



Published in final edited form as:

Arthritis Rheum. 2009 October ; 60(10): 3028–3037. doi:10.1002/art.24799.

Functional Characterization of TRPV4 As an Osmotically Sensitive Ion Channel in Articular Chondrocytes

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Abstract

Objective—Transient receptor potential vanilloid 4 (TRPV4) is a Ca²⁺ permeable channel that can be gated by tonicity (osmolarity) and mechanical stimuli. Chondrocytes, the cells in cartilage, respond to their osmotic and mechanical environments; however, the molecular basis of this signal transduction is not fully understood. The objective of this study was to demonstrate the presence and functionality of TRPV4 in chondrocytes.

Methods—TRPV4 protein expression was measured by immunolabeling and Western blotting. In response to TRPV4 agonist/antagonists, osmotic stress, and interleukin-1 (IL-1), changes in Ca²⁺ signaling, cell volume, and prostaglandin E₂ (PGE₂) production were measured in porcine chondrocytes using fluorescence microscopy, light microscopy, or immunoassay, respectively.

Results—TRPV4 was expressed abundantly at the RNA and protein level. Exposure to 4αPDD, a TRPV4 activator, caused Ca²⁺ signaling in chondrocytes, which was blocked by the selective TRPV4 antagonist, GSK205. Blocking TRPV4 diminished the chondrocytes' response to hypo-osmotic stress, reducing the fraction of Ca²⁺ responsive cells, regulatory volume decrease (RVD), and PGE₂ production. Ca²⁺ signaling was inhibited by removal of extracellular Ca²⁺ or depletion of intracellular stores. Specific activation of TRPV4 restored defective RVD caused by IL-1. Chemical disruption of the primary cilium eliminated Ca²⁺ signaling in response to either 4αPDD or hypo-osmotic stress.

Conclusion—TRPV4 is present in articular chondrocytes, and chondrocyte response to hypo-osmotic stress is mediated by this channel, which involves both an extracellular Ca²⁺ and intracellular Ca²⁺ release. TRPV4 may also be involved in modulating the production or influence of pro-inflammatory molecules in response to osmotic stress.

Articular cartilage, the avascular connective tissue that covers diarthrodial joint surfaces, provides a low-friction surface that supports and distributes mechanical loads. Cartilage comprises a hydrated extracellular matrix (ECM) of proteoglycans and collagen fibrils, as well as chondrocytes, the cells responsible for maintaining the ECM. The chondrocytes' metabolic function is influenced by a number of factors in the microenvironment, including soluble mediators, ECM composition, and mechanical loading (1,2). The transduction of biomechanical factors to intracellular signals appears to involve changes in other biophysical

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parameters secondary to compression of the ECM (3), but such pathways have not been fully elucidated.

The ECM of cartilage is inherently negatively charged due to the large concentration of the anionic proteoglycan aggrecan which attracts cations to counterbalance the charge. The resulting increase in interstitial osmolarity causes the tissue to imbibe water (4). Upon joint loading, water is exuded from the tissue but is reabsorbed when the tissue is no longer compressed (5,6). Thus, chondrocytes experience large fluctuations in their osmotic environment as a result of normal joint loading, in addition to changes in other biophysical parameters, such as cell deformation, fluid flow, pH, and fluid pressure (1,7).

Chondrocytes respond to osmotic stress with the initiation of intracellular signaling cascades and acute volume change (8-10), followed by volume regulation involving cytoskeletal F-actin restructuring (9) and/or solute transport (10,11). Osmotic stimulation of chondrocytes also elicits a cytoplasmic Ca^{2+} -signal originating from both extracellular Ca^{2+} -influx as well as intracellular Ca^{2+} release from stores (8,9). This Ca^{2+} -signal may play a role in volume regulation (12), cell metabolism, gene expression (13), and restructuring of the F-actin cytoskeleton (9). While the response of chondrocytes to mechanical and osmotic stresses has been extensively characterized, the molecular mechanisms by which these cells sense changes in the biophysical environment are not well understood (1,2).

Osteoarthritis, the primary disease afflicting cartilage, is characterized by the gradual breakdown of the cartilage ECM. In this disease, damage to the collagen network results in swelling and increased water content of the tissue, thus decreasing the interstitial osmotic pressure (1). Osteoarthritis is also characterized by a significant inflammatory component at the molecular level. In particular, the pro-inflammatory cytokine, interleukin 1 (IL-1) has been shown to elicit Ca^{2+} transients in chondrocytes and to affect the response to hypo-osmotic stress by preventing volume regulation (14). Furthermore, IL-1 is a potent activator of cyclo-oxygenase 2 (COX2) and associated pro-inflammatory autacoids such as prostaglandin-E2 (PGE_2) (15), which serve as the primary pharmacologic targets for combating pain and inflammation in osteoarthritis (16).

One potential candidate involved in chondrocyte osmo-sensation, and thus potentially mechanotransduction, is the Ca^{2+} -permeable, nonspecific cation channel Transient Receptor Potential Vanilloid 4 (TRPV4). Although the permeability of TRPV4 is relatively similar for Ca^{2+} , Sr^{2+} , Ba^{2+} , Mg^{2+} (17,18), under physiologic conditions calcium is the primary ion traversing the channel. TRP channels act as cellular sensors in a variety of settings (19). TRPV4 was the first osmotically sensitive vertebrate ion channel to be discovered and subsequently has been found expressed in a variety of tissues (17,19-23). TRPV4 is activated by hypo-osmotic cell swelling (17,20,23), warmth ($>27^\circ\text{C}$) (24-26), phorbol esters (e.g., 4 α -phorbol 12,13-didecanoate, 4 α PDD, a specific synthetic activator of TRPV4) (27), and arachidonic acid and its metabolites (28,29). TRPV4 activation has also been shown to promote chondrogenesis by inducing SOX9 transcription through a Ca^{2+} /calmodulin pathway (22). Furthermore, a TRPV4 gain-of-function mutation has recently been shown to underlie a familial form of brachyolmia, a developmental skeletal dysplasia (30), which suggests a potentially critical role for TRPV4 in skeletal development.

The goal of this study was to characterize the expression and function of TRPV4 in porcine articular cartilage. As an osmotically gated ion channel, we investigated whether TRPV4 would conduct extracellular Ca^{2+} in response to osmotic stress, and whether it would participate in osmotically-induced volume regulation. We also addressed whether TRPV4 plays a role in chondrocytes' response to IL-1, and in the release of PGE_2 , which is a pathogenic, pro-nociceptive downstream effect of IL-1-receptor signaling. Finally, because

ciliary expression of TRP channels has been implicated in signal transduction in a variety of cell types and organisms (31-33), we examined the role of chondrocytic cilia in TRPV4 function.

Materials and Methods

Porcine cell culture

All cell culture reagents were obtained from Gibco (Invitrogen Corp., Grand Island, NY), unless otherwise stated. Cartilage was harvested from the femoral condyles of skeletally mature pigs (*Sus scrofa*), and chondrocytes were isolated from the tissue in a sequential pronase (Calbiochem, San Diego, CA) and collagenase (Worthington Biochemical Corp., Lakewood, NJ) digestion as previously described (34). Chondrocytes were cultured overnight at 37°C in 5% CO₂ on glass coverslips (1.0-1.5E6 cells/mL) in DMEM, 10% FBS, 15 mM HEPES, 0.1mM non-essential amino acids. Measurements of intracellular Ca²⁺, volume, and PGE₂ experiments were performed at 37°C in medium containing only DMEM and 15 mM HEPES.

Immunohistochemistry

Samples of porcine articular cartilage were fixed in paraformaldehyde and embedded in paraffin (35). An anti-TRPV4 rabbit antibody (33) was applied to 5 µm sections and detected using a fluorescent secondary antibody (Alexa Fluor 555; Molecular Probes, Invitrogen, Eugene, OR). A negative control and a pre-immune control using serum from the antibody-producing rabbit were also run.

Western blotting

We followed standard methods described previously (33). Briefly, cells were lysed with RIPA buffer and separated on an 8% SDS polyacrylamide gel, then transferred to PVDF filters (0.45 mm pore; Millipore, Amherst MA) by semi-dry blot using Tris-glycine/20% methanol transfer buffer. Blotted proteins were immuno-detected using a two-step antibody-mediated chemoluminescence assay, employing a specific antibody against the TRPV4 (36), and secondary peroxidase-coupled antibodies (Jackson ImmunoResearch, West Grove, PA). As a control to assure specificity of detected bands, the antibodies were pre-incubated with the immunogenic peptide (17 C-terminal amino acids of rat TRPV4).

RT-PCR and Sequencing

A BLAST (<http://www.ncbi.nlm.nih.gov/>) search among porcine expressed sequence tags (ESTs) was done to search for sequences homologous to the human TRPV4 gene (NCBI Accession # NM_021625.1). Two sets of custom primers (Integrated DNA Technologies, Coralville, IA) based on ESTs similar to the TRPV4 gene were developed. RNA was extracted from porcine chondrocytes and reverse-transcribed using commercial kits (RNeasy Mini Kit; Qiagen, Valencia, CA, and SuperScript™ First-Strand Synthesis System for RT-PCR; Invitrogen, Carlsbad, CA). PCR was performed using reagents from Qiagen, and porcine GAPDH (33,37) was used as a housekeeping gene. The PCR products were submitted to a core facility (Duke University DNA Analysis Facility, Durham, NC) for sequencing.

Sequencing porcine TRPV4

The 5' and 3' sequence ends of the porcine TRPV4 gene were determined via RACE PCR and cloning from porcine brain and spleen SMART cDNA (Clontech Mountain View, CA). Primers were designed based on an alignment between the human (NM_021625), and mouse (AF208026) sequences and RACE PCR was performed to amplify these ends. Once the 5'

and 3' sequence ends were confirmed between the 2 different tissue sources, primers were then designed to amplify the full length cDNA ORF. The full length ORF was obtained by PCR from porcine brain SMART cDNA. This sequence was confirmed by comparison with cDNA clones obtained from multiple tissue sources. The final clone was flanked with a 5' CACC for directional cloning into the pcDNA3.1_V5-His-TOPO vector. The sequence was deposited in GenBank.

Ligand-gated porcine TRPV4 activation

Porcine articular chondrocytes were loaded with Fura-2-AM and the intracellular concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$) was measured in a multiwell format using a FlexStation (Molecular Devices) following exposure to TRPV4 agonist (4 α PDD). Inhibition of ligand-gated (4 α PDD) activation by Ruthenium Red or GSK-205 was also demonstrated.

Osmotic stress

Media osmolarity was determined by a freezing point osmometer (Osmette 2007; Precision Systems, Inc., Natick, MA), using 380 mOsm as the iso-osmotic point for chondrocytes (2). Hypo-osmotic (280 mOsm) and hyper-osmotic media (480 mOsm) were prepared by adding distilled water or sucrose (EMD Chemicals, Gibbstown, NJ) to iso-osmotic experimental medium (DMEM/HEPES).

Calcium Measurements

Changes in $[\text{Ca}^{2+}]_i$ were measured by previously described fluorescence ratio imaging of Fura Red and fluo-3 using a laser scanning microscopy (8,38). Changes in $[\text{Ca}^{2+}]_i$ at 37°C were measured in response to: 1 μM 4 α PDD (MP Biomedicals, Inc., Aurora, OH, and Sigma, St. Louis, MO), a TRPV4 agonist. The Ca^{2+} response to 1 μM 4 α PDD was also evaluated in chondrocytes pre-stimulated with the following compounds: 1 μM 4 α PDD with a 30 minute pre-incubation in 1 μM Ruthenium Red (RR; non-selective TRPV4 inhibitor, Sigma); 10 μM GSK205 (selective TRPV4 inhibitor, GlaxoSmithKline); 3 μM thapsigargin (thaps, inhibitor of intracellular Ca^{2+} release; Calbiochem); 10 μM gadolinium (Gd^{3+} , non-specific inhibitor of mechanosensitive ion channels, Sigma); Ca^{2+} -free medium containing EGTA (10 mM, Calbiochem); and 1 μM capsazepine (selective TRPV1 inhibitor, Sigma). Cells were exposed to all of the aforementioned compounds, except for 4 α PDD, for a pre-imaging incubation period ranging from a few minutes to an hour. Cells were also exposed to 480 mOsm media and 280 mOsm media alone or with 1 μM RR or 10 μM GSK205. After the iso-osmotic medium was withdrawn from the perfusion chamber, 1 mL of experimental solution was perfused on to the cells by a syringe pump.

$[\text{Ca}^{2+}]_i$, as measured by the intensity ratio of the two Ca^{2+} indicators, was normalized to baseline levels, and a positive response was defined as an increase of $[\text{Ca}^{2+}]_i$ greater than three standard deviations over the average response to control. Vehicular controls, ethanol in 380 mOsm DMEM/HEPES, were also run to confirm a lack of response. The time to the first peak $[\text{Ca}^{2+}]_i$ and the normalized height of this peak were also measured using a custom-written Matlab (The MathWorks, Natick, MA) program.

Cell volume measurements

Cell surface area was measured using a custom-written digital image analysis program (39). Eight baseline images were obtained prior to infusing experimental medium. Experimental media tested were: 280 mOsm, 280 with 1 μM RR, 280 with 10 μM GSK205, 280 with 10 ng/mL IL-1 α (R&D Systems, Minneapolis, MN), 280 with IL-1 and 1 μM 4 α PDD, 380 mOsm, 380 with 4 α PDD, 380 with 4 α PDD and RR, 380 with 4 α PDD and GSK205, and 480 mOsm. Cell volumes were derived from these data and normalized to baseline levels.

Maximum volume change within the first 200 s, final volume (average of the last 20 images) and recovered volume (maximum-final) were computed.

PGE₂ Assay

Isolated chondrocytes were plated directly onto tissue culture-treated 12-well plates (1E6 cells per well) and incubated overnight. The cells were treated with 280 mOsm media, 280 plus RR (1 μ m), 280 plus GSK205 (10 μ m), 380 mOsm media, 380 plus 4 α PDD (1 μ M), 380 plus 4 α PDD plus RR, 380 plus 4 α PDD plus GSK205, or 480 mOsm media. Samples of media were taken from the wells at 16 and 30 min. for the PGE₂ assay, which was used to the manufacturer's specifications (PGE₂ Parameter Immunoassay; R&D Systems). The assay sample absorbances were evaluated in a GENios plate reader (Tecan US, Inc., Research Triangle Park, NC).

Cilia and TRPV4

Freshly isolated porcine chondrocytes were fixed with cold methanol and labeled with the following antibodies: α -tubulin (Sigma) to detect primary cilia and TRPV4. To determine whether cilia affected the function of TRPV4, freshly isolated porcine chondrocytes were cultured for 24 hours in either normal feed media or chemical deciliation media (380 mOsm feed media plus 4 mM chloral hydrate) (31), which acts by disrupting ciliary microtubules (40). Changes in [Ca²⁺]_i were measured (as described above) in response to 380 mOsm, 280 mOsm or 380 mOsm plus 1 μ M 4 α PDD. Positive controls with 380 mOsm plus 10 μ M ionomycin were also run.

Statistical analysis

Comparisons of continuous values among treatment groups were made using a one-factor ANOVA and Fisher's PLSD post-hoc analysis. Nominal data were compared between treatment groups using the chi-square test. PGE₂ data were analyzed with a repeated measures ANOVA with time as the repeated measure. All data reported are mean \pm standard error with statistical significance at the 95% confidence level. Statistical analysis was performed with StatView (SAS Institute, Cary, NC) or Statistica (Statsoft, Tulsa, OK)

Results

Chondrocyte TRPV4 Expression

Immunohistochemistry using TRPV4 antibodies clearly demonstrated localization of the channel to the cell membrane with some intracellular labeling of *in situ* porcine chondrocytes (Fig. 1a), while the negative and pre-immune controls exhibited little to no fluorescence. Western blotting confirmed the presence of TRPV4 in protein extracts from chondrocytes (Fig. 1b). The porcine sequence (genbank accession# EU784669) is 97% similar to human TRPV4 at the amino acid level. RT-PCR from porcine chondrocyte RNA yielded two DNA fragments that showed 94.4% homology to the human TRPV4 cDNA (Fig. 1c).

Chemical activation of TRPV4

In isolated porcine chondrocytes the TRPV4 agonist, 4 α PDD induced an influx of Ca²⁺ (EC₅₀~2 μ M; Fig. 2). The Ca²⁺ influx induced by 10 μ M 4 α PDD was inhibited by the TRP-blocker Ruthenium Red (RR, IC₅₀~5 nM). A selective TRPV4 channel blocker, GSK-205 (N-(4-{2 [methyl(phenylmethyl)amino]ethyl}phenyl)-5-(3-pyridinyl)-1,3-thiazol-2-amine hydrobromide), which was identified using a FLIPR-based high throughput screen and has been shown by electrophysiology and Ca²⁺ imaging studies to block both ligand-gated and

hypo-osmotic activation of endogenous and transiently expressed human TRPV4 channels, was found to inhibit Ca^{2+} influx ($\text{IC}_{50} \sim 600 \text{ nM}$; Fig. 2).

Ca^{2+} signaling in chondrocytes

Control experiments demonstrated that iso-osmotic medium or vehicular controls did not result in significant increases in $[\text{Ca}^{2+}]_i$. Cell stimulation with 4 α PDD caused either sharp increases (often with subsequent oscillations) or sustained elevations in intracellular Ca^{2+} in 49% of the cells (Fig. 3). Treatment with RR prior to and during 4 α PDD stimulation significantly decreased the percentage of cells signaling to only 29%. Treatment with the TRPV4 antagonist, GSK205, decreased Ca^{2+} signaling in response to 4 α PDD even further with 26% of cells responding. Thirty minute pre-incubation with Gd^{3+} (10 μM), a purported stretch-activated channel blocker (41), significantly attenuated the response to 4 α PDD with 30% of cells responding. The response to 4 α PDD was clearly dependent upon extracellular Ca^{2+} , as only 6% of the cells exposed to 4 α PDD in Ca^{2+} -free medium signaled. Moreover, 4 α PDD-induced elevations in $[\text{Ca}^{2+}]_i$ were facilitated by Ca^{2+} release from intracellular stores because only 3% of the cells treated with thapsigargin showed increased $[\text{Ca}^{2+}]_i$. Capsazepine, a TRPV1 channel antagonist, did not significantly block the 4 α PDD response, as 43% of the cells signaled in its presence.

Ca^{2+} signaling in chondrocytes was also induced by osmotic stress (Fig. 3). Hypo-osmotic stress (280mOsm) invoked increases in $[\text{Ca}^{2+}]_i$ in 91% of the cells, whereas hyper-osmotic stress (480mOsm) elicited responses in only 26% of the cells. Both hypo- or hyper-osmotic stress caused significantly more Ca^{2+} signaling than iso-osmotic control. In the presence of GSK205, the hypo-osmotic response was strikingly attenuated, with only 11% of cells signaling respectively (RR: 65%).

Changes in cell volume

Joint loading can cause changes in the osmotic environment of the chondrocytes as fluid is forced out and reabsorbed into the cartilage. These fluctuations can cause regulatory volume changes as well as Ca^{2+} signaling (8,9), thus we further examined whether TRPV4 plays a role in volume regulation. Both osmotic and chemical stimuli altered the volume of the chondrocytes (Fig. 4). Chondrocyte maximum volume increased in hypotonic (280mOsm) medium, decreased in hypertonic (480mOsm) medium, and remained constant in isotonic (380mOsm) medium. Cells exposed to 4 α PDD in iso-osmotic conditions underwent a more modest, but nonetheless significant volume increase to a maximum volume that was significantly diminished by RR and GSK205. IL-1 receptor signaling significantly increased the maximum volume in response to hypo-osmotic stress, and that increase was inhibited by stimulating TRPV4 channels with 4 α PDD. The addition of RR or GSK205 decreased the maximum volume in response to hypo-osmotic stress.

The chondrocytes exhibited regulatory volume decrease (RVD) in response to hypo-osmotic stimuli, and that RVD was significantly inhibited by blocking TRPV4 activation with RR or GSK205 (Fig. 4). The IL-1-treated chondrocytes showed greatly impaired RVD which was eliminated when TRPV4 channels were stimulated with 4 α PDD.

PGE₂ Release

We monitored stimulus-induced release of PGE₂ because of the important role of this pro-inflammatory autacoid in joint inflammation and pain. Hypo-osmotic stimulation significantly increased the short-term (15-30minutes) release of PGE₂ in a TRPV4-dependent manner, as indicated by the strong inhibitory effect of GSK205 (also by RR; Fig. 5). Specific chemical stimulation of TRPV4 with 4 α PDD, under isotonic conditions, did not induce significant PGE₂ release.

TRPV4 and Cilia

Because TRP channel-mediated signal transduction has been linked to primary cilia in a variety of cell types and organisms (31-33), we examined co-localization of cilia and TRPV4 in articular chondrocytes and asked whether TRPV4 activation required intact cilia. Tubulin (α and γ) labeling clearly showed the presence of a primary cilium on all cells (Fig. 6a; data not shown). TRPV4 labeling was relatively uniform on the periphery of the cells. Careful inspection demonstrated TRPV4 decorating the cilium itself. Therefore, TRPV4 labeling in chondrocytes, while co-localizing with cilia, was not restricted to cilia.

In order to examine the function of ciliary TRPV4 in chondrocytes, they were subjected to treatment with chloral hydrate, which de-ciliates cells. This treatment almost fully eliminated Ca^{2+} signaling in response to TRPV4-activating stimuli, exposure to $4\alpha\text{PDD}$ and hypo-osmotic stress (Fig. 6b). Control experiments validated this provocative result, inasmuch that iso-osmotic media caused negligible Ca^{2+} signaling. Chondrocytic Ca^{2+} signaling appeared basically unaffected, since intracellular store depletion using ionomycin was fully functional.

Discussion

The goal of this study was to demonstrate and characterize the functional expression of the TRPV4 channel in primary articular chondrocytes, the cell population responsible for maintenance of joint function and disease. Using a reductionist cellular model, we demonstrated that TRPV4 is expressed robustly in porcine articular chondrocytes at both the RNA and protein levels. Channel function was confirmed using both chemical and physical activators of TRPV4. For example, $4\alpha\text{PDD}$, a known TRPV4 agonist, induced Ca^{2+} transients, which were blocked by a novel TRPV4-selective small molecule inhibitor, GSK205. We also established that specific blocking of TRPV4 inhibits the volume-regulatory and Ca^{2+} signaling responses to hypo-osmotic stress, suggesting a pivotal role for TRPV4 in osmotically-mediated signal transduction in chondrocytes. Our data also reveal a potentially central role for TRPV4 in mediating the inflammatory response of chondrocytes; activation of TRPV4 counteracts several tested effects of IL-1, while TRPV4 block decreases short-term release of PGE_2 . Finally, our data suggest that chondrocytic TRPV4 activation is critically dependent on the integrity of the primary cilium.

These experiments were conducted in primary cells isolated from tissue expressing normal levels of TRPV4. Previous studies have suggested that cell-ECM interactions may be important for proper signal transduction (14,42), but chondrocytes appear to exhibit similar signaling responses to osmotic stress whether isolated or *in situ* (43). Furthermore, isolated chondrocytes represent a heterogeneous population, as studies have shown that cells from the different zones of cartilage may respond to stimuli differently (14,44). Thus, further *in situ* studies will be important in developing a full understanding of the potentially site-specific role of TRPV4 in articular cartilage.

TRPV4 was localized to the cell membrane, and pharmacological activation of TRPV4 with $4\alpha\text{PDD}$ caused a significant Ca^{2+} influx, which is consistent with the reported role of TRPV4 as a Ca^{2+} permeable channel (45). Our data also show that stimulation of Ca^{2+} signals with $4\alpha\text{PDD}$ is blocked by both RR and by the TRPV4-selective compound GSK205 (Fig. 2). The effects of selective inhibition of TRPV4 using GSK205 suggest that it is TRPV4, and not other possible RR-targets, that is necessary for osmotically-mediated signal transduction and volume regulation in chondrocytes.

Specifically activating the TRPV4 channel with $4\alpha\text{PDD}$ in Ca^{2+} -free medium did not elicit Ca^{2+} signaling, implicating extracellular influx as an initiating signal in the Ca^{2+} response

(Fig. 3). Furthermore, a novel finding of this study was that thapsigargin, an inhibitor of Ca^{2+} -ATPases on the endoplasmic reticulum, strongly diminished Ca^{2+} signaling in response to $4\alpha\text{PDD}$. Taken together, these results suggest that the TRPV4 response is dependent upon both Ca^{2+} influx from the extracellular milieu and subsequent Ca^{2+} release from intracellular stores suggesting a form of “ Ca^{2+} -induced Ca^{2+} release”. Previous studies have shown that the chondrocytes' response to hypotonicity also occurs through this mechanism, consistent with the hypothesis that the effects of hypotonicity are mediated by TRPV4 (8,9).

Chondrocyte volume regulation in response to osmotic stress appears to be mediated by TRPV4, since specifically inhibiting TRPV4 with GSK205, to a lesser degree also with the less specific RR, prevented normal RVD in response to hypo-osmotic stress (Fig. 4). The mechanism is likely a block of TRPV4-mediated Ca^{2+} influx in response to hypotonicity (Fig. 3). According to previous results, the chondrocytic RVD response to hypotonic stress depends not only on Ca^{2+} , but also on actin remodeling which in turn is evoked by influx of extracellular Ca^{2+} (9,14,46). Therefore, it is plausible that Ca^{2+} influx mediated by TRPV4 may influence cytoskeletal reorganization following an osmotic stimulus (9). Furthermore, other studies have also posited a link between the cytoskeleton and TRPV4 (21,47).

Osteoarthritis is increasingly recognized as a human disease with a significant inflammatory component at the molecular level, involving the action of cytokines such as IL-1 (15). In the present study, hypotonic exposure in the presence of IL-1 was shown to result in a significant impairment of physiological RVD, consistent with previous studies (48). However, specific chemical activation of TRPV4, under the same conditions, was able to fully compensate for this deficit. This suggests that TRPV4-mediated Ca^{2+} influx is downstream of IL-1 receptor signaling and that IL-1 receptor mediated changes are capable of disabling osmotic TRPV4 activation. In chondrocytes, IL-1 has been shown to induce stabilization of cortical F-actin (14). Presumably, this increased stabilization of the actin cytoskeleton inhibits normal RVD, and thus the specific chemical activation of TRPV4 induces Ca^{2+} transients which might be necessary for F-actin remodeling, which in turn will counteract the F-actin stabilization evoked by IL-1 receptor signaling (9).

TRPV4 may also be involved in release of pathogenic autacoid mediators, such as prostaglandins, a final effector pathway leading to tissue damage and stimulation of nociceptor neurons' peripherals. Hypo-osmotic stress was found to increase PGE_2 release through a TRPV4-dependent mechanism, since it was blocked by GSK205. However, specific chemical activation of TRPV4 in isotonicity had no effect on PGE_2 production. Similar to our results, Le *et al.* have also shown little to no PGE_2 production in response to iso- or hyper-osmotic stimuli (49). The observed PGE_2 release in response to hypo-osmotic stress may be related to the RVD cascade because taurine efflux mediates RVD in response to hypotonicity (50), and taurine efflux is down-stream of cAMP, which is known to be produced in response to PGE_2 in chondrocytes (51). The mechanism of TRPV4 activation by hypo-osmotic stimulation is not fully understood but could possibly involve the activation of PLA_2 by cell swelling, which then releases arachidonic acid (AA) from membrane phospholipids; AA is converted by cytochrome p450 epoxygenase into EETs, which can function as endogenous activators of TRPV4 (45). AA can also be metabolized by COX enzymes to form PGE_2 (15). Thus, our data demonstrating an increase in PGE_2 release in response to hypo-osmotic stress in chondrocytes appear seemingly consistent with activation of an arachidonic acid pathway up-stream of TRPV4. However, specific inhibition of TRPV4 using GSK205 blocked PGE_2 production in response to hypo-osmotic medium, which apparently argues against critical involvement of the PLA_2 -P450-epoxygenase signaling pathway.

TRP channels have been implicated in the osmo-mechanosensory functions of cilia in a variety of settings (31-33). Chondrocytes have been shown to express primary cilia (52), and we confirmed this observation for porcine cells. We also demonstrated that TRPV4 was highly expressed in the periphery of the cell and did not localize specifically the cilium. Seemingly counterintuitive to these expression data, we found that an intact cilium was required for TRPV4-mediated Ca^{2+} signaling in response to 4 α PDD or hypo-osmotic stress. One explanation is that ciliary TRPV4 is in an active state, whereas its abundant expression in the remainder of the cell is not. Other sensory systems function optimally with only a few thousand channels for all the processes of one cell (53), which is consistent with the low level of expression of TRPV4 on the cilium. However, it also raises the question of the functional role for non-ciliary TRPV4. The primary cilium has been suggested to have a mechanical function in chondrocytes (52) as well as bone cells (54), our data suggest a role in osmotic signal transduction as an underlying mechanism.

In summary, we have demonstrated that functional TRPV4 channels are present in porcine articular cartilage and that they are involved in responding to hypo-osmotic stress and inflammation. Because of the unique structure of cartilage, mechanical loading is directly coupled to interstitial osmolarity. Thus, in acting as an osmosensor, TRPV4 may in fact be acting as a “mechanosensor” that regulates cellular response to changes in joint loading. In addition to physiological short-term changes in osmolarity with loading, the pathologic loss of proteoglycan content and increased hydration of cartilage with osteoarthritis may also result in chronic changes in the osmotic environment of the chondrocytes. Given the potential role of TRPV4 in sensing extracellular osmotic changes and in the inflammatory response of cartilage, further insight on the expression and function of this channel may provide new insights in the regulation of cartilage development, physiology, and disease.

Acknowledgments

Supported by GlaxoSmithKline, Inc., the NIH (AG15768, AI07217, AR48852, AR50245), and the Arthritis Foundation.

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1 Phan ↑

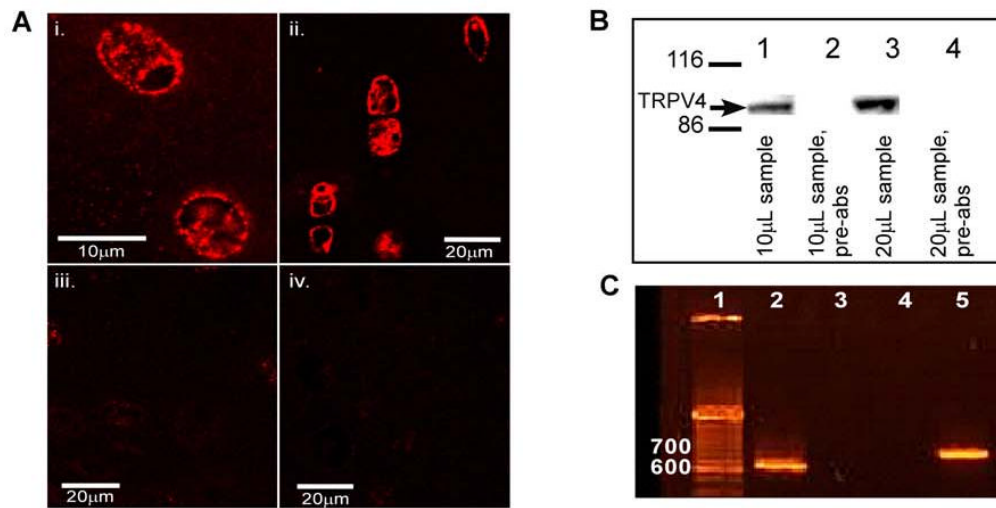


Figure 1.

A. Micrographs showing binding of the anti-TRPV4 antibody to chondrocytes in porcine tissue. Surface zone cells labeling positive for TRPV4 (i); deep zone cells showing positive labeling for TRPV4 primarily on the cell membrane (ii); pre-immune control shows negligible labeling (iii); negative control shows little labeling (iv). B. Western blot showing binding of the anti-TRPV4 antibody to protein extracts from porcine chondrocytes. Lanes 1 and 3 show 10 μl and 20 μl of protein extract respectively; lanes 2 and 4 show the TRPV4 peptide pre-absorbed antibody controls for lanes 1 and 3. All lanes were run on the same gel. C. Agarose gel electrophoresis of RT-PCR products from porcine articular cartilage showing amplification of TRPV4. Lane 1 contains a DNA ladder, lane 2 is product amplified with primers to TRPV4 Segment 1 (620 bp), lanes 3 and 4 are empty, and lane 5 is product amplified with primers to TRPV4 Segment 2 (740 bp).

2 Phan ↑

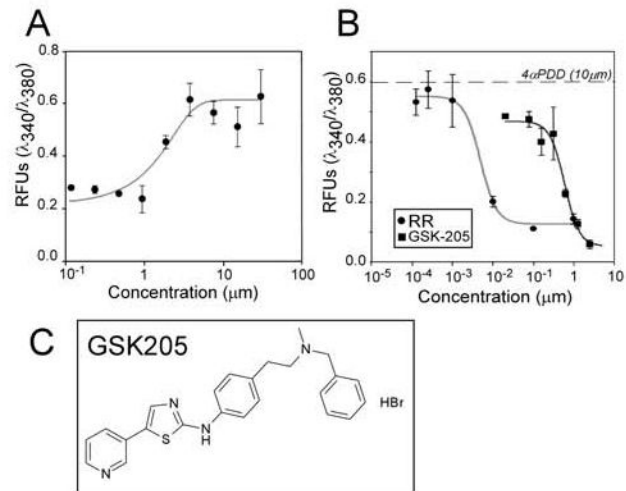
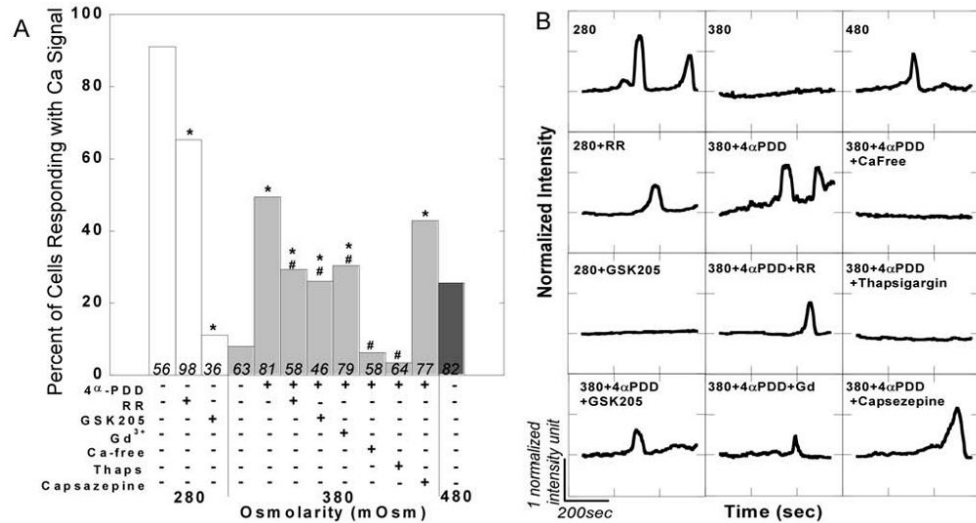


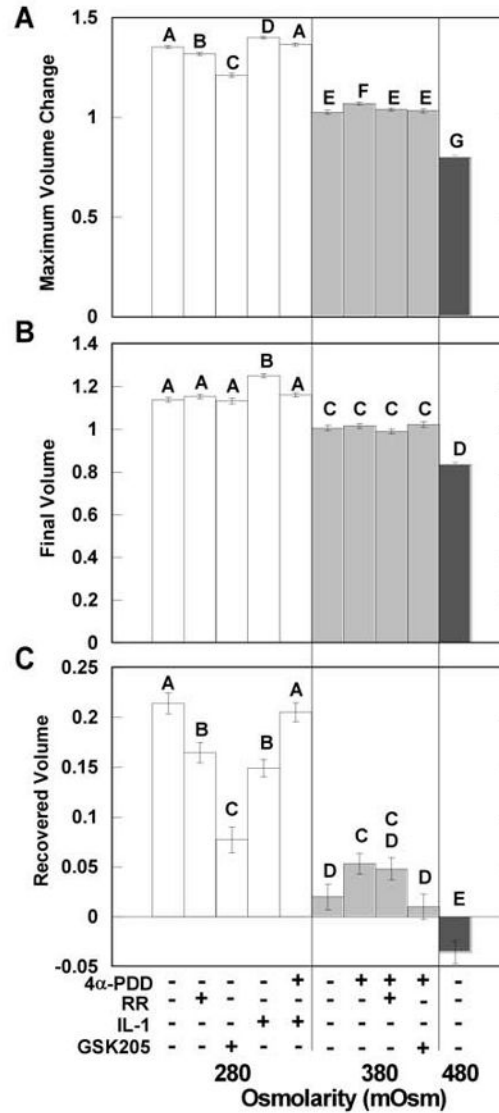
Figure 2. Ca^{2+} response of porcine articular chondrocytes to increasing concentrations of TRPV4 agonist, 4αPDD, (A) and antagonists, RR and GSK205 (B). Lines show best fit curve (Logistic 4-parameter). Data points represent the mean (\pm SD) from triplicate determinations in 2 independent experiments. Structure of GSK205 (C).

3 Phan ↑

**Figure 3.**

A. Percentage of cells responding to osmotic and chemical stimuli with increases in intracellular Ca^{2+} . Asterisks denote experimental groups that are significantly different from control within a given osmolarity (chi-squared test, $p < 0.05$); number signs specify bars within the 380 mOsm group that are significantly different from 380+4 α PDD (chi-squared test, $p < 0.05$). Both the 280 and 480 mOsm controls are significantly different from the 380 mOsm control (chi-squared, $p < 0.05$). Numbers in bars show n ; bars do not have error bars because the percent responding metric does not have an error associated with it. B. Representative Ca^{2+} traces for each condition showing normalized intensity versus time.

4 Phan ↑

**Figure 4.**

Mean (\pm sem) maximum volume change (swelling or shrinking) (A), final volume (average of the last 117 seconds of the experiment) (B), and recovered (maximum-final) (C) volume after stimulation with different osmotic and chemical stimuli. Bars with different letters are significantly different from one another (ANOVA, $p < 0.05$).

5 Phan ↑

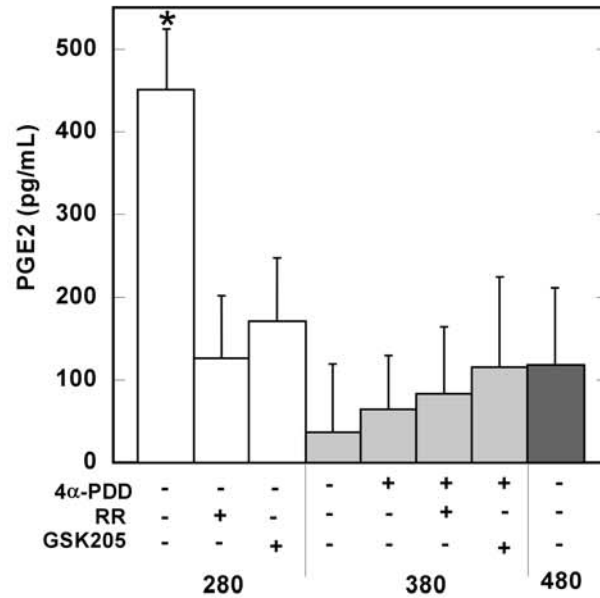


Figure 5. Mean (+sem) levels of PGE₂ in culture medium after treatment with different osmolarities and TRPV4 agonists/antagonists. Measurements at 16 and 30 minutes of exposure were combined as there was no significant effect of time. Bars with different letters are significantly different from one another (Repeated Measures ANOVA, $p > 0.05$).

6 Phan ↑

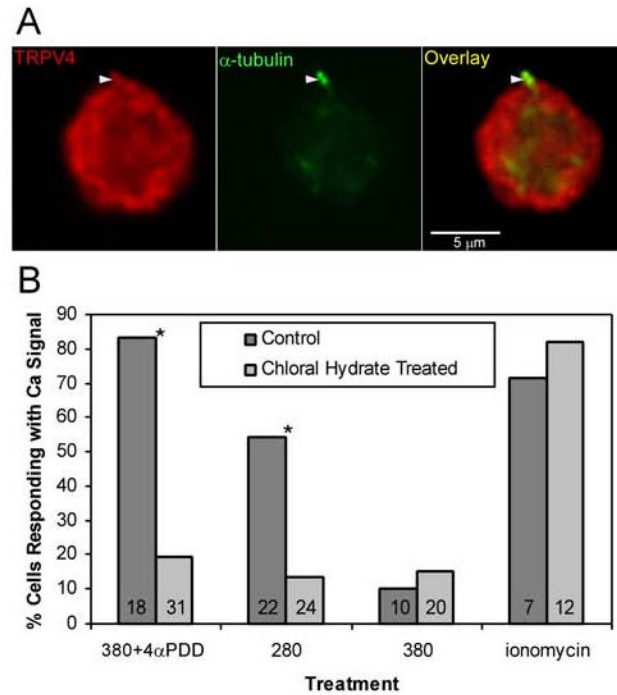


Figure 6.

A: Micrograph of porcine chondrocytes labeled for TRPV4 and α -tubulin (to show primary cilia). Arrowhead shows primary cilium with both tubulin and TRPV4 labeling. B: Percent of cells responding with a Ca^{2+} signal to 4 α PDD or hypo-osmotic stress significantly decreases when the cilia are disrupted with chloral hydrate treatment. Bars show means, numbers in bars show *n*, asterisks indicate significant difference between chloral hydrate treated and controls (chi-squared, $p < 0.05$).