+} mice, ASIC3 localised to articular cartilage, growth plate, meniscus and type B synoviocytes. In cultured FLS, *ASIC3* mRNA and protein was also expressed. In FLS cultures, pH 5.5 increased hyaluronan release in *ASIC3*^{+/+} FLS, but not *ASIC3*^{−/−} FLS. In FLS from *ASIC3*^{+/+} mice, approximately 50% of cells (25/53) increased intracellular calcium while only 24% (14/59) showed an increase in *ASIC3*^{−/−} FLS. Of the cells that responded to pH 5.5, there was significantly less intracellular calcium increases *ASIC3*^{−/−} FLS compared to *ASIC3*^{+/+} FLS.

Conclusion—ASIC3 may serve as a pH sensor in synoviocytes and be important for modulation of expression of hyaluronan within joint tissue.

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INTRODUCTION

Decreases in pH occur in a variety of inflammatory exudates, including those from people with rheumatoid arthritis, and can reach as low as pH 5.4.¹⁶ The severity of pain and joint damage correlates with the degree of acidity in synovial fluid from arthritic joints.¹⁷ The primary acid sensors in peripheral tissues are acid-sensing ion channels (ASICs).⁸ The expression of ASICs in the peripheral and central nervous system is pivotal to their role in a variety of disease states including: multiple sclerosis, musculoskeletal pain, learning and memory disorders, and anxiety.^{9,12} In accord, we have shown that *ASIC3*^{-/-} mice exhibit reduced hyperalgesia compared to *ASIC3*^{+/+} mice.^{10,13} There is also increased expression of ASIC3 in primary afferent fibres innervating the joint, and in retro-gradely labelled dorsal root ganglia in response to joint inflammation.¹⁴ Multiple investigators report the expression of ASICs in non-neuronal cells: monocytes, arthritic chondrocytes, osteoblasts and osteoclasts, carotid body glomus cells, nucleus pulposus cells and muscle cells,^{15,18} suggesting diverse roles for ASICs. These findings are of particular interest as we recently demonstrated ASIC3 protein in synoviocytes.¹³

Synoviocytes are responsible for maintaining homeostasis by secreting lubricating synovial fluid into, and absorbing waste from, the joint cavity. Type A synoviocytes are of macrophage lineage and act to phagocytose joint cavity debris and waste; while type B synoviocytes, derived from fibroblasts, secrete the protective synovial fluid into the joint cavity.^{19,20} The two cell types synchronously work to maintain proper synovial fluid balance, a regulation which is crucial in protecting the joint from destruction inherent in inflammatory disease.²¹ Of the synovial fluid constituents, hyaluronan is the most abundant²² and in articular cartilage, forms a cushion for the joint.²³ Increased synthesis of hyaluronan occurs in response to mechanical stretch of intact joints²⁴ and from cultured fibroblast-like synoviocytes (FLS).^{25,26} Further, proinflammatory cytokines or nitric oxide donors also induce a rapid increase in hyaluronan production in cultured FLS.^{27,23} Thus, hyaluronan plays a critical role in normal function of the joint, as well as after joint inflammation.

The purpose of the current study was to determine the functional role of ASIC3 in synoviocytes and to test the hypothesis that ASIC3 acts as a pH sensor in these non-neuronal cells. We, therefore, examined localisation of ASIC3 in joint tissues, responses of synoviocytes to decreases in pH and interactions of ASIC3 with hyaluronan.

MATERIALS AND METHODS

Mice

All experiments were approved by the University of Iowa Animal Care and Use Committee and conducted in accordance with National Institutes of Health guidelines. Congenic *ASIC3*^{-/-} and *ASIC3*^{+/+} mice on a C57Bl/6J background²⁹ were bred at the University of Iowa Animal Care Facility. Male mice, 9–10 weeks of age, were used in these studies.

Isolation and culturing of fibroblast-like synoviocytes

FLS were isolated from *ASIC3*^{+/+} and *ASIC3*^{-/-} mice according to previously published methods³⁰ (see supplementary material).

Cell and tissue preparation for immunohistochemistry (figures 1–5)

FLS were plated onto chamber slides at 24 000 cells/well (see supplementary material) incubated 2 days in Dulbecco's modified Eagle medium (DMEM) supplemented media, serum starved for 24 h and fixed with 2% paraformaldehyde, 15 min, room temperature

(figure 2). Two different protocols for tissue collection were used (figures 1, 3–5). The 20 μm cryosections in figure 1 were obtained by removal of the anterior synovial tissue, which was snap frozen at -160°C and postfixed on the slide with 2% paraformaldehyde. Figures 3–5 were obtained by perfusing mice with 4% paraformaldehyde and, removal of the whole leg, prior to decalcification. Sections were cryosectioned at 14 μm (see supplementary material). For hyaluronan and immunohistochemical staining, sections were obtained and tested from *ASIC3*^{+/+} (n=6) and *ASIC3*^{-/-} mice (n=6).

Immunohistochemistry

Immunohistochemical staining was carried out using rabbit polyclonal antibodies for ASIC3 (1:500; Alomone, Jerusalem, Israel) and heat shock protein (Hsp)25 (1:500; Stressgen, Ann Arbor, Minnesota, USA), and mouse anti-rat ED1 (1:250; AbD Serotec, Oxford, UK) using standard procedures (see supplementary material). For double labelling, antibodies were checked by omitting either antibody; results confirmed that there was no crossreactivity. Hyaluronan was stained using biotinylated hyaluronan binding protein (bHABP; 4 $\mu\text{g}/\text{ml}$; Northstar Bioproducts, East Falmouth, Massachusetts, USA). Nuclear staining was with TO-PRO3 (1:5000, 20 min; Invitrogen, Carlsbad, California, USA). After blocking, tissue was incubated in primary antibody overnight at 4°C . Secondary antibodies included donkey anti-mouse Cy5 (Jackson ImmunoResearch, West Grove, Pennsylvania, USA), biotinylated goat anti-rabbit Jackson ImmunoResearch), streptavidin Alexa568 (Invitrogen, Carlsbad, California, USA) and goat anti-rabbit IgG-Alexa647 (Invitrogen). Images of fluorescent stained sections were collected with an MRC-1024 confocal imaging system. Multiple images (3–5) per animal were examined to confirm immunohistochemistry results. All staining procedures included ‘no primary’ controls to confirm that there was no non-specific binding of the secondary antibody.

Quantitative reverse transcription (RT)-PCR

FLS of *ASIC3*^{+/+} and *ASIC3*^{-/-} mice used in RNA analyses were between passages 2 and 5 and at >90% confluence using standard procedures (see supplementary material). Total RNA was extracted from the FLS cell pellet using the RNeasy mini purification kit (Qiagen, Valencia, California, USA). First strand cDNA was synthesised from 1 μg of each RNA sample using Superscript III reverse transcriptase (Invitrogen). All PCRs were performed with known dilutions of rodent cDNA to allow construction of a standard curve relating cycle threshold to template copy number. ASIC3 (Mm00805460_m1), transient receptor potential vanilloid-1 ion channel (TRPV1) (Mm01246282_s1) and the mouse control assay for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Applied Biosystems (Foster City, California, USA). Relative quantities of gene expression were determined by comparing relative cycle thresholds, normalised to GAPDH.

Knee joint inflammation

Knee joint inflammation was induced by injecting 20 μl of 3% carrageenan dissolved in sterile saline into the left knee joint cavity while the mice were briefly anaesthetised with isofluorane (3%). At 24 h after injection mice were killed, transcardially perfused and tissue removed for immunohistochemistry.

ASIC3 binding to hyaluronan

Cultured Chinese hamster ovary (CHO-K1) cells, which do not express endogenous ASIC3, were transfected with hae-magglutinin-tagged mouse ASIC3 cDNA (15 $\mu\text{g}/10^6$ cells) with Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA) and cell lysates were harvested 48 h after transfection for immunoprecipitation experiments (see supplementary material). Lysates were incubated with either biotinylated rooster comb hyaluronic acid (HA),

biotinylated HA-74KDa, biotinylated HA-12KDa or phosphate-buffered saline (PBS) for 1 h, and then incubated with Neutravidin sepharose beads for an additional 3 h at 4°C. Biotinylated material was collected, the beads washed with lysis buffer, and eluted with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) buffer. Samples were separated by gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked and incubated with anti-haemagglutinin-horseradish peroxidase (HRP) conjugate monoclonal antibody and developed with the ECL Plus western detection system (GE Healthcare, Piscataway, New Jersey, USA). Additional experiments were also conducted to assess binding (see supplementary material).

Quantitative hyaluronic acid assay

pH 7.4 and pH 5.5 external physiological pH solutions used for the hyaluronic acid and calcium imaging were prepared according to previously published procedures³¹ (see supplementary material). FLS cultures plated at 100 000 cells/well (*ASIC3*^{+/+} n=9; *ASIC3*^{-/-} n=8) were exposed to either pH 7.4 or pH 5.5 for 60 min. Supernatants were removed, and assayed in triplicate for hyaluronan concentration (Corgenix, Denver, Colorado, USA) (see supplementary material).

Calcium imaging

Cells were plated with 30 000 cells/dish (6-well plate), loaded with cell permeant Oregon Green Bapta-1 AM (OGB-1) in pH 7.4 for 1 h. Either pH 5.5 or 30μM cyclopiazonic acid (CPA; Sigma Aldrich, St Louis, Missouri, USA) was introduced into the culture dish using a syringe pump. Fluorescence was measured 15 s before, 69 s during and 10 s after application of pH 5.5, or CPA on an Olympus 1X81 motorised inverted microscope (Leeds Precision Instruments, Minneapolis, Minnesota, USA). Percentage change in fluorescence intensity at a given time point was calculated as follows: $\% \Delta F = (F - F_0) / F_0 * 100$, where F is current fluorescence intensity and F₀ is fluorescence intensity under control conditions (pH=7.4).

Statistical analysis

Values are presented as mean±SEM. Data were analysed with one-way analysis of variance (ANOVA) for the RT-PCR and hyaluronan assay. For measurement of calcium imaging repeated measures ANOVA compared values between responses in FLS from *ASIC3*^{+/+} and *ASIC3*^{-/-} mice, and a t test compared the area under the curve differences in FLS from *ASIC3*^{+/+} and *ASIC3*^{-/-} mice. The χ^2 test was used to calculate differences between the number of responders in *ASIC3*^{+/+} and *ASIC3*^{-/-} mice. p<0.05 was considered significant.

RESULTS

ASIC3 is expressed in type B synoviocytes

In knee joint synovium from *ASIC3*^{+/+} mice, ASIC3 colocalised with the fibroblast (type B) marker, Hsp25²⁰ (figure 1A–C) but not with the macrophage (type A) marker ED-1²⁰ (figure 1D–F). Further, ASIC3 staining did not occur in synovium from *ASIC3*^{-/-} mice (figure 1A, inset).

In cultured FLS from *ASIC3*^{+/+} mice, immunostaining confirmed the presence of ASIC3 protein in FLS (figure 2A); ASIC3 staining was absent in FLS from *ASIC3*^{-/-} mice (figure 2B). Analysis of mRNA from *ASIC3*^{+/+} FLS showed abundant expression of *ASIC3* mRNA (figure 2C). Because FLS express the TRPV1 channel where it has been shown to be activated by decreases in pH,^{32,33} we compared the relative expression of *TRPV1* and *ASIC3* mRNA in *ASIC3*^{+/+} and *ASIC3*^{-/-} FLS. *TRPV1* mRNA was detected in similar quantities in FLS from *ASIC3*^{+/+} and *ASIC3*^{-/-} mice, but its expression was 40-fold less than that of *ASIC3* in the *ASIC3*^{+/+} FLS.

ASIC3 is expressed in cartilage

To examine ASIC3 expression in other joint tissues, we prepared tissue sections from whole leg segments and immunohistochemically stained for ASIC3. ASIC3 was expressed in articular cartilage and growth plate of the tibia and femur of *ASIC3*^{+/+} mice (figure 3A,B). No ASIC3 immunoreactivity was observed in the subchondral bone. Immunoreactivity was negative in the corresponding tissue of *ASIC3*^{-/-} mice (figure 3C,D).

ASIC3 modulates the expression and release of hyaluronan

In tissue sections from whole leg segments, there was decreased hyaluronan staining in *ASIC3*^{-/-} mice when compared to *ASIC3*^{+/+} mice. This reduction in hyaluronan staining occurred in the synovium, growth plate, articular cartilage and meniscus in animals without inflammation (figure 4). Since synoviocytes rapidly alter the amount of hyaluronan secreted upon inflammation,³⁴ we examined the degree of hyaluronan staining in the synovium 24 h after carrageenan was injected into the knee joint cavity. At 24 h after inflammation the thickness of the synovium was increased in tissue from *ASIC3*^{+/+} and *ASIC3*^{-/-} mice (figure 5A,B). This increased thickness is a result of increased connective tissue without an increase in the number of cells. After inflammation, hyaluronan expression increases in *ASIC3*^{+/+} mice (figure 5B and inset). However, hyaluronan expression in the synovium from *ASIC3*^{-/-} 24 h after inflammation is less than either uninflamed or inflamed synovium from *ASIC3*^{+/+} mice (figure 5A,B and insets).

To confirm and quantify the regulation of hyaluronan by ASIC3, we next tested if acidic pH altered the amount of hyaluronan released from cultured FLS from *ASIC3*^{+/+} and *ASIC3*^{-/-} mice. The average amount of HA released from *ASIC3*^{+/+} FLS under acidic conditions (10.94 ng/ml) was greater than that released under physiological pH (6.99 ng/ml) ($p < 0.05$) (figure 5C).

Decreases in pH increase intracellular calcium release in FLS through ASIC3

Because a rapid increase in intracellular calcium is indicative of signalling pathway activation,^{35,36} and ASICs are permeable to Ca^{2+} and are activated by decreases in pH,⁸ we monitored changes in intracellular Ca^{2+} concentration [Ca^{2+}]_i in cultured FLS from *ASIC3*^{+/+} and *ASIC3*^{-/-} mice in response to pH 5.5. As a control, application of CPA, a sarcoplasmic reticulum pump inhibitor, increased [Ca^{2+}]_i which indicated viable and loaded FLS (see supplementary material). In response to pH 5.5, there was a rapid increase in [Ca^{2+}]_i in almost half of *ASIC3*^{+/+} FLS (25 of 53 cells) (figure 6), when compared to pH 7.4. In contrast, only 24% of *ASIC3*^{-/-} FLS (14 of 59 cells) responded to pH 5.5 with a rise in [Ca^{2+}]_i ($p < 0.001$) (figure 6). Of those that responded to pH 5.5 there was a smaller increase in [Ca^{2+}]_i in FLS from *ASIC3*^{-/-} mice compared to those from *ASIC3*^{+/+} mice ($*p = 0.0001$). Quantification of the area under the curve showed that [Ca^{2+}]_i elevations of cells that responded to pH 5.5 was less in *ASIC3*^{-/-} FLS compared to *ASIC3*^{+/+} FLS ($p = 0.0001$) (figure 6).

ASIC3 does not bind to the extracellular matrix component, hyaluronan

Because degerin (DEG)/epithelial Na^{+} channels (ENaC) family members can bind extracellular proteins in *Caenorhabditis elegans*^{37,42} and ASICs contain putative, hyaluronan binding motifs in their extracellular domain, we examined whether ASIC3 would bind to hyaluronan in vitro. Binding of ASIC3 and hyaluronan was examined with lysates from CHO-K1 cells expressing haemagglutinin-tagged ASIC3 (figure 5D). Immunoprecipitation, conducted by incubating the protein lysate with biotinylated hyaluronan and subsequently with Neutravidin beads, showed that no precipitate contained the haemagglutinin-tagged ASIC3, indicating a lack of binding (figure 5D).

DISCUSSION

ASIC3 serves as a pH sensor in non-neuronal cells

The current study shows expression of ASIC3 in type B FLS, chondrocytes and the epiphyseal plate from *ASIC3*^{+/+} mice. Importantly, no immunostaining for ASIC3 was detected in tissues from *ASIC3*^{-/-} mice, serving as a negative control. These data extend prior studies that show expression of ASIC3 in non-neuronal joint cells¹⁵ including synoviocytes²⁴ by showing protein in joint tissue and mRNA in FLS of *ASIC3*^{+/+} mice.

Decreases in pH occur in a variety of inflammatory exudates, including those from people with rheumatoid arthritis and this pH decrease correlates with severity of joint destruction and pain.¹⁷ ASICs detect decreases in pH with ASIC3 being most sensitive in the physiological range. Increases in intracellular calcium, which regulate activity of intracellular pathways, occur upon activation of a variety of ion channels, including *N*-methyl-D-aspartic acid (NMDA) receptors, transient receptor potential (TRP) channels, cholinergic receptors, or ATP receptors on synoviocytes, each of which can modulate production of inflammatory mediators.³²⁴³⁴⁶ In neuronal cells, proton activation of ASICs increase intracellular calcium⁴⁷⁴⁸ and ASIC1a gates calcium directly.⁴⁹ In FLS, activation of TRPV1³²³³ and a G-protein-coupled receptor⁵⁰ by protons also increases $[Ca^{2+}]_i$, suggesting ASIC3 is not the sole pH sensor in FLS. However, we show a 40-fold greater expression of *ASIC3* over *TRPV1* mRNA in FLS; and *ASIC3*^{-/-} FLS are less likely to be activated by protons than *ASIC3*^{+/+} FLS, despite equal expression of *TRPV1* mRNA. In agreement with the current study, human FLS, isolated from patients with inflammatory arthropathies or tumour derived, increase $[Ca^{2+}]_i$ in response to low pH.⁴⁶ These findings suggest that pH modulates FLS activity and that ASIC3 is a prominent pH sensor in the synoviocytes.

FLS play a crucial role in enhancement of the inflammatory process,⁵¹ are a major source of cytokines and are the primary producers of matrix metalloproteinases (MMPs) in rheumatoid arthritis. Recent data in *cadherin-11*^{-/-} mice confirm that FLS contribute to the inflammatory component of arthritis as well as cartilage degradation.⁵² As decreases in pH are associated with inflammation, it is reasonable to assume that inflammatory mediators, such as tumour necrosis factor α (TNF α) or Interleukin 1 (IL1), could sensitise the pH response in FLS. In sensory neurons, application of inflammatory mediators induces an upregulation of ASIC1, ASIC2 and ASIC3 mRNA in the dorsal root ganglion (DRG) suggesting inflammatory mediators can modulate expression of ASICs in DRG.⁵³⁵⁴ Recently, we confirmed an upregulation of ASIC3 protein and mRNA in sensory neurons after carrageenan-induced inflammation.¹⁴⁵⁵ In synoviocytes, a similar process is likely to occur. For example, in FLS, the heat sensor TRPV1 shows an enhanced responsiveness to increases in temperature and an increase in expression after prior treatment with TNF α .⁵⁶ Thus, future experiments should investigate an interaction between inflammatory mediators and the pH response in FLS.

While pH can reach as low as 5.4 in some disease conditions, the pH is commonly between 6.0 and 6.9 after inflammatory arthritis. Our study used pH 5.5 as the test stimulus for examining the role of ASICs in decreases in pH because prior published studies examining pH effects in synoviocytes showed the greatest effect with pH 5.5.⁵⁰ In sensory neurons, routine electrophysiological studies of ASICs and pH show that responses to pH occur in a dose-dependent manner with the greatest effect occurring around pH 5.0.⁵⁷ Future experiments should examine the pH response effect across multiple acidic pHs to determine the role of ASIC3 at all of these ranges.

ASIC3 modulates expression and release of hyaluronan

There was a decrease in hyaluronan staining in joint structures, synovium and cartilage, from *ASIC3*^{-/-} mice compared to *ASIC3*^{+/+} mice. We also show increased hyaluronan release in FLS in response to decreases in pH and that this release is dependent on the presence of ASIC3. FLS secrete hyaluronan in response to the inflammatory cytokines TNF α , IL1 β , and interferon γ and to mechanical stimulation.²⁷ Further, in FLS, activation of TRP channels, NMDA, cholinergic and ATP receptors modulates the production of inflammatory mediators.³²⁴³⁴⁶ Our data suggests a direct or intermediate role for ASIC3 to modulate hyaluronan release to the inflammation-induced decrease in pH, making it a potential target in prevention of inflammatory induced joint destruction.

It is possible that ASIC3 could interact with hyaluronan in the extracellular domain of the channel. The extracellular domains of ASICs are large, encompassing the majority of the channel.⁵⁸ Several ligands interact with the extracellular domain including calcium, lactate, arachidonic acid and redox reagents. As ASICs are vertebrate homologues of the mechanosensitive DEG/ENaC channels in *C. elegans*, they could act in mechanotransduction, or respond to mechanical stimuli. Binding of the extracellular domain of ASICs to extracellular components, could functionally form a tether to physically open the channel.⁵⁹ The extracellular domain of ASIC3 contains a sequence (amino acids 382–398 of mouse ASIC3, accession number Q6X1Y6, NCBI Entrez protein database) that is consistent with potential receptor for hyaluronic acid-mediated motility (RHAMM)-like binding motifs for hyaluronan.⁶⁰⁶¹ However, the current data show no binding of hyaluronan to ASIC3.

Conclusions

In summary, the current study shows expression of ASIC3 in FLS and chondrocytes in adult tissue from *ASIC3*^{+/+} mice and not from *ASIC3*^{-/-} mice. Alterations in hyaluronan expression occur in *ASIC3*^{-/-} mice suggesting that ASIC3 plays a significant role in joint function. Decreases in pH activate FLS by increasing intracellular calcium and releasing hyaluronan. We conclude that ASIC3 in non-neuronal joint cells regulates pH responsiveness and controls release of the extracellular matrix polysaccharide hyaluronan. We propose ASIC3 as a potential therapeutic target not only to treat the symptoms of pain associated with diseases of the joint, but more significantly, to attenuate the underlying causes of such diseases.

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Figure 1.

Acid-sensing ion channel 3 (ASIC3) is located in type B, but not type A synoviocytes. Whole mount preparations of anterior synovium from an *ASIC3*^{+/+} mouse knee joint, were double labelled for ASIC3 (red; A,D), and either the type B synoviocyte marker, heat shock protein 25 (Hsp25) (green; B) or the type A marker, ED1 (green; E). C. Merged image of ASIC3 (A) and Hsp25 (B), and shows colocalisation of ASIC3 protein with Hsp25. F. Merged image of ASIC3 (D) and ED1 (E). The insets of (A) and (B) show the staining of whole mount anterior synovium of *ASIC3*^{-/-} mice with ASIC3 and Hsp25 antibodies, respectively. The inset of (C) is the overlay of the (A) and (B) insets.

Figure 2.

Cultured synoviocytes express acid-sensing ion channel 3 (ASIC3) protein and mRNA. A,B. Fibroblast-like synoviocytes (FLS) were fixed and stained with an antibody to ASIC3 (red). Nuclei were stained with TO-PRO3 (blue). The FLS of (A) and (B) were derived from *ASIC3*^{+/+} and *ASIC3*^{-/-}, respectively. C. Quantitative reverse transcription (RT)-PCR of *ASIC3* and *TRPV1* mRNA expression in FLS cultures. Normalised gene expression (arbitrary units) for *ASIC3* and *TRPV1* mRNA is plotted for *ASIC3*^{+/+} (black bars) and *ASIC3*^{-/-} (white bars) FLS cultures. Quantitative reverse transcription (RT)-PCR data were normalised with *GAPDH* mRNA levels. Each bar represents 12 measurements.

*Significantly different from *ASIC3*^{-/-} ; +significantly different from *TRPV1* mRNA. Data are mean±SEM.

Figure 3.

Acid-sensing ion channel 3 (ASIC3) immune-reactivity is found in cartilaginous structures of the knee joint. Sagittal sections through legs from [A,B) *ASIC3*^{+/+} and [C,D) *ASIC3*^{-/-} mice were immunohistochemically stained for ASIC3. Immunoreactivity for ASIC3 was found in epiphyseal cells of the femoral growth plate (A) and in the femoral articular cartilage (B) in *ASIC3*^{+/+} mice. No staining is seen in the corresponding tissue of *ASIC3*^{-/-} mice (C,D). See supplementary material for colour images.

Figure 4.

Hyaluronan expression is decreased in knee joint structures from *ASIC3*^{-/-} mice compared to tissues from *ASIC3*^{+/+} mice. Sagittal sections stained for hyaluronan (green; blue = nuclear dye TO-PRO3) in whole leg segments from *ASIC3*^{+/+} (A-D) and *ASIC3*^{-/-} (E,F) mice. Hyaluronan staining is reduced in the synovium (A,E), the epiphysial cells of the femoral growth plate (B,F), the femoral articular cartilage (C,G) and in the meniscus (D,H) of *ASIC3*^{-/-} mice when compared to staining in *ASIC3*^{+/+} mice.

Figure 5. Hyaluronan expression after inflammation and release in response to decreases in pH are reduced in *ASIC3*^{-/-} mice. Sagittal sections of whole leg segments through the knee joints of *ASIC3*^{+/+} (A) and *ASIC3*^{-/-} (B) mice 24 h after carrageenan induced inflammation, were stained for hyaluronan, green. The cell nucleus is depicted in blue. For comparison, the insets of (A) and (B) show uninflamed synovium cropped from the images in figure 4A and E, respectively. Note the increase in the thickness of the synovium after inflammation synovium from *ASIC3*^{+/+} and *ASIC3*^{-/-} tissues (dotted white line/double arrows). Hyaluronan expression is increased in the inflamed synovium of *ASIC3*^{-/-} (B) mice compared to the uninflamed *ASIC3*^{-/-} synovium (inset, B) but still appears less than either inflamed (A) or uninflamed (inset, A) *ASIC3*^{+/+} synovium. (C) Supernatants of fibroblast-like synoviocyte (FLS) cultures exposed 1 h to pH 5.5 show increases in hyaluronan concentration compared to control conditions exposed to pH 7.4. Inset shows the percentage

increased release of hyaluronan in *ASIC3*^{+/+} (n=9) was significantly greater than that from *ASIC3*^{-/-} (n=8) mice ($p<0.05$). Data are mean $\pm$ SEM. (D) Lysates containing haemagglutinin-tagged acid-sensing ion channel 3 (ASIC3) incubated with either no hyaluronan (lane 3), or biotinylated hyaluronan, high molecular weight (lane 4), 74 K (lane 5) and 12 K (lane 6). None of the hyaluronans interacted with ASIC3. The precleared lysate alone shows expression of ASIC3 (lane 1). The haemagglutinin-tagged ASIC3 protein did not directly bind Neutravidin beads (unbound fraction, lane 2).

Figure 6. Increased calcium release in response to decreases in pH is reduced in *ASIC3*^{-/-} mice. **A.** Time course profile of all cells for the percentage change in $[Ca^{2+}]_i$ from baseline for *ASIC3*^{+/+} fibroblast-like synoviocytes (FLS) and *ASIC3*^{-/-} FLS in response to (arrow indicates pH or cyclopiazonic acid (CPA) application). *Significantly different from *ASIC3*^{+/+} (repeated measures analysis of variance (ANOVA)). Data are mean±SEM. **B.** The area under curve for each responding FLS, shows a statistically significant difference between groups ($p=0.0001$, t test). Data are mean±SEM. See supplementary material for more calcium imaging results.