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The role of BK_{Ca} channels on hyperpolarization mediated by hyperosmolarity in human articular chondrocytes

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Abstract. Chondrocytes, the only cell in cartilage, are subjected to hyperosmotic challenges continuously since extracellular osmolarity in articular cartilage increases in response to mechanical loads during joint movement. Hyperosmolarity can affect membrane transport, and it is possible that load modulates matrix synthesis through alterations in intracellular composition. In the present study, the effects of hyperosmotic challenges were evaluated using the whole-cell patch clamp technique, whole cell mode on freshly isolated human and bovine articular chondrocytes. In human chondrocytes, hypertonicity induced the activation of outward Ca^{2+}-sensitive K^+ currents, which were inhibited by iberiotoxin and TEA-Cl. The current induced by hypertonic switching (osmolarity from 300 to 400 mOsm/l) caused cell hyperpolarization (from –39 mV to –70 mV) with a reversal potential of –96 ± 7 mV. These results suggest a role for Ca^{2+}-activated K^+ channels in human articular chondrocytes, leading to hyperpolarization as a consequence of K^+ efflux through these channels. These channels could have a role in the articular chondrocyte's response to a hyperosmotic challenge and matrix metabolism regulation by load.

Key words: Chondrocyte — Electrophysiology — Calcium-activated potassium channels — Hyperosmolarity

Introduction

During joint movement, articular chondrocytes are subjected to fluctuations in extracellular osmolarity, which arise from the fluid flux secondary to changes in mechanical load (Urban 1994; Wilkins et al. 2000a,b). These osmotic changes can affect turnover of the extracellular matrix since chondrocytes are solely responsible for matrix synthesis and breakdown (Muir 1995). Furthermore, changes in the intracellular composition in response to these osmotic challenges have been shown to affect metabolism of the extracellular matrix (Gray et al. 1988; Behrens et al. 1989; Urban 1994; Wilkins et al. 2000b; Hopewell and Urban 2003), which also may be regulated for ion channel function (Mouw et al. 2007).

Hyperosmolarity occurs when mechanical load increases because of the expression of fluid from the matrix, which concentrates extracellular solutes, mainly cations, which are attracted by the fixed negative charges in matrix proteoglycans (Quinn et al. 1998; Sun et al. 2004). Articular chondrocytes respond to hypertonic shock with a rise in intracellular Ca^{2+} concentration, due to increase of Ca^{2+} influx through the Na^+-Ca^{2+} exchanger (NCX) and annexins (Sanchez and Wilkins 2004). Hypertonicity also causes hyperpolarization of articular chondrocytes (Sanchez and Wilkins 2004) and can modulate the function of the Na^+-H^+ exchanger (NHE), producing intracellular alkalinization (Yamazaki et al. 2000). However, to date, the electrophysiological effects of hyperosmolarity in articular chondrocytes have not been evaluated in spite of their relevance because chondrocytes from osteoarthritic cartilage exhibit altered electrophysiological responses to load in comparison to chondrocytes from healthy cartilage (Millward-Sadler et al. 2000), and changes in membrane potential can decrease cell proliferation and matrix production (Wohlrab et al. 2001; Mouw et al. 2007).

In the present study, we have characterized the effects of hyperosmolarity on whole-cell currents and membrane potential in human articular chondrocytes and present

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evidence for the operation of a big conductance Ca^{2+}-activated K^+ channel (BK_{Ca}), which is responsible for the hyperpolarization secondary to hyperosmolarity we observed.

Materials and Methods

Isolation of chondrocytes

Cells were isolated from human articular cartilage using a method described in detail by Browning et al. (1999) for bovine chondrocytes. Briefly, cells were isolated by type I collagenase digestion (2000 U/ml in DMEM for 18 h at 37°C) from healthy knee or hip cartilage obtained from patients undergoing orthopedic surgery, who signed an informed consent. After filtration of the isolation solution, cells were resuspended in fresh Dulbecco's modified Eagle's medium (DMEM) prior to resuspension in experimental media. The study was conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and was approved by the Committee of Bioethics of Universidad Tecnológica de Pereira.

Media and chemicals

All chemicals and solutions were obtained from Sigma-Aldrich (St Louis, MO, USA), unless otherwise stated. Cartilage slices and isolated chondrocytes were incubated in DMEM, supplemented with glutamine (2 mmol/l) and antibiotics (penicillin 100 μM and streptomycin 100 μM).

In patch clamp experiments, the standard external solution contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 15 HEPES, and 10 glucose, with pH adjusted to 7.4 with 1 M NaOH. The standard pipette solution contained (in mM): 110 KCl, 20 CsCl, 20 NaCl, 4 $CaCl_2$, 4 $MgCl_2$, 10 HEPES, 5 glucose, and 20 BAPTA, with pH adjusted to 7.1 with 1 M NaOH. Osmolarity of these solutions was adjusted to 300 mOsm/l with aliquots of a 5 M mannitol solution. An estimate of free Ca^{2+} concentration was calculated using 'Maxchelator' software (Dr. C. Patton, John Hopkins University) and was 102 nM in the intrapipette solution. In K^+-free solutions, KCl was replaced by CsCl in equimolar concentrations. EGTA 1 M was added to the Ca^{2+}-free solution to chelate contaminant traces of this ion.

Solutions were made hyperosmotic by the addition of aliquots of mannitol (5 M, in distilled water) until the osmolarity required was reached. Osmolarities of all solutions were measured by an Advanced Model 3320 Micro-Osmometer (Advanced Instruments, Norwood, MA, USA). Solutions were changed using the cFlow V2.x flow controller (Cell Microcontrols, Norfolk, VA, USA).

Electrophysiological recording

Standard whole-cell patch clamp techniques (Hamill et al. 1981) were used to record membrane currents (voltage clamp) and membrane potential (current clamp) in human articular chondrocytes. Borosilicate patch pipettes (Clark PG150T glass, from Harvard Apparatus Ltd, Edenbridge, Kent, UK) were pulled and polished (P-97, Sutter Instrument, Novato, CA, USA) to resistances of 4–10 MΩ. After a seal of resistance greater than 5 GΩ was obtained, recordings of membrane currents were made using an Axopatch 200B amplifier with a CV203BU headstage (Axon Instruments Inc. Union City, CA, USA). Voltage clamp signals were generated by a Digidata 1440A interface (Axon Instruments Inc.), applying a ramp or step protocol according to the experiment. Membrane currents were filtered at 2 kHz and digitized at a sampling rate of 10 kHz. Acquisition and analysis of the signal was made using pCLAMP 10.0 (Axon Instruments Inc.). At the beginning of each experiment, the junction potential between the pipette solution and bath solution was electronically adjusted to zero. Macroscopic current values were normalized as pA/pF. All experiments were performed at room temperature (20°C).

Statistics

Results are shown as means ± standard errors of the mean (SEM), where n is the number of cells tested. Each experimental observation was repeated in at least ten cells from at least five different samples. Significant differences were determined by Student's unpaired t-tests.

Results

Using a whole-cell mode patch clamp technique, a descending ramp protocol was applied to human chondrocytes from healthy cartilage, stepping membrane potential to –100 mV for 100 ms and then ramped to +100 mV over 2 s, from a holding potential of –40 mV. This protocol was applied to cells suspended in solutions with osmolarity of 300 mOsm/l (control) or of 450 mOsm/l, current-voltage relations were obtained and the current activated by hyperosmolarity was determined by subtraction (Figure 1). In contrast to control conditions, for cells bathed in the hyperosmotic extracellular solution a predominantly outward current was apparent at the voltages tested. This current exhibited a reversal potential E_{rev} of –89 ± 5 mV (n = 15), which suggest that K^+ efflux is responsible for this current.

To evaluate the osmotic sensitivity of the current recorded in hyperosmotic conditions, the current at –40 mV in cells resuspended in solutions with a range of osmolarities from 300 to 500 mOsm/l was recorded (Figure 2). As extracel-

lular osmolarity was increased, outward current increased in a linear fashion. This effect was fully reversible when the hyperosmolar solution was replaced by an iso-osmolar solution.

In order to explore which channel could be responsible for these K^+ currents, Figure 3 (A, B) shows the effect of TEA-Cl (5 mM) and iberiotoxin (200 nM) on the current induced by hypertonic shock. The current was abolished in the presence of either inhibitor at –40 mV. Gd^{3+} (at 10 and 100 μM), an inhibitor of stretch-activated channels (Hamill and McBride 1996), another candidate to be involved, had no effect on these currents (data not shown).

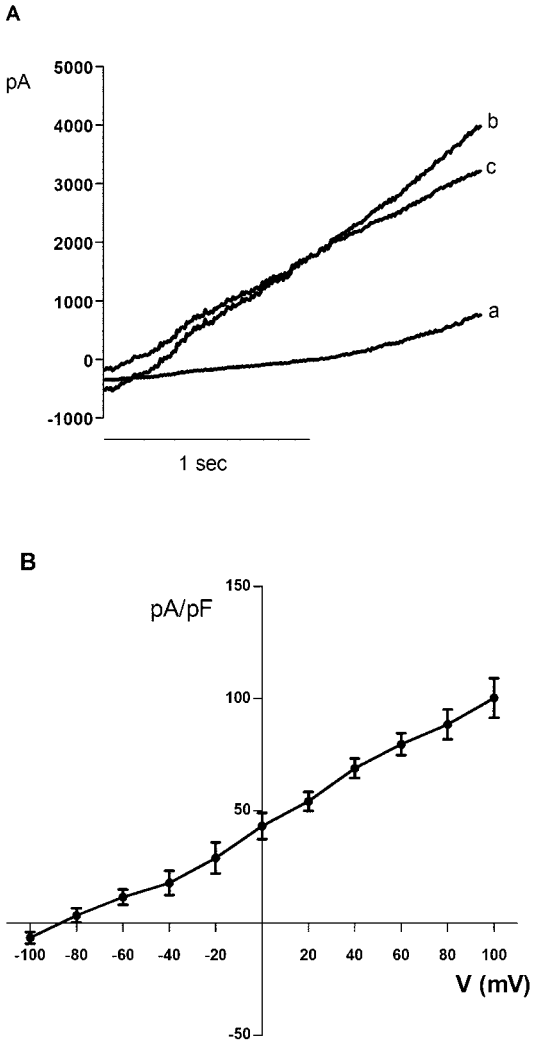

Figure 1. A. Typical current traces from human articular chondrocyte in isotonic solution (300 mOsm/l, control) (a), following switching to a hypertonic solution (from 300 to 450 mOsm/l) (b) and the current obtained subtracting b from a (c). Currents were obtained by stepping membrane potential to –100 mV for 100 ms and then ramped to +100 mV over 2 s, from a holding potential of –40 mV. **B.** I-V relationship of the current obtained by subtraction from human articular chondrocytes (n = 15).

Given the sensitivity of the current to TEA-Cl and iberiotoxin and the recording of a negative reversal potential close to the Nernst potential for K^+, experiments in which Cs^+ replaced this ion in the intrapipette solution were performed in order to evaluate the dependence of the current on intracellular K^+ ions. At –40 mV, no significant current was recorded following hypertonic shock in these conditions. Substitution of K^+ with Cs^+ in the extracellular solution does not affect the current significantly (Figure 3C and D).

In order to characterize the K^+ current activated by hyperosmolarity, the sensitivity of this current to intracellular Ca^{2+} was evaluated. In experiments in which intrapipette solution was nominally Ca^{2+}-free and had a high concentration of BAPTA (20 mM), the current induced by hyperosmolarity was almost completely inhibited (Figure 3E and F). Attenuation of the current was also observed when a Ca^{2+}-free extracellular solution was employed. However, when these experiments were repeated in the absence of Ca^{2+} ions in both, the intrapipette and extracellular solution, the current was abolished.

To establish whether Ca^{2+} release from intracellular stores had a role in the activation of the K^+ current following a hyperosmotic challenge, experiments were performed using cells preincubated in the presence or absence of thapsigargin (1 μM, 30 min preincubation in Ca^{2+}-free HEPES-buffered saline solution (HBS)) in Ca^{2+}-free intrapipette and extracellular solutions (Figure 3E and F). In these experiments, BAPTA was not added to intrapipette solutions in order to allow for significant increases in $[Ca^{2+}]_i$. At –40 mV, in thapsigargin-pretreated cells the current following hypertonic shock was significantly attenuated.

To assess the effects of this current on membrane potential, experiments were performed in current clamp mode to record membrane potential before and following hypertonic shock of different magnitudes (Figure 4). Chondrocyte membrane potential in control conditions was –39 ± 3 mV (n = 14). The membrane potential hyperpolarized rapidly after hypertonic shock, and the magnitude of this hyperpolarization depended on the magnitude of the increase in extracellular osmolarity. After restoring control osmolarity, the membrane potential returned to its basal value (–38 ± 4 mV (n = 8), no significant difference with the initial value). TEA-Cl and iberiotoxin inhibited this effect of hypertonic shock on membrane potential.

Discussion

The experiments described here were undertaken to characterize the effects of hyperosmotic challenges on whole-

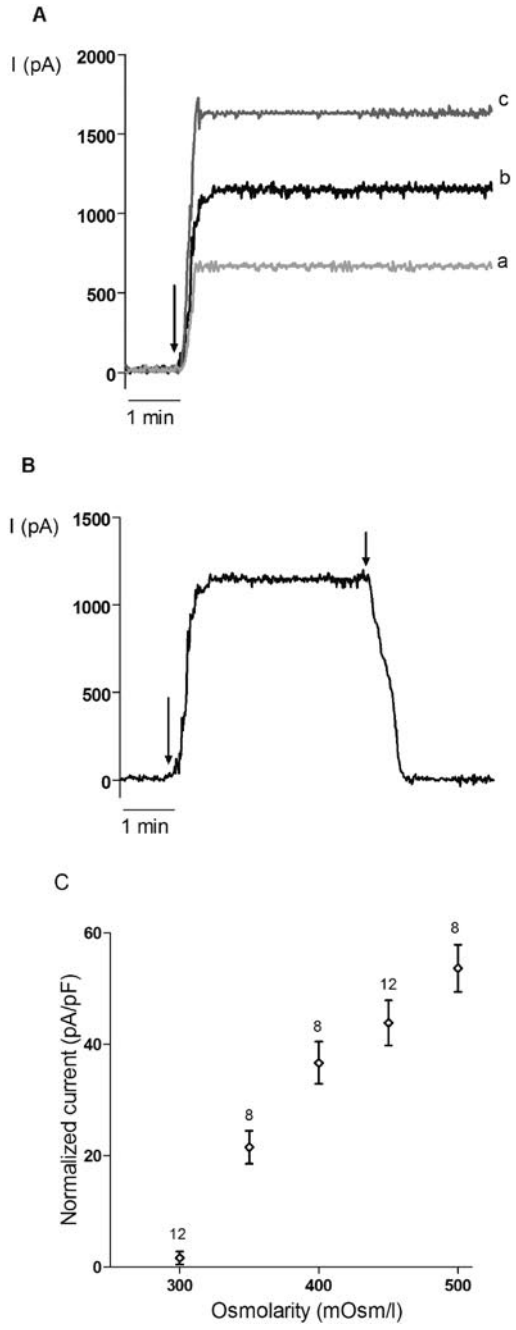

Figure 2. A. Typical current recordings at –40 mV in bath solutions at 300 mOsm/l control osmolarity and following switching to hypertonic solutions 375 mOsm/l (a), 450 mOsm/l (b) and 500 mOsm/l (c) from human articular chondrocytes. The arrow indicates the point at which solution switching was initiated. **B.** Reversibility of the inward current at –40 mV, elicited by a hypertonic bath solution (from 300 mOsm/l to 450 mOsm/l). The large arrow indicates the point at which the solution switching was produced, and the small arrow indicates the point at which osmolarity was restored to 300 mOsm/l. In the traces shown in A and B, the baseline was adjusted to zero to subtract the resting current. **C.** Relation between extracellular osmolarity and maximal current at –40 mV; *n* values are indicated in each case.

cell currents and membrane potential in human articular chondrocytes. In the results presented in a previous paper, fluorescence measurements showed that hyperosmotic shock can elicit an increase in $[Ca^{2+}]_i$ and hyperpolarization in bovine chondrocytes (Sanchez and Wilkins 2004); the present results using whole-cell patch clamp techniques support those findings and provide evidence for a role of Ca^{2+}-activated K^+ (K_{Ca}) channels and Ca^{2+} release from intracellular stores in the hyperpolarization induced by hyperosmolarity in human chondrocytes.

Hyperosmolarity elicited a current with a reversal potential near to that for K^+ in the experimental conditions employed. The results obtained suggest that this current was dependent on K^+ efflux through K_{Ca} channels since it could be inhibited when K^+-free intrapipette solutions were employed and was dependent on the presence of both intracellular and extracellular Ca^{2+} ions. Moreover, this current was not affected by Gd^{3+}, a typical inhibitor of stretch-activated channels, which are the other candidates to be responsible for these currents. Although solute efflux is not expected in a hyperosmotic challenge, it is possible that chondrocytes activate this mechanism in order to modulate matrix metabolism by hyperpolarization.

The K_{Ca} grouping represent a large family of channels highly selective for K^+ and the gating of which is dependent on intracellular Ca^{2+}, with their open P_o being rapidly increased by rises in $[Ca^{2+}]_i$, even in the submicromolar range (Vergara et al. 1998; Sah and Davies 2000). The activation of these channels causes rapid membrane hyperpolarization, and they have been implicated in a wide variety of physiological processes including neurosecretion, maintenance of muscle tone, action potential modulation and neuronal firing frequency adaptation (Sah 1996). Although they have been studied principally in neurons and in muscle cells, they also have been found to operate in a wide range of different non-excitable cell types. K_{Ca} channels are grouped in three subfamilies, which exhibit different single-channel conductances and pharmacological profiles: small conductance (SK_{Ca}), intermediate conductance (IK_{Ca}) and large conductance (BK_{Ca}) K_{Ca} channels (Vergara et al. 1998; Sah and Davies 2000).

BK_{Ca} are perhaps the best-studied subtype of K_{Ca} channels. Their single-channel conductance is between 100–300 pS, and they are characteristically voltage-sensitive; they can be blocked by TEA^+ at low concentrations (1 mM) and by charybdotoxin and iberiotoxin, with the latter highly specific for these kinds of channels (Haylett and Jenkinson 1990). There is evidence for the operation of BK_{Ca} channels in a number of non-excitable cells including breast cancer cells (Ouadid-Ahidouch et al. 2004), neutrophils, eosinophils (Ahluwalia et al. 2004), alveolar epithelial cells (Jovanovic et al. 2003), juxtaglomerular cells (Friis et al. 2003), renal proximal tubule epithelial cells (Tauc et al. 1993), endothe-

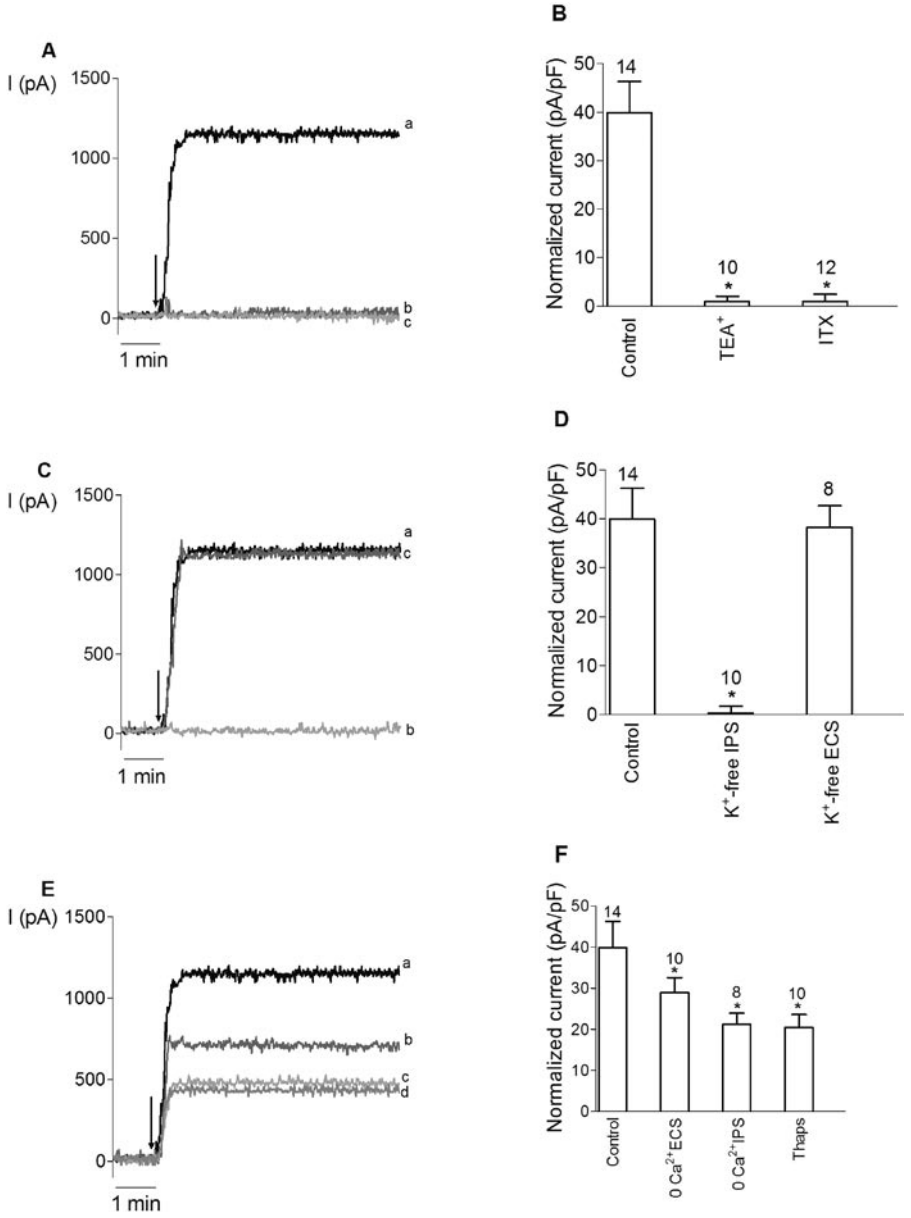

Figure 3. A. Typical current recordings at –40 mV in solutions at 300 mOsm/l control osmolarity and following switching to a 450 mOsm/l hypertonic solution without drugs (a) and in the presence of tetraethylammonium (TEA^+, 5 mM) (b) and iberiotoxin (ITX, 200 nM) (c) from human articular chondrocytes. The arrow indicates the point at which bath solutions were switched. The baseline was adjusted to zero to subtract the resting current. **B.** Mean maximal normalized currents from the same conditions in human articular chondrocytes obtained after subtraction of resting current from maximal current following solution switching. *n* values are indicated in each case above each bar. * denotes significant difference from control, $p < 0.05$. **C.** Typical current recordings at –40 mV in solutions at 300 mOsm/l control osmolarity and following switching to a 450 mOsm/l hypertonic solution in control (a), with K^+-free intrapipette solution (b) and with K^+-free extracellular solution (c) human articular chondrocytes. The arrow indicates the point at which bath solutions were switched. The baseline was adjusted to zero to subtract the resting current. **D.** Mean steady maximal currents from the same conditions obtained after subtraction of resting current from maximal current following solution switching; *n* values are indicated in each case above each bar. * denotes significant difference from control, $p < 0.05$. **E.** Typical current recordings at –40 mV in solutions at 300 mOsm/l control osmolarity and following switching to a 450 mOsm/l hypertonic solution in control (a), high BAPTA (20 mM), Ca^{2+}-free intrapipette solution (b), Ca^{2+}-free extracellular solution (c) and in the thapsigargin-treated cells (d). The arrow indicates the point at which bath solutions were switched. The baseline was adjusted to zero to subtract the resting current. **F.** Mean steady maximal currents from the same conditions obtained after subtraction of resting current from maximal current following solution switching; *n* values are indicated in each case. * denotes significant difference from control, $p < 0.05$.

lium (Kestler et al. 1998; Brakemeier et al. 2003) and growth plate chondrocytes (Grandolfo et al. 1992).

Given that in the experiments described here, iberiotoxin attenuated the current to the same degree as the use of K^+-free intrapipette solutions, BK_{Ca} channels are most likely to be the class responsible for the current recorded. Stretch sensitivity of this type of channel has been reported in cardiac myocytes (Iribe et al. 2010).

This finding is not surprising because BK_{Ca} is probably the most widely expressed subtype of K_{Ca}; Grandolfo et al. (1992) and Long and Walsh (1994) have provided evidence for the operation of these channels in growth plate chondrocytes. In agreement with our findings, recently Mobasheri et al. (2010) reported evidence for the osmotic sensitivity of a BK_{Ca} channel in equine articular chondrocytes, although they found that these channels where activated by hypoosmolarity and were sensitive to higher doses of TEA and IBX which could reflect a functional difference in equine chondrocytes. It is worthy to note that they did not explore the effect of hyperosmolarity and the method used to evoke those currents was mechanical stretch on the cells induced by negative pressure. Further experiments to evaluate the single-channel activity of BK_{Ca} need to be performed in order to determine the biophysical properties and single-channel conductance of the channels operating here and to clarify the functional differences of these currents in different species.

BK_{Ca} channels are activated by an increase in $[Ca^{2+}]_i$, and the results presented here suggest that Ca^{2+} influx is responsible for the increase in $[Ca^{2+}]_i$, which activates BK_{Ca} currents following exposure to hyperosmotic conditions; in a previous paper we demonstrated that a hypertonic shock produces an increase in $[Ca^{2+}]_i$ following the activation of reverse mode NCX and annexins (Sanchez and Wilkins 2004), which may also be the pathways involved here, although further research about this topic is necessary. However, a role for release from intracellular stores is also suggested because currents activated by hyperosmolarity can be attenuated by thapsigargin treatment.

Following hyperosmotic shock, hyperpolarization of the membrane potential was observed, and the magnitude of this hyperpolarization reflected the rise in external osmolarity. This effect was reversible after restoration to the control osmolarity; it was almost completely inhibited by TEA^+ and iberiotoxin, which suggests that K^+ efflux through BK_{Ca} is responsible for this effect. Hyperpolarization in response to hyperosmolarity has been reported in atrial myocytes (Beyer et al. 1986) and bovine articular chondrocytes (Sanchez and Wilkins 2004) and hyperosmolarity as a consequence of BK_{Ca} activation has been reported in a variety of cells such as neurons and endothelial cells (Song et al. 2010; Vang et al. 2010) and also in equine articular chondrocytes (Mobasheri et al. 2010). Although in this last study the BK_{Ca}-induced hyperpolarization was smaller, the recordings were

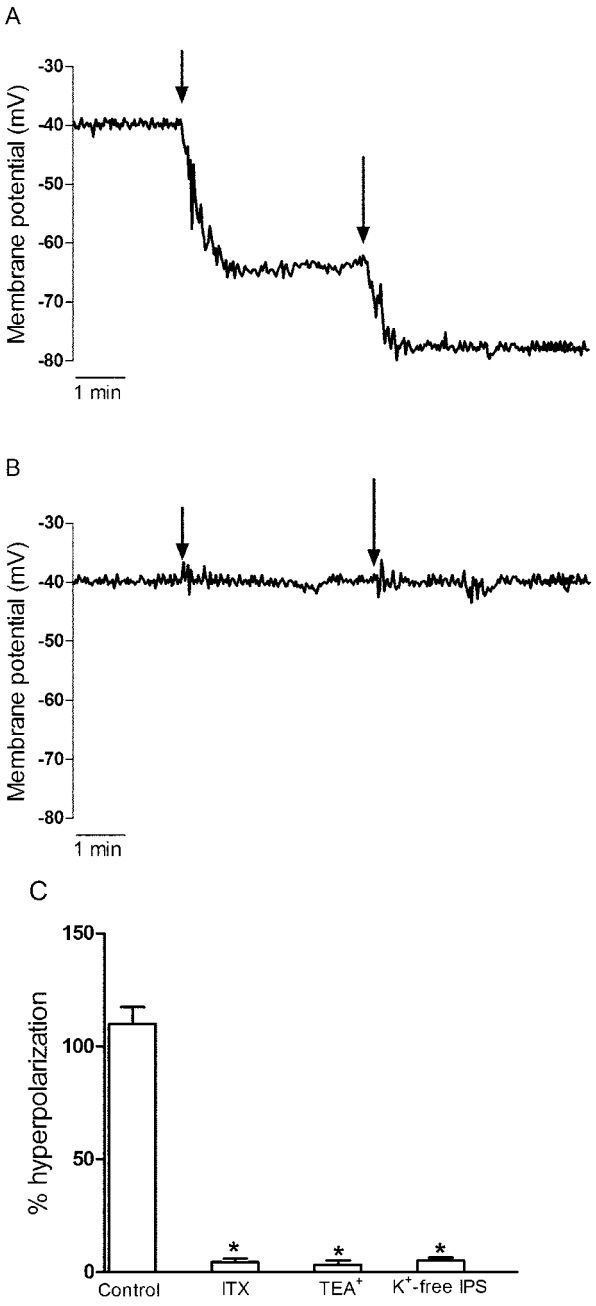

Figure 4. Effect of hypertonicity on membrane potential. **A.** Typical membrane potential traces obtained at different extracellular osmolarities as indicated by the arrows. The small arrow indicates switching from 300 to 375 mOsm/l and the large arrow indicates switching from 375 to 450 mOsm/l. **B.** Membrane potential recorded in a 300 mOsm/l solution followed by switching to a 450 mOsm/l solution, indicated by the small arrow in the presence of iberiotoxin (200 nM). The large arrow indicates the point at which extracellular osmolarity was restored to 300 mOsm/l. **C.** Mean steady percentage of hyperpolarization induced by switching osmolarity from 300 to 450 mOsm/l in control conditions, under iberiotoxin (ITX, 200 nM) and tetraethylammonium (TEA^+, 5 mM) treatment and recorded in K^+-free intrapipette solution. * denotes significant difference from control, $p < 0.05$.

performed on equine chondrocytes and the currents were evoked by applying positive pressure on cells, which could explain the discrepancy.

In summary, the results presented here provide evidence that hyperosmotic challenges can activate BK_{Ca} channels, and as a consequence, efflux of K^+ occurs, causing membrane hyperpolarization in human articular chondrocytes. This mechanism may be important in the chondrocyte's response to hyperosmotic challenges and could be one of the pathways through which hypertonicity-induced Ca^{2+} increase affects matrix turnover since hyperpolarization may affect a number of metabolic pathways.

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