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Original Paper

The Role of Swelling-Activated Chloride Currents ($I_{CL,swell}$) in the Regulatory Volume Decrease Response of Freshly Dissociated Rat Articular Chondrocytes

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Key Words
Articular chondrocytes • Hyposmotic challenge • Regulatory volume decrease • Swelling activated chloride currents • Patch clamp

Abstract
Background/Aims: Articular chondrocytes dwell in an environment that is continuously changing its osmolarity as a consequence of mechanical loading, yet their volume regulation capabilities (RVD) are not fully understood. This work aimed to determine the osmotic sensitivity of freshly isolated rat chondrocytes, their RVD capabilities and to study the properties of any anion currents associated. ***Methods:*** Cell volume responses were determined by microscopy. Whole cell patch clamp was used to record ion currents. ***Results:*** Chondrocytes showed to be osmotically sensitive and capable of RVD in a size-dependent manner. RVD was accompanied by activation of outwardly rectifying chloride currents, featuring time and voltage independent activation and inactivation at most depolarizing voltage levels, with an anion selectivity sequence of: $SCN^- > I^- \cong NO_3^- > Br > Cl^- > F^- >>$ Gluconate$^-$ > Methanesulphonate$^-$, corresponding to Eisenman's sequence I. They were also permeable to taurine. These currents were blocked by DIDS, SITS, 9-AC and NPPB and by drugs that block $I_{Cl,swell}$ such as fluoxetine, phloretin, DCPIB and tamoxifen. RT-PCR assays show the presence of mRNA for CLC-3 and TMEM16A, that had been proposed as molecular determinants of $I_{Cl,swell}$ currents. ***Conclusions:*** These findings indicate that freshly isolated rat articular chondrocytes have $I_{Cl,swell}$. The further finding that $I_{Cl,swell}$ blockers alter the RVD response suggest that $I_{Cl,swell}$ is involved in the RVD response of rat articular chondrocytes.

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Introduction

Articular chondrocytes, the only cells found in synovial joints are mainly responsible for the production and maintenance of articular cartilage [1, 2]. In response to mechanical loads, they regulate the synthesis and degradation of extracellular matrix components such as proteoglycans and collagen, which are fundamental for the integrity and properties of this tissue [3]. Chondrocytes survive in a rather unusual environment: Mechanical loads on cartilage, both static and dynamic, produce changes in matrix hydration, which provoke variations on the osmolarity and ionic composition of the extracellular media. Mechanical loading influences the synthesis of new matrix components by articular chondrocytes [4, 5]. So far, the molecular components involved on the mechanical sensitivity of chondrocytes have not been clearly described, although there are some studies suggesting that ion channels may likely be playing a role [6, 7]. It has been shown that chondrocytes possess a wide diversity of ion channels [8], including voltage-gated Na^+ channels [9], voltage-gated K^+ channels [10-14], calcium-dependent K^+ channels [15], voltage-gated calcium channels [16], TRPV channels [17], aquaporins [18] and voltage-gated Cl^- channels [19].

Because of their permeability to water, most cells behave as osmometers, modifying their volume in response to osmolarity variations, either swelling after hyposmotic shocks or shrinking after hyperosmotic challenges. It has been found that many cell types have the ability to resume their original volume by mechanisms that involve the transport of solutes (osmolytes) either organic or inorganic, across the plasma membrane. In order to recover from a hyperosmotic challenge, osmolytes are taken in from the external media, a process known as regulatory volume increase (RVI), whereas osmolytes are extruded to recover from a hyposmotic challenge, a process named regulatory volume decrease (RVD). The volume regulatory properties of a given cell type depends on the presence of specific membrane pathways of permeability that become activated in response to volume changes [20-24].

It has been observed that chondrocytes, both isolated and "in situ", are sensitive to osmotic challenges [25, 26]. They also can regulate their cell volume after variations on external osmolarity, being capable of both RVI and RVD responses [27-29], although the permeability pathways involved have not been fully described yet. The RVD response involves the efflux of osmolytes, either organic or inorganic. Several membrane transport pathways have been found to be involved in the RVD response of chondrocytes, including (1) the loss of taurine (2-amino-ethanesulphonic acid), a non-essential sulphonic amino acid, through a swelling-activated transport pathway [30, 31]; (2) The efflux of potassium, which may be through a stretch activated potassium channel or through a large-conductance voltage-dependent potassium channel [32, 33]; (3) The efflux of cations through a gadollinum-sensitive conductance dependent of intracellular calcium [34]. An efflux of anions is expected to occur along with the efflux of cations in order to preserve electrical neutrality and swelling activated chloride currents ($I_{Cl,Swell}$) have been described in a wide variety of cell types in association with the RVD response [35-37]. However little is known on the presence and characteristics of such kind of currents in articular chondrocytes [38].

In this work we aimed: (1) To describe the regulatory volume decrease (RVD) response from freshly isolated rat articular chondrocytes; (2) To find out whether these cells express anion currents in response to hyposmotic challenges and if so, to determine their selectivity, as well as their kinetic and pharmacological properties and (3) To establish whether such currents participate in the RVD response of chondrocytes.

Materials and Methods

Cell culture

Articular chondrocytes were obtained from rat articular cartilage as previously described [10], briefly: Juvenile Wistar male rats, weighting 160-180 grams, were deeply anesthetized by intramuscular injection of pentobarbital and the hip joints dissected, minced and incubated with 0.2% type II collagenase in PBS

for 2-3 hrs at 37°C. After digestion, cells were collected by mild centrifugation and re-suspended in DMEM supplemented with 3% calf serum. Cells were further dissociated mechanically with a tip polished Pasteur pipette. Chondrocytes were plated at 1-3 x10^5 cells/ml on glass coverlips and kept under sterile conditions at 37°C in a humidified atmosphere with 5% CO_2 until use.

Whole cell membrane current recordings

Patch pipettes were made from borosilicate glass capillary tubing (34500, KIMAX), pulled with a horizontal Brown-Flaming type puller (model p87, Sutter Instruments, Novato, CA, USA). Pipettes with a tip electrical resistance of 2-6 MΩ were selected. Ion currents were recorded using a Dagan 8900 amplifier (Dagan Instruments) coupled to a Digidata 1200 A/D-D/A converter (Axon Instruments). Current signals were filtered at 1 or 10 KHz using a four-pole Bessel filter. Voltage stimulation protocols and data acquisition were computer-controlled through dedicated software (pClamp suite version 8.0, Molecular Devices, Sunnyvale, CA). In some experiments the protocol of stimulation consisted of square voltage pulses of -60 to +100 mV with steps of +20 mV, from a holding potential of -40 mV. In other instances voltage ramps from -80 to +80 mV (dV/dt=200 mV/sec) were used. To study the time course of the activation of ion currents, cells were stimulated with repetitive alternating step voltage pulses (every 8 seconds), from -80 and +100 mV from a holding potential of -40 mV. Continuous recording of data was made, after digitizing the signal with a Digidata1200 (Molecular Devices) , on a desk computer with the program Axoscope (from pClamp suite) whereas a second computer-Digidata 1200 set was used to deliver the stimulation protocol.

Capacitance measurements

After seal rupture, a capacitive transient current was elicited by a hyperpolarizing step voltage from -80 to -90 mV and recorded at 10 KHz. Membrane capacitance was estimated offline, integrating the area of the capacitive transient, starting at the onset of the pulse. Cell membrane capacitance (Cm) was calculated dividing the integrate of the capacitive current by the amplitude of the voltaje pulse (−10 mV). The membrane surface area was estimated, assuming a specific membrane capacitance of 0.01 pF/μm^2 . The Clampfit module of the pClamp software suite was used to obtain the integrate of the capacitive transient current.

Measurement of cell volume

Glass coverslips containing recently isolated chondrocytes were gently deposited on a recording chamber which was placed on the stage of an inverted microscope (Axiovert 200M, Carl Zeiss). Cells having a spherical shape were randomly selected and images were taken at regular intervals to follow the timecourse of volume changes after hyposmotic challenges. Posteriorly the diameter of individual cells was measured from images using an appropriate software (Axiovision 4.8, Carl Zeiss) and their volume calculated using the formula for a sphere ($V = \frac{4}{3}\pi r^3$).

Reverse transcription polymerase chain reaction (RT-PCR) analysis of TMEM16A and ClC-3

Tissue samples were obtained from juvenile male Wistar rats. Rat brain was used as positive control. Rat hip condyles were collected and kept under frozen liquid nitrogen. After grinding, total RNA was isolated from either tissue, using the Trizol reagent (Invitrogen) and quantified by UV spetroscopy. cDNA was produced by reverse transcription using Superscript III and oligo $(dT)_{12-18}$ as a primer (Invitrogen). PCR assays were carried out using a PCR system 2400 (Perkin Elmer). Primers were designed using the Primer-BAST public software (NCBI, USA). Primers for ClC-3, obtained from Rattus norvegicus Clcn3 mRNA sequence (ID:NM 053363.2) were: Forward (TGTCGCCGACGCAAGTCCAC); Reverse (GGCGAGCTGCTCCACAGCAA) to give a product of 405 bp. Those for TMEM16A, from Rattus norvegicus anoctamin-1 mRNA (ID:NM 001107564.1) were: Forward (CCGCAGCGTCCACATCGTGA) and reverse (CTGCGGGTCCCCAGGACCAT) to give a product of 240 bp. B-actin was used as a PCR control and the sequences of primers (from sequence ID: NM_031144.2) were: Forward (AAGATGACCCAGATCATGTT) and reverse (GAGTACTTGCGCTCAGGAGG), to give a product of 662 pb.

Solutions and chemicals

The composition of solutions is described in Table 1. The composition of the pipette solution for whole cell recordings was (mM): CsOH 100, HCl 100, TEACl 20, $MgCl_2$ 2, Na_2ATP 5, EGTA 1, Na_3GTP 1, Hepes

Cell Volume Measurements								
	NaCl	KCl	$CaCl_2$	$MgCl_2$	glucose	Hepes	Mannitol	Osmolarity
Isosmotic	73.3	5	1.8	1	5	10	150	330
Hyposmotic-280	73.3	5	1.8	1	5	10	100	280
Hyposmotic-230	73.3	5	1.8	1	5	10	50	230
Hyposmotic-180	73.3	5	1.8	1	5	10	0	180
Whole Cell Recordings								
	NaCl	TEACl	$CaCl_2$	$MgCl_2$	glucose	Hepes	Mannitol	Osmolarity
Ext. Isosmotic	110	18.3	1.8	1	5	10	50	330
Ext. Hyposmotic	110	18.3	1.8	1	5	10	0	280
Chloride Replacement Assays								
	NaCl	TEACl	$CaCl_2$	$MgCl_2$	glucose	Hepes	Na-gluconate	Osmolarity
Cl-101 mM	77	18.3	1.8	1	5	10	33	280
Cl-61 mM	37	18.3	1.8	1	5	10	73	280
Cl-28 mM	4	18.3	1.8	1	5	10	106	280

Table 1. Composition of solutions. Numbers indicate concentration (in mM). The pH was adjusted to be 7.4.

10, Mannitol 30. For experiments to determine the relative permeabilities of anions, 110 mM NaCl of the external standard hyposmotic solution were substituted by the sodium salt of the correspondent anion. For taurine permeability measurements the following external solutions were used: (1) cesium external solution (180 mosM) (in mM): CsCl 85, $MgSO_4$ 2.0, glucose 5, Hepes 10 (pH adjusted to 8.2 with CsOH); (2) taurine external solution (180 mosM): taurine 140, $MgSO_4$ 2.0, glucose 5.5, Hepes 10 (pH adjusted to 8.2 with CsOH). At this pH level, the concentration of anionic taurine is calculated to be 37.0mM considering a pKa for taurine of 8.78 [39]. The osmolarity of solutions was verified with a vapor pressure osmometer (WESCOR, 5500) . All salts and drugs employed in this work were from Sigma-Aldrich (St Louis, MO, USA), including SITS (4-Acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid disodium salt hydrate, cat A0554), DIDS (4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate, cat D3514), 9-AC (9-Anthracenecarboxylic acid, cat A89405), NPPB (5-nitro-2-(3-phenylpropylamino)-benzoate , cat N4779), Tamoxifen ((Z)-2-[4-(1,2-diphenylbut-1-enyl)phenoxy]-N,N-dimethyl-ethanamine), cat T5648), DCPIB (4-(2-butyl-6,7-dichloro-2-cyclopentylindan-1-on-5-yl)oxybutyric acid, cat D4068), fluoxetine (cat F132) and phloretin (cat P7912). Concentrated stock solutions were in dimethyl sulphoxide (DMSO) and stored in aliquots at -20°C until use. In all cases, the stock solutions were made up so that the final concentrations of DMSO was not greater than 0.1%.

Data analysis

Graphics and statistical analysis, as well as curve fitting procedures were all carried out using the Sigmaplot 10 software (Systat Inc). In cases satisfying a normality test (Kolmogorov-Smirnoff), group comparisons were evaluated by Student's test, otherwise a nonparametric test (Mann-Whitney) was used instead. Multiple comparisons of proportions was made by $\div^2$ test. All reported values are given as mean±S.E.

Results

Regulatory volume decrease response from rat articular chondrocytes

Chondrocytes were dissociated from hip cartilage slices. After dissociation they were incubated on isosmotic media (330 mOsm) for at least 15 minutes. Under this condition chondrocytes had a mean volume of 531.4±12.2μm^3 (n=150,N=4), ranging from 235 to 854 μm^3 . The histogram of frequencies (Fig. 1A) shows a three modal behavior, suggesting three populations of chondrocytes, therefore for further purposes they were categorized as small (250-450 μm^3), medium (455-700 μm^3) and large (705-850 μm^3). Next, to evaluate its osmotic sensitivity, the volume of chondrocytes (medium size) was measured before and after 10 minutes of hyposmotic challenges to 280, 250 and 130 mOsm. REV5901 (100 μM)

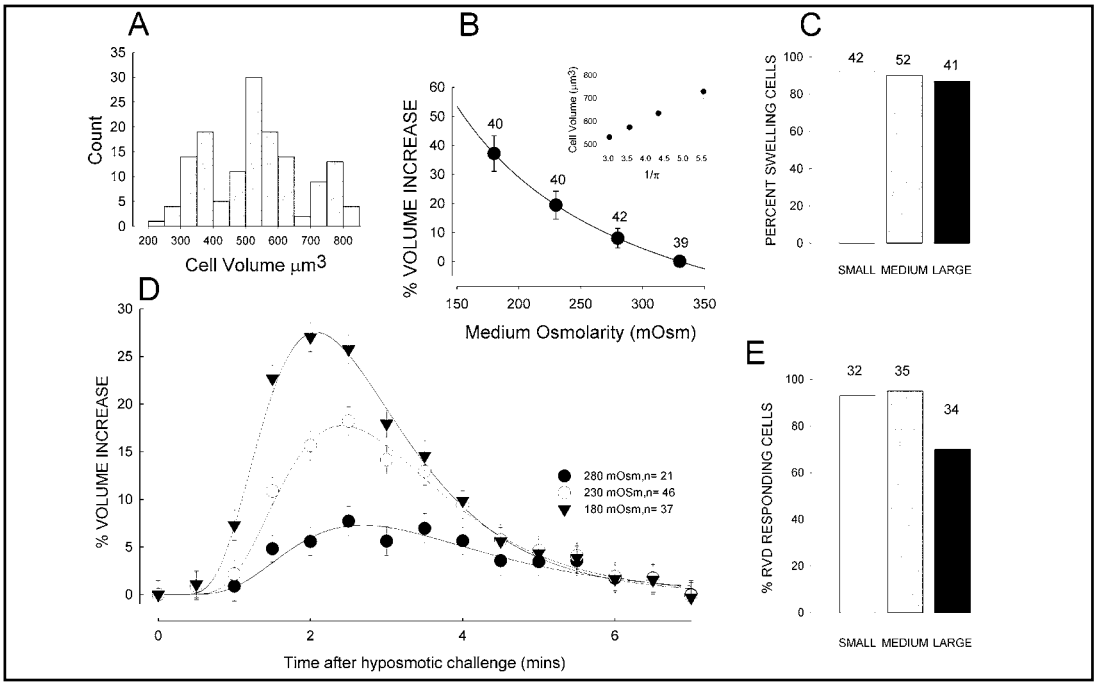

Fig. 1. Osmotic sensitivity and Regulatory Volume Decrease (RVD) response of freshly isolated rat articular chondrocytes. (A) Histogram of frecuencies of condrocyte´s volume under isosmotic conditions describes a three modal behavior. (B) The relation between %volume increase and medium osmolarity shows a trend that can be described by the Boyle-Van Hoff Law; The inset shows a plot of the average volume versus the reciprocal of the osmolarity of the external medium($1/\pi$), made to fit data to a linear transformation of the Boyle-Van´t Hoff equation; Regression analysis resulted in r^2 of 0.9995, indicating a perfect fit . (C) The percentage of swelling chondrocytes in response to a hyposmotic challenge of 130 mOsm is not statistically different among cells because of their size. (D) Time course of volume increase of chondrocytes following hyposmotic shock. Cells were challenged to 180, 230 or 280 mOsm at zero time and their volume measured every 30 seconds over the time shown, percent volume increase is calculated from the volume a cell has at a given time related to their volume before the challenge. Number of data is indicated in the inset. (E) The percentage of RVD responding cells to a hyposmotic challenge of 180 mOsm and was statistically different because of their size (χ^2 test, $p<0.005$).

was added to the external medium to block the RVD response [25]. As Figure 1B shows, chondrocytes increased its volume as a function of external media, as predicted by the Boyle-Van't Hoff law [40], this result indicates that freshly isolated rat chondrocytes are osmotically sensitive over the osmolar range tested. It was observed that not all cells were osmotically sensitive, as some did not swell when challenged. To evaluate if the likelyhood of a given cell to be osmotically sensitivity is related to their size, chondrocytes were challenged to 130 mOsm in the presence of REV5901 and the proportion cells that swollen was counted from samples of each size category. As Figure 1C show, no significant difference was found ($p=0.89$). To observe the time course of the RVD response, the volume of chondrocytes was measured at regular intervals after hyposmotic challenges of 280, 230 and 130 mOsm. As shown in Figure 1D, cells increased their volume until reaching a peak value within 2 to 2.5 minutes after the onset of the hyposmotic challenge, and the rate and the amplitude of swelling was proportional to the amplitude of the hyposmotic challenge. As it can be observed, chondrocytes were capable of RVD response, resuming their volume to their initial values within 7 minutes after the onset of the challenge. It was noticed that not all swollen chondrocytes were able to regulate their volume. To determine if the likelihood of a given swollen chondrocyte to do RVD is related to their size, chondrocytes were challenged in the absence of REV5901 and the proportion of RVD responding cells, from among those that

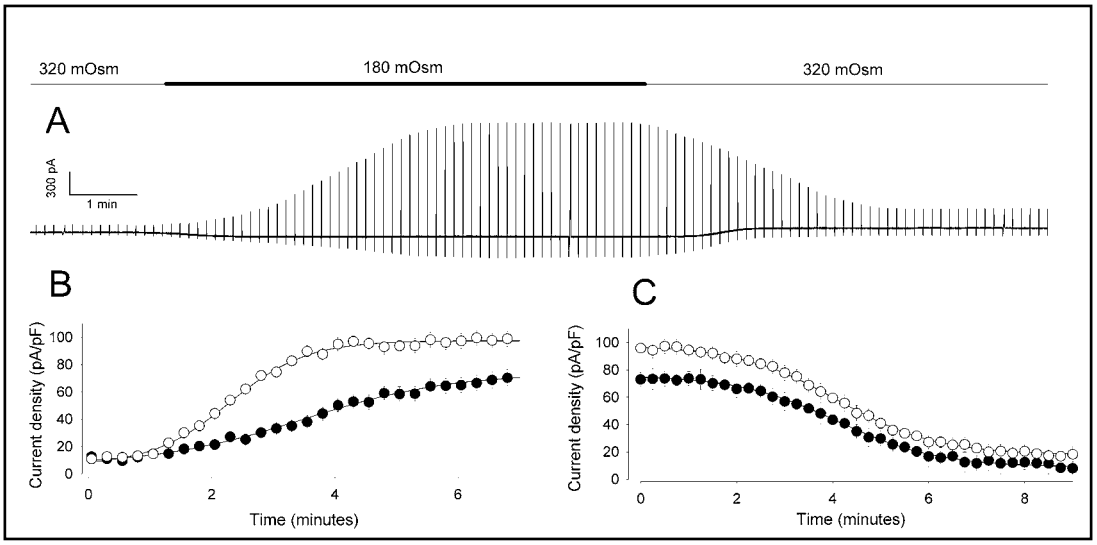

Fig. 2. Ion currents activated after hyposmotic challenge of rat chondrocytes. (A) A representative example shows progressive activation of ion currents after switching from isosmotic (330 mOsm) to hyposmotic (180 mOsm) media. These currents gradually diminish after external media is switched back to isosmotic. From a holding potential of -60 mV, an alternating step voltage protocol, from -80 and +100 mV was delivered to cells every 8 seconds. (B) Time course of activation of swell induced currents . Symbols represent the averaged values±s.e. of current density at +100 mV after hyposmotic challenge (at time zero). Open circles are from 13 chondrocytes challenged to 180 mOsm, filled circles are form 12 chondrocytes challenged to 280 mOsm. (C) Time course of deactivation of currents, obtained as in (B) after switching back to an isosmotic external medium. Continuous lines in B and C show the best fit to a sigmoidal equation.

had previously swollen, was obtained for each size category. This proportion was found to be related to size ($\div^2$ test , $p<0.005$). As Figure 1E shows, no significant difference was found ($p=0.12$) between small (92%, n=42) and medium cells (90%, n=52), however large cells were significantly ($p<0.001$) less likely to restore their volume after swelling (87%, n=41).

Anion currents are activated after hyposmotic challenge

To know whether any ion currents are activated after hyposmotic challenges, the whole cell variant of the patch clamp technique was used (on medium sized chondrocytes only). To follow the time course of the activation of ion currents, cells were stimulated with a repetitive step voltage protocol, consisting of alternating pulses of -80 and +100 mV every 8 seconds. As Figure 2A shows, no significant currents were observed under isosmotic conditions (4.3±3.7 pA/pF at +100 mV, n=20, N=3, p=0.13), however hyposmotic challenges (280 or 180 mOsm) induced the onset of ion currents, that activated gradually, to a maximum level, several minutes after replacement of external media. Chondrocytes challenged with 180 mOsm had a mean maximum current density of 98.0±7.4 pA/pF at +100 mV (n=11, $p<0.001$) whereas a maximum value of 72.9±6.2 pA/pF at +100 mV (n=9, $p<0.001$) was measured from chondrocytes challenged with a 280 mOsm solution. The activation of currents after hyposmotic challenge was faster when media was replaced with a 180 mOsm that when it was replaced with a 280 mOsm (Fig. 2B). In order to measure the rate of activation, data were fitted to a sigmoidal, 4 parameter equation:

$$I_{t=}I_{min} + \frac{I_{max} - I_{min}}{1 + e^{-\left(\frac{t-t_0}{\tau_{on}}\right)}}$$

Where I_{min} is the membrane current level at the onset of the hyposmotic challenge, I_{max} is the level of membrane current at steady state and τ_{on} is the time constant of activation.

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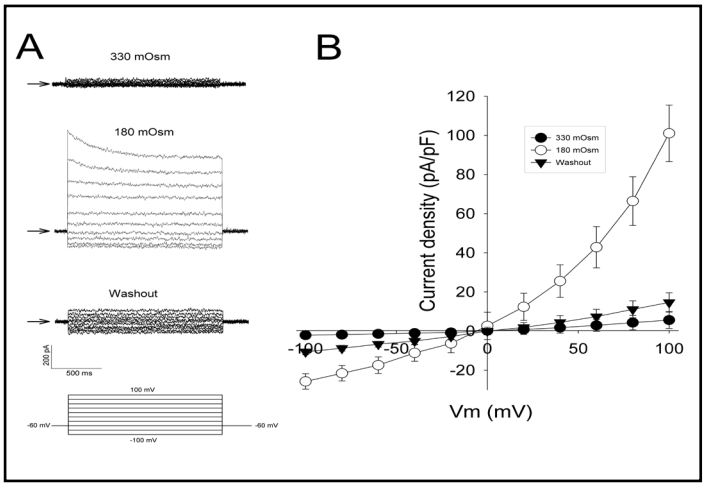

Fig. 3. Kinetic properties of hyposmotically activated ion currents from rat chondrocytes. (A) Representative series of currents obtained from a chondrocyte before (top), during (middle) and after (bottom) a hyposmotic challenge (180 mOsm). (B) I-V Relationships. Current values were measured 50 miliseconds after the onset of pulse and divided by their membrane capacitance. Data shown are mean±s.e. of 23 cells from 4 rats.

The time constant of activation of ion currents after a 180 mOsm challenge ($\tau_{on, 180}$) was 0.64 ± 0.04 min(n=13) whereas that obtained after a 280 mOsm challenge ($\tau_{on,280}$) was 1.21± 0.03 min (n=12) which was significantly slower (p<0.001). Currents activated after hyposmotic challenge, gradually vanished upon returning to isosmotic conditions (Fig. 2C). The rate of deactivation of these currents was roughly the same in both conditions. From fitting of the process of deactivation from a hyposmotic challenge of 280 mOsm, a time constant ($\tau_{off, 280}$) of 0.99 ± 0.02 min (n=12) was estimated, which was non-significantly distinct (p=0.33) of that obtained from a 180 mOSm challenge ($\tau_{off, 180}$=1.03 ± 0.03 min, n=13).

To study the kinetic properties of those currents, another voltage protocol was used, consisting of depolarizing steps from -80 to 100 mV in steps of 20 mV, from a holding of -60 mV. Currents were recorded under isosmotic media, ten minutes after hyposmotic challenge to 180 mOsm and ten minutes after resuming isosmotic media. Figure 3A shows a representative series of currents under each condition, whereas Figure 3B describes the relationship between the mean current density ± S.E. and the membrane potential. Currents activated by hyposmotic challenge showed no voltage dependent activation. Outward rectification and inactivation was observed at most depolarizing voltage levels. At +80 mV the mean current density values obtained were 4.3±3.7 pA/pF (n=15, p=0.13) under isosmotic conditions; 64.4±12.4 pA/pF (n=15, p<0.0001) under hyposmotic (180 mOsm) conditions and 11.1±4.3 pA/pF (n=13, p=0.02) after returning to isosmotic media.

Ion currents activated after hyposmotic shock are selective to chloride

To determine whether those currents are due to chloride anions being carried across the membrane, recordings were made to find the relationship between reversal potential (Erev) and chloride concentration of the external media. Current recordings were obtained from hyposmotically challenged chondrocytes (180 mOsm), in response to depolarizing voltage ramps from -80 to +80 mV (dV/dt=200 mV/sec). Reversal potential measurements were obtained from this data after fitting to a simple exponential function in the range close to inversion of currents (Fig. 4A). Replacement of chloride on the external solution by a relatively impermeant anion (gluconate) caused a shift of reversal potential to more positive values (Fig. 4B), as expected if those currents were due to the flux of chloride anions. Plotting of the reversal potential versus the log10 of the relation of internal to external chloride concentration shows a linear trend whose slope, obtained by linear regression is 52.5, a value that is barely less steep than the ideal value of 58.8 at 22°C from the Nernst equation [41]. These results show that currents activated after hyposmotic challenges are due to activation of a chloride selective pathway. To determine the selectivity sequence, the same procedure was followed after partial replacement of external chloride by monovalent anions:

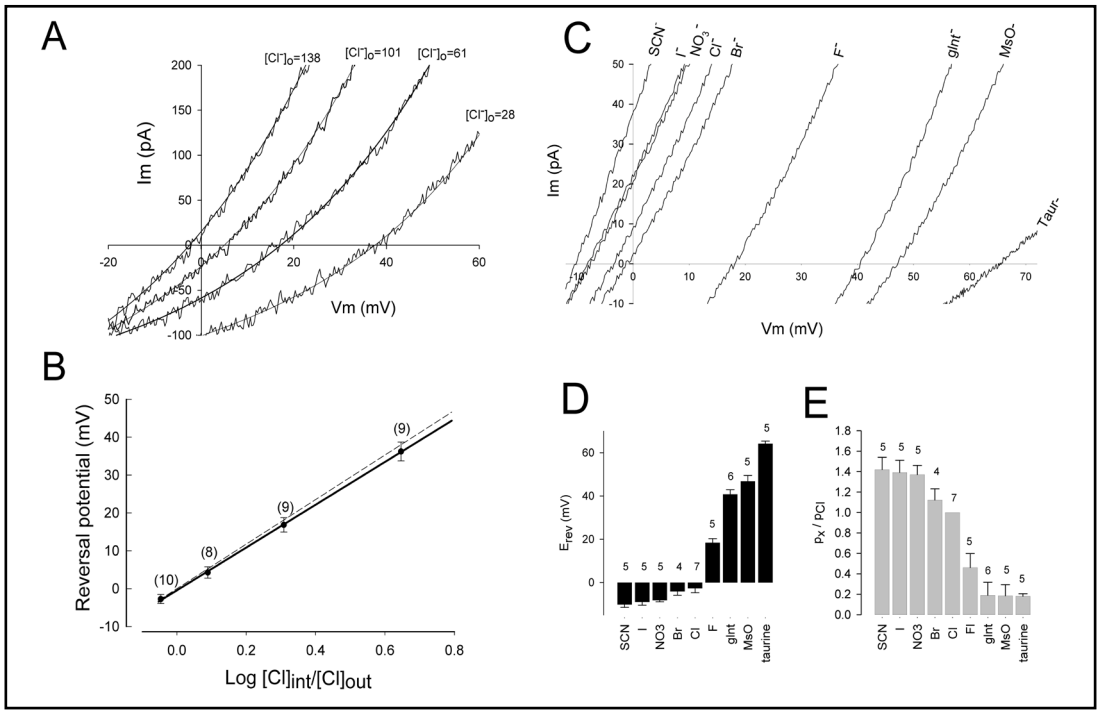

Fig. 4. Anion selectivity properties of swelling activated currents from rat articular chondrocytes. A) Representative currents obtained in response to a ramp voltage protocol at several different external chloride concentrations. Continuous lines are fits to determine the reversal potential. B) A semi-log plot of reversal potential versus the log10 of the relation of internal to external chloride concentration shows a linear trend with a slope of 52.5; Dashed line indicates the expected relationship for a perfectly selective chloride pathway; Parenthesis indicate number of repeats . C) Representative currents, in response to ramp voltage protocols, obtained from chondrocytes immersed in an external media where chloride was substituted by either anion (SCN^-,I^-,Br^-,F^-,NO_3^-, $glnt^-$, MsO^- or taurine). D) Bar chart showing mean±S.E. of the reversal potential of each case. E) Bar chart showing mean±S.E. of relative permeabilities to chloride. Numbers above bars indicate number of repeats.

Iodide(I^-), bromide (Br^-), fluoride (F^-), thiocyanate (SCN^-), nitrate (NO_3^-), gluconate ($glnt^-$) and methanesulphonate (msO^-). While Figure 4C shows a series a representative traces, the mean inversion potential of each anion was (Fig. 4D): I^- (-9±1.5 mV, n=4); Br^- (-4.1±1.8 mV, n=5); Cl^- (-2.7±2 mV, n=4); F^-(18.4±1.9 mV, n=4); SCN^- (-10.8±1.5 mV, n=5); NO_3^- (-8.4±1.9 mV, n=4); $glnt^-$ (40.8±2.1 mV, n=5); MsO^- (46.8±2.7 mV, n=4). In order to calculate the relative permeability of each of these anions to chloride (px/pCl), the following formula, derived from the Goldman-Hodgkin-Katz equation was used:

$$\frac{p_x}{p_{Cl}} = \frac{\left([Cl_o]e^{\left(\frac{\Delta EzF}{RT}\right)} - [Cl_{res}]\right)}{[X_o]}$$

Where $[X_0]$ is the concentration of the substitute anion (110 mM), $[Cl_{res}]$ is the remaining chloride (28 mM), whereas other constants have their usual meanings. From this procedure the following data were obtained (Fig. 4E): pSCN/pCl=1.51±0.1, n=5; pI/pCl=1.39±0.1, n=5; pNO3/pCl=1.37±0.1, n=5; pBr/pCl=1.1±0.1, n=5; pF/pCl=0.4±0.1, n=5; pglnt/pCl=0.1±0.1, n=5; pMsO/pCl=0.1±0.1, n=5. This gives the anion selectivity sequence: SCN^->I^-≅NO_3^->Br^->Cl^- >Fl^->> $glnt^-$> MsO^-, corresponding to Eisenman's sequence I [42].

Taurine permeability of hyposmotically induced chloride currents

In many cell types efflux of taurine, has been found to be associated with the volume regulatory decrease phenomenon [43-45]. In some cases it has been shown that taurine permeates through volume sensitive chloride channels [46, 47]. Therefore we sought to investigate whether this non-essencial aminoacid permeates through the same pathway that produces volume sensitive chloride currents in rat chondrocytes. For this purpose whole cell currents were obtained in response to ramp protocols under hyposmotic conditions as with the other anions (Fig. 4C) and measured its relative permeability (Fig. 4 E). In this case the composition of the external media was (in mM): taurine 180, mannitol 100, $MgSO_4$ 2.0, glucose 5, Hepes 10, pH 8.2), equivalent to 40.2 mM of anionic taurine at this pH value, considering a p_{Ka} of 8.78 [38]. The pipette solution was the same as in the previously described assays. The permeability ratio of taurine to chloride (Ptau/Pcl) was calculated from the reversal potential (Erev) based on the Goldman-Hodgkin-Katz equation, which in its general form is:

$$E_{rev} = \frac{RT}{zF} \ln \frac{\frac{p_{tau}}{p_{cl}}[taurine^-]_o + [Cl^-]_o + \frac{p_{cs}}{p_{cl}}[Cs^+]_i}{\frac{p_{tau}}{p_{cl}}[taurine^-]_i + [Cl^-]_i + \frac{p_{cs}}{p_{cl}}[Cs^+]_o}$$

However , the first summand of the denominator reduces to zero, since the pipette solution contains no $taurine^-$. The permeability ratio of cesium to chloride (PCs/PCl) was calculated from recordings with an external solution containing no taurine (in mM): CsCl 100, mannitol 90, $MgSO_4$ 2.0, glucose 5, Hepes 10 (pH adjusted to 8.2 with CsOH), resulting in an almost negligible value of 0.027±0.015 (n=6, p=0.16). Currents obtained from cells bathed with taurine external solution had a mean Erev of 64.18±1.23 mV (n=9), from which a ptau/pCl of 0.187±0.012 was calculated (p<<<0.01).

Pharmacological properties of hyposmotically activated chloride currents

The effect of several Cl^- channel blockers on these currents was studied, including the stilbene-derivatives 4-acetamido-4'-isothiocyanostilbene (SITS) and 4,4-diisothiocyanatostilbene-2,2-disulfonate (DIDS), as well as the carboxylate analogues, 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) and 9-Anthracenecarboxylic acid (9-AC). For this purpose chloride currents were activated by a 180 mOsm challenge and drugs were added to external media at increasing concentrations. Recording of currents in response to ramp voltage protocols were made, for each concentration of blocker, ten minutes after its addition to media (Fig. 5A). From the membrane current values at +80 and -60 mV were obtained at each drug concentration and compared to control values in order to calculate a percent inhibition index (Pi) as follows:

$$P_{IX} = 100 * (1 - \frac{I_X}{I_0})$$

The dose-response curve of each drug was fitted with the following Hill, dose-response equation:

$$Pi_x = Pi_{min} + \frac{(\mathrm{Pi_{max}} - \mathrm{Pi_{min}})}{1 + 10^{(IC_{50} - x) * HS}}$$

Where Pi_{max} is the maximal percent inhibition, IC_{50} is the drug concentration producing a half blocking effect and HS is the Hill slope, a parameter that determines the steepness of the relationship. All drugs effectively reduced the hyposmotically induced currents as shown on Figure 5. Pimax was significantly distinct from a zero value for all of them (p<0.001). DIDS blocked currents at +80 mV, with a Pi_{max} of 73.8±1.2% (n=5), and IC_{50} of 28.0±4.0μM; At -60 mV these parameters were roughly the same: Pi_{max}=80.7±2.3% (p=0.03) and IC_{50} =28.0±6.0 μM (p=1.0), indicating that blocking is non-voltage dependent. SITS produced a larger Pi_{max} (87.3±2.1%) at +80 mV than DIDS (p<0.001), although the IC_{50} was larger too (225±16 μM); The fitted values at -60 mV were statistically not different of those at +80 mV (IC_{50} =225±16

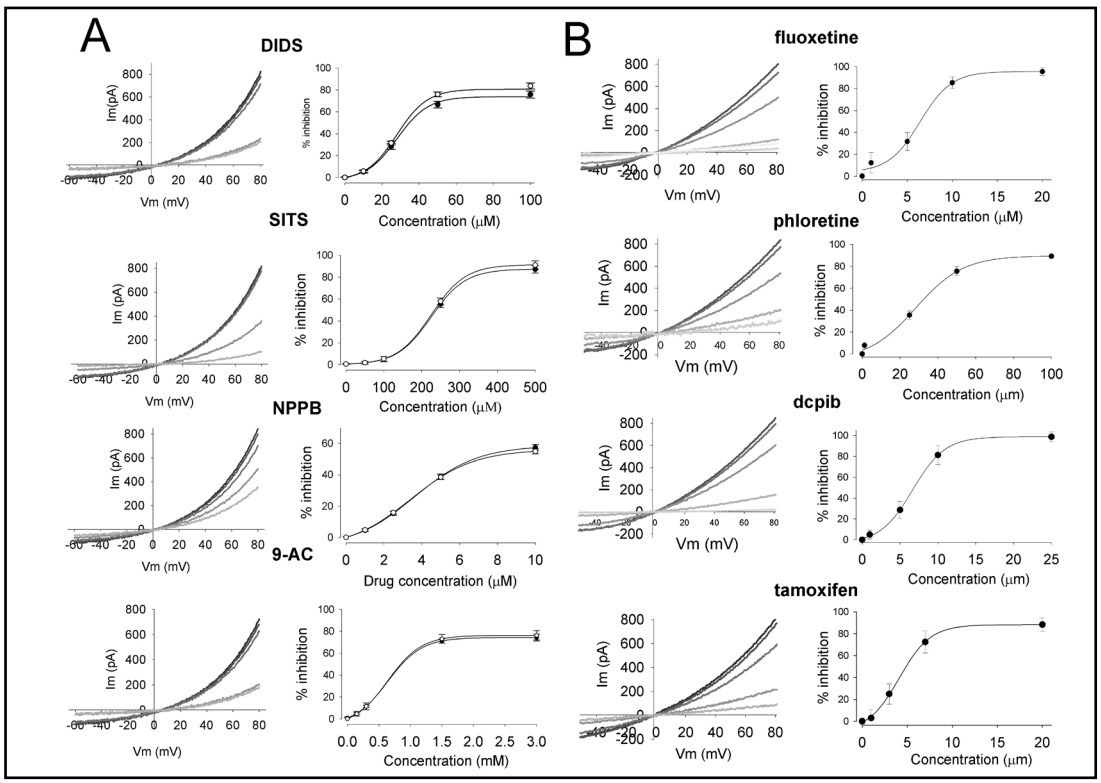

Fig. 5. Pharmacological properties of swelling activated currents from rat articular chondrocytes. (A) Inhibition of swelling activated currents from rat articular chondrocytes by blockers of chloride channels (DIDS, SITS, NPPB, 9-AC); For each blocker, left graph shows a representative series of currents in response to a ramp voltage protocol, increasing trace darkness indicate increasing concentration; Right graph shows the relationship between percent of inhibition and drug concentration as calculated from inward (-60 mV, empty circles) and outward (+80 mV, filled circles) currents. (B) Inhibition of swelling activated currents from rat articular chondrocytes by VRAC selective blockers. Left column shows representative series of currents in response to ramp voltages at distinct blocker concentrations. Right column shows the relationship between percentage inhibition of current at +80 mV and blocker concentration.

μM, Pi_{max}=91.3±2.8%). 9-AC produced a Pi_{max} of 75.3±3.6 at +80 mV which was non distinct of 76.3±6.2 obtained at -60 mV; IC_{50} values were 640.3±24.2 and IC_{50}=638.3±12.6 μM at +80 and -60 mV correspondingly.NPPB showed the lower percent of inhibition: 58.7±3.2% at +80 mV and 56.5±3.7% at -60 mV; whereas IC_{50} was 3.6±0.01 μM and 3.4±0.14 μM correspondingly.

Inhibition of hyposmotically activated chloride currents by $I_{Cl,swell}$ blockers

The effect of several drugs (fluoxetine, phloretin, DCPIB and tamoxifen), which had been shown to block $I_{Cl,swell}$ currents, was evaluated (Fig. 5B). Fluoxetine (also known as Prozac), a substituted propylamine, commonly prescribed as antidepressant, has been shown to block $I_{Cl,swell}$ currents at micromolar range concentrations [48]. Extracellular addition of fluoxetine inhibited the hyposmotically activated chloride currents, with a Pi_{max} of 95.6±1.8% (n=4, p<0.001). IC_{50}= 6.42±0.94 μM (n=4, p<0.01). Phloretin, a bisphenol derivative, has also been found to block $I_{Cl,swell}$ currents at low concentrations (below 100 μM) [49]. This drug inhibited the hyposmotically activated currents from rat chondrocytes with a $P_{i,max}$= 89.6±1.5% (n=6, p<0.001) and IC_{50}=28.33±4.39 μM (p<0.01). DCPIB, an ethacrynic-acid derivative, has been proven to be a potent blocker of volume-sensitive chloride currents in a variety of cells [50]. Extracellular addition of DCPIB reduced the amplitude of the hyposmotically activated currents from rat chondrocytes with a $P_{i,max}$= 98.87±1.2% (n=6, p<0.001) and IC_{50}=6.7±0.2

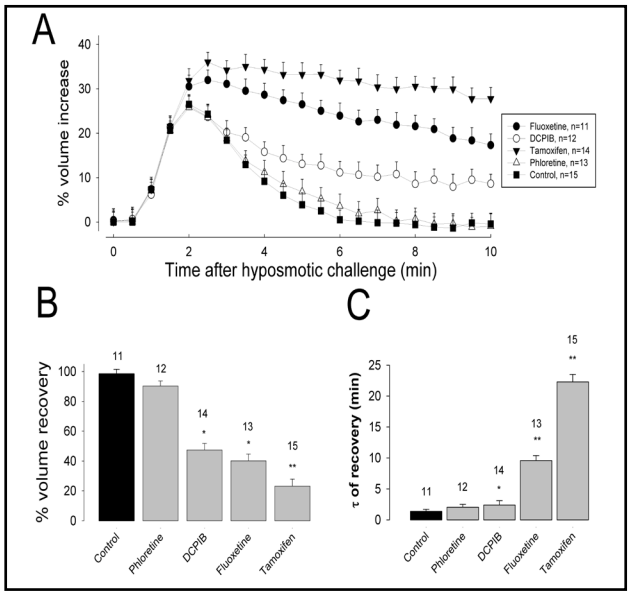

Fig. 6. Effect of vrac blockers on the regulatory volume decrease response of rat articular chondrocytes. A)Timecourse of regulatory volume decrease response of freshly dissociated rat articular chondrocytes following a hyposmotic challenge of 180 mOsm. Cell volume measurements were taken every 30 seconds from individual cells. B) Percent volume recovery of chondrocytes under treatment of VRAC blockers as compared to control. C) Comparison of the time constant of the volume recovery phase of chondrocytes under treatment of VRAC blockers. The number of repeats in B and C are the same as in A.

μM ($p<0.01$). Tamoxifen is an antagonist of the estrogen receptor, used against breast cancer. In several cell lines, it has been found to be a reversible, high-potency blocker of volume-regulated chloride currents, without affecting other types of chloride currents [51, 52]. Tamoxifen diminished hyposmotically activated currents from rat chondrocytes with a $P_{i,max}$= 88.2±1.7% (n=5, $p<0.005$) and IC_{50}=4.1±0.3 μM ($p<0.01$). For all of these drugs, maximal blocking effect of each concentration was achieved within 1-2 minutes and currents fully recovered after washout.

Effect of volume sensitive chloride channel blockers on the regulatory volume decrease capacity of rat chondrocytes

The effect of the $I_{Cl,swell}$ blockers tamoxifen (20 μM), phloretin (100 μM), fluoxetine (20 μM) and DCPIB (20 μM) on the ability of cells to recover their volume after an hyposmotic challenge was assayed. To this end, drugs were added to external media, five minutes before and kept throughout a 180 mOsm hyposmotic challenge and the timecourse of the volume response was followed (Fig. 6A). The rate of swelling was not notably distinct under any treatment and the peak volume increase was significantly distinct only with tamoxifen (36±2.2%, n=14, $p=0.005$) as compared to control (26.5±2.2, n=15). Nonetheless the volume recovery capacity of rat chondrocytes was notably affected by DCPIB, tamoxifen and fluoxetine although not by phloretin: Chondrocytes without treatment recovered 98.5±1.2% of their original volume at ten minutes after challenge. Those treated with phloretin recovered 95.3±3.4% after the same time, a value which was not significantly different from control ($p=0.49$). Nonetheless a significant difference was observed for those treated with DCPIB ($p<0.001$), fluoxetine ($p<0.001$) and tamoxifen ($p<0.00001$), which recovered 47.5±4.3%, 40.2±4.5% and 23.2±4.7% respectively (Fig. 6B). A similar result was found when comparing the time constant of volume recovery (τ_{rvd}) (Fig. 6C): A value of 1.4±0.3 min was found for control cells; A non-significant difference ($p>0.2$) was found for those treated with phloretin (2.0±0.4 minutes) or DCPIB (2.4±0.7 min); Cells incubated with fluoxetine or tamoxifen recovered with a significantly ($p<0.001$) slower rate (9.6±0.8 and 22.3±1.2 minutes respectively).

Rat articular chondrocytes express TMEM16A and ClC-3

So far the molecular identity of VRAC (Volume Regulated Anion Channel), the ion channel that accounts for $I_{Cl,swell}$ remains unresolved, however several known chloride channels have been proposed as candidates, including TMEM16A, a calcium-dependent chloride channel [53, 54] and ClC-3, a member of the voltage dependent chloride channel family [55, 56].

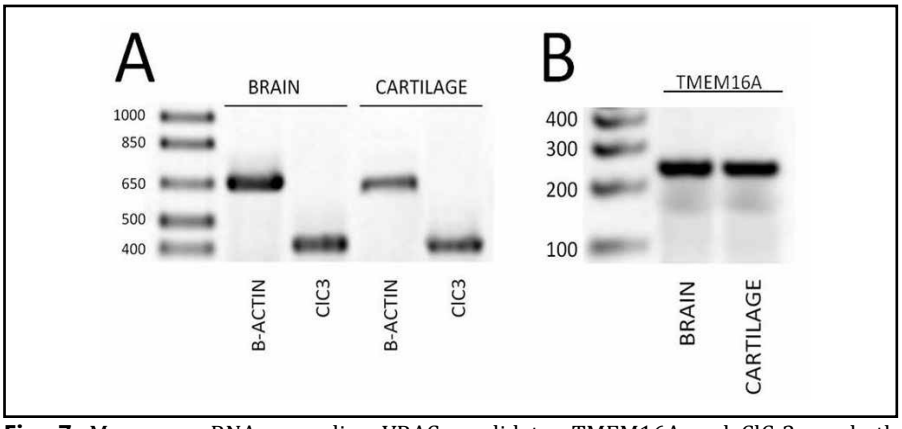

Fig. 7. Messenger RNAs encoding VRAC candidates TMEM16A and ClC-3 are both present in rat articular chondrocytes. (A) and (B) shows RT-PCR results for ClC-3 and TMEM16A respectively in (A) β-actin was used as RT-PCR control. In both cases rat brain RNA was assayed as positive control, along with rat cartilage.

Therefore we resorted to RT-PCR analysis to know if rat articular chondrocytes express either one or both of them. As shown in Figure 7, our results indicate that mRNA for both TMEM16A and ClC-3 are present in rat chondrocytes.

Discussion

The main purpose of this work was to determine if there are anion currents activated after hyposmotic challenges in freshly isolated rat articular chondrocytes and if so, how those currents compare to $I_{Cl,Swell}$ as described in other cell types. Before that, it was necessary to know whether freshly isolated rat articular chondrocytes are sensitive to hyposmotic variations and if they are capable of a regulatory volume decrease (RVD) response. We found that, under this experimental condition, most chondrocytes were in fact osmotically responsive since they swollen proportionally to the amplitude of the osmotic challenge, as predicted by the Boyle-Van Hoff equation. We found also that these cells are capable of an RVD response: Periodical measurements of cell volume showed that, when exposed to hyposmotic challenges, most chondrocytes swollen within 2 minutes, then exhibited rapid RVD and restored their former volume within approximately 10 minutes. The rate and efficiency of cell swelling and subsequent volume recovery are fairly comparable to those obtained from chondrocytes from other mammal species [27]; However these abilities seem not to be intrinsic, as some cells did not swell and some of those that swollen could not restore their volume, suggesting some variability in the expression of the different membrane pathways involved. Our results further suggest that the ability of chondrocytes to recover its volume is somehow related to their size because large chondrocytes were less likely to exhibit an RVD response than small and middle ones.

The articular cartilage is a stratified tissue, where three major zones can be distinguished across its thickness: the superficial, the middle and the deep zone. These zones are structural, physical and biochemically different [57]. It has been observed, for instance that both ionic strength and osmolarity increase with depth, this depending strongly on the density of fixed negative charges within the tissue, which is less at the surface compared with the middle and deep zones. Several biochemical properties such as the quantity and proportion of glucosaminoglycans, hexosamine content or the distribution of type I and type II collagens also change within these zones [58-60]. The histomorphological, biological, and mechanical characteristics of these zones are in turn correlated with the distributions of pressures, deformations, and pressure-induced fluid flow that are created *in vivo* by mechanic loads

[61]. In the intact cartilage the size of chondrocytes varies according to their location, with the smaller ones found preferentially on the surface and their size increasing with depth [62].It is clear then that chondrocytes dwell in an environment whose properties are related to their depth and therefore they are expected to show differences in their physiological properties, which would include their response to hyposmotic challenges. In these regard our finding, that the likelihood on a chondrocyte to exhibit RVD is related to size sustain this hypothesis.

As described before, we found anion currents that were activated by hyposmotic challenges. The properties of these currents resemble most of properties described for $I_{Cl,swell}$, including: (1) The fact that it is activated by cell swelling resulting from hyposmotic challenges; (2) Their anionic selectivity sequence: $SCN^->I^-\cong NO_3^->Br^->Cl^->F^->>$ glnt$^-$> MsO$^-$; (3) The time course of currents in response to step voltage pulses, showing outward rectification, voltage independent activation, and inactivation at most depolarizing voltage levels [63, 64]; (4) The fact that they are blocked by drugs that had been described to block chloride channels, including DIDS, SITS, 9-AC and NPPB, as well as substances that had been described to specifically inhibit $I_{Cl,swell}$, including tamoxifen, fluoxetine, phloretin and DCPIB. The half maximal inhibitory concentration (IC_{50}) of almost all those drugs was within the range reported for $I_{Cl,swell}$ currents, except DCPIB, whose IC_{50} (6.7 μM) was slightly over the reference range (1-5 μM) observed in other cells [65]. Additionally, these results coincide with a previous report describing $I_{Cl,swell}$ in rabbit chondrocytes [66]. However our outcomes indicating that DIDS, SITS and NPPB do block $I_{Cl,swell}$ in a non voltage-dependent manner diverge from other reports showing a voltage dependent inhibition for these drugs [65, 67, 68]. In this regard is worth to consider that $I_{Cl,swell}$ is a functional attribute, whose properties vary widely among distinct tissues and mammalian species. So far the molecular entity that accounts for $I_{Cl,swell}$ has not been elucidated and, given the functional diversity, it is conceivable that several molecularly distinct variants may be participating on distinct tissues and species.

On the other hand, in order to know whether these currents participate in the RVD process, we probed the effect of tamoxifen, fluoxetine, phloretin and DCPIB on the volume regulatory response of rat chondrocytes. The concentration tested was the one that inhibited around 80% of chloride currents. As shown before, except for phloretin, they slowed down the RVD. These results suggest that such currents have a role on the RVD process, however the fact that the RVD process was no fully abolished by any of those drugs suggest further that, in addition to $I_{Cl,swell}$, there are other membrane pathways conjunctly participating during the RVD response. In order to preserve the membrane potential, an efflux of cations, more likely potassium, is expected to accompany anions, although so far no volume, dependent potassium currents have been demonstrated in these cells. There however had been described voltage dependent K currents [10], as well as stretch activated K channels [69] and TRPV5 [70] that could be likely candidates of such cationic currents. Additionally there can be other pathways to allow the flux of osmolytes, other than ions [30, 31]. It is possible that such pathways can be also be blocked by the drugs tested. For instance it has been found that tamoxifen blocks also delayed rectifier potassium channels as well as voltage dependent calcium channels in myocites [71]. DCPIB has been found to modulate KATP channels in beta pancreatic cells [72]. Fluoxetine inhibits A-type potassium currents and two pore potassium channels TREK-1 [73, 74]. Therefore the different results obtained with the different drugs tested can be explained considering that those drugs, in addition to block $I_{Cl,swell}$ currents could block other pathways participating in the RVD process.

We probed as well whether $I_{Cl,swell}$ could be a pathway to account for the flux of taurine associated with RVD, as it has been described elsewhere [30] and measured its relative permeability ratio (ptau/pCl). The value found (0.187) is rather low compared to 0.75 described in MDCK cells [75], however is fairly comparable to values reported in several other types of cells such as rat C6 glioma cells (0.2), 9HTEo- human tracheal (0.31) and rat IMCD cells (0.15) [45, 76, 77]. Furthermore, it is closely comparable to 0.21 reported in isolated rabbit articular chondrocytes [38]. Thus $I_{Cl,swell}$ may be a pathway that partially

accounts for the transport of taurine, however other pathways not yet described may also be involved.

It is important to mention that, for the sake of uniformity, most of electrophysiological and pharmacological studies were performed on medium sized cells. Nonetheless we made some whole cell recordings on small and large sized cells, which revealed no conspicuous differences on some of the properties addressed to medium sized, such as membrane current density and the profile of anion currents, however a further thorough comparison of all of their kinetic and pharmacological properties may help to explain the relation of RVD responsiveness to size.

Also it is worth to highlight our RT-PCR results, showing that mRNA for both TMEM16A and ClC-3 are present in rat articular chondrocytes; Even when the molecular identity of the ion channels that account for $I_{Cl,swell}$ is still a debatable issue, there exist evidence for both TMEM16A and ClC-3 to be the molecular determinants of $I_{Cl,swell}$ currents [53-56]; the former, also known as anoctamin-1 (ANO1) encodes for calcium activated chloride channels (CaCC) [54] and it is been shown that mice lacking it reduce their $I_{Cl,swell}$ currents [53]. Since intracellular calcium has been described to be related to RVD in chondrocytes [20, 56, 57] TMEM16A could be a likely candidate to produce VRAC channels in chondrocytes. On the other hand, the fact that $I_{Cl,swell}$ and ClC-3 currents have similar biophysical and pharmacological properties has led to think of ClC-3 as the molecular determinant of $I_{Cl,swell}$. This hypothesis has been supported by experiments showing that decreasing the expression of ClC-3 significantly decreases the rate of RVD in bovine nonpigmented ciliary epithelial cells [78], smooth muscle cells [79] and Xenopus laevis oocytes [56]. Our findings could support the idea that either one of them or both could be contributing to the expression of the observed $I_{Cl,swell}$ currents in rat articular chondrocytes.

In summary, in this work we showed that rat articular chondrocytes freshly dissociated express $I_{Cl,swell}$ currents and that they have a role in the ability of chondrocytes to effectively regulate their volume after hyposmotic variations.

References

1 Hall AC, Horwitz ER, Wilkins RJ: The cellular physiology of articular cartilage. Exp Physiol 1996;81:535-545.

2 Buckwalter JA, Mankin HJ: Articular cartilage: tissue design and chondrocyte-matrix interactions. Instr Course Lect 1998;47:477-486.

3 Wilkins RJ, Browning JA, Urban JP: Chondrocyte regulation by mechanical load. Biorheology 2000;37:67-74.

4 Lane Smith R, Trindade MC, Ikenoue T, Mohtai M, Das P, Carter DR, Goodman SB, Schurman DJ: Effects of shear stress on articular chondrocyte metabolism. Biorheology 2000;37:95-107.

5 Adams MA: The mechanical environment of chondrocytes in articular cartilage. Biorheology 2006;43:537-545.

6 Wu QQ, Chen Q: Mechanoregulation of chondrocyte proliferation, maturation, and hypertrophy: ion-channel dependent transduction of matrix deformation signals. Exp Cell Res 2000;256:383-391.

7 Mobasheri A, Carter SD, Martin-Vasallo P, Shakibaei M: Integrins and stretch activated ion channels; putative components of functional cell surface mechanoreceptors in articular chondrocytes. Cell Biol Int 2002;26:1-18.

8 Barrett-Jolley R, Lewis R, Fallman R, Mobasheri A: The emerging chondrocyte channelome. Front Physiol 2010;1:135.

9 Sugimoto T, Yoshino M, Nagao M, Ishii S, Yabu H: Voltage-gated ionic channels in cultured rabbit articular chondrocytes. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 1996;115:223-232.

10 Ponce A: Expression of voltage dependent potassium currents in freshly dissociated rat articular chondrocytes. Cell Physiol Biochem 2006;18:35-46.

11 Walsh KB, Cannon SD, Wuthier RE: Characterization of a delayed rectifier potassium current in chicken growth plate chondrocytes. Am J Physiol Cell Physiol 1992;262:C1335-C1340.
12 Mozrzymas JW, Visintin M, Vittur F, Ruzzier F: Potassium channels of pig articular chondrocytes are blocked by propofol. Biochem Biophys Res Commun 1994;202:31-37.
13 Wilson JR, Duncan NA, Giles WR, Clark RB: A voltage-dependent K^+ current contributes to membrane potential of acutely isolated canine articular chondrocytes. J Physiol 2004;557:93-104.
14 Mobasheri A, Gent TC, Womack MD, Carter SD, Clegg PD, Barrett-Jolley R: Quantitative analysis of voltage-gated potassium currents from primary equine (Equus caballus) and elephant (Loxodonta africana) articular chondrocytes. Am J Physiol Regul Integr Comp Physiol 2005;289:R172-180.
15 Mobasheri A, Lewis R, Maxwell JE, Hill C, Womack M, Barrett-Jolley R: Characterization of a stretch-activated potassium channel in chondrocytes. J Cell Physiol 2010;223, 511–518.
16 Shao Y, Alicknavitch M, Farach-Carson MC: Expression of voltage sensitive calcium channel (VSCC) L-type Cav1.2 (alpha1C) and T-type Cav3.2 (alpha1H) subunits during mouse bone development. Dev Dyn 2005;234:54-62.
17 Sanchez JC, Danks TA, Wilkins RJ : Mechanisms involved in the increase in intracellular calcium following hypotonic shock in bovine articular chondrocytes. Gen Physiol Biophys 2003;22:487–500.
18 Mobasheri A, Trujillo E, Bell S, Carter SD, Clegg PD, Martin-Vasallo P, Marples D: Aquaporin water channels AQP1 and AQP3, are expressed in equine articular chondrocytes. Vet J 2004;168:143-150.
19 Tsuga K, Tohse N, Yoshino M, Sugimoto T, Yamashita T, Ishii S, Yabu H: Chloride conductance determining membrane potential of rabbit articular chondrocytes. J Membr Biol 2002;185:75-81.
20 Eveloff JL, Warnock DG: Activation of ion transport systems during cell volume regulation. Am J Physiol 1987;252:F1-10.
21 Sarkadi B, Parker JC: Activation of ion transport pathways by changes in cell volume. Biochim Biophys Acta 1991;1071:407-427.
22 Wehner F, Olsen H, Tinel H, Kinne-Saffran E, Kinne RK: Cell volume regulation: osmolytes, osmolyte transport, and signal transduction. Rev Physiol Biochem Pharmacol 2003;148:1-80.
23 Lang F: Mechanisms and significance of cell volume regulation. J Am Coll Nutr 2007;26:613S-623S.
24 Hoffmann EK, Lambert IH, Pedersen SF: Physiology of cell volume regulation in vertebrates. Physiol Rev 2009;89:193-277.
25 Bush PG, Hall AC: The osmotic sensitivity of isolated and in situ bovine articular chondrocytes. J Orthop Res 2001;19:768-778.
26 Guilak F, Erickson GR, Ting-Beall HP: The effects of osmotic stress on the viscoelastic and physical properties of articular chondrocytes. Biophys J 2002;82:720-727.
27 Bush PG, Hall AC: Regulatory volume decrease (RVD) by isolated and in situ bovine articular chondrocytes. J Cell Physiol 2001;187:304-314.
28 Kerrigan MJ, Hook CS, Qusous A, Hall AC: Regulatory volume increase (RVI) by in situ and isolated bovine articular chondrocytes. J Cell Physiol 2006;209:481-492.
29 Lewis R, Feetham CH, Barrett-Jolley R: Cell volume regulation in chondrocytes. Cell Physiol Biochem 2011;28:1111-1122.
30 Hall AC: Volume-sensitive taurine transport in bovine articular chondrocytes. J Physiol 1995;484:755-766.
31 Hall AC, Bush PG: The role of a swelling-activated taurine transport pathway in the regulation of articular chondrocyte volume. Pflugers Arch 2001;442:771-781.
32 Martina M, Mozrzymas JW, Vittur F: Membrane stretch activates a potassium channel in pig articular chondrocytes. Biochim Biophys Acta 1997;1329:205-210.
33 Mozrzymas JW, Martina M, Ruzzier F: A large-conductance voltage-dependent potassium channel in cultured pig articular chondrocytes. Pflugers Arch 1997;433:413-427.
34 Yellowley CE, Hancox JC, Donahue HJ: Effects of cell swelling on intracellular calcium and membrane currents in bovine articular chondrocytes. J Cell Biochem 2002;86:290-301.
35 Nilius B, Eggermont J, Voets T, Droogmans G: Volume-activated Cl^- channels. Gen Pharmacol 1996;27:1131-1140.
36 Strange K, Emma F, Jackson PS: Cellular and molecular physiology of volume-sensitive anion channels. Am J Physiol 1996;270:C711.

37 Sardini A, Amey JS, Weylandt KH, Nobles M, Valverde MA, Higgins CF: Cell volume regulation and swelling-activated chloride channels. Biochim Biophys Acta 2003;1618:153-162.
38 Okumura N, Imai S, Toyoda F, Isoya E, Kumagai K, Matsuura H, Matsusue Y: Regulatory role of tyrosine phosphorylation in the swelling activated chloride current in isolated rabbit articular chondrocytes. J Physiol 2009;587:3761-3776.
39 Christensen JJ, Hansen LD, Izatt RM: Handbook of Proton Ionization Heats and Related Thermodynamic Quantities, Wiley-Interscience, New York, 1976
40 Katkov II: On proper linearization, construction and analysis of the Boyle-van't Hoff plots and correct calculation of the osmotically inactive volume. Cryobiology 2011;62:232-241.
41 Hille B: Ion Channels of Excitable Membranes (3rd ed.). Sunderland, Mass: Sinauer Associates. 2001, ISBN 0-87893-321-322.
42 Wright EM, Diamond JM: Anion selectivity in biological systems. Physiological Reviews 1977;57,109-186.
43 Jackson PS, Strange K: Volume-sensitive anion channels mediate swelling-activated inositol and taurine efflux. Am J Physiol 1993;265:C1489-1500.
44 Pasantes-Morales H, Quesada O, Morán J: Taurine: an osmolyte in mammalian tissues. Adv Exp Med Biol 1998;442:209-217.
45 Shennan DB: Swelling-induced taurine transport: relationship with chloride channels, anion-exchangers and other swelling-activated transport pathways. Cell Physiol Biochem 2008;21:15-28.
46 Nilius B, Eggermont J, Voets T, Buyse G, Manolopoulos V, Droogmans G: Properties of volume-regulated anion channels in mammalian cells. Prog Biophys Mol Biol 1997;68:69–119.
47 Mulligan SJ, MacVicar BA: VRACs CARVe a path for novel mechanisms of communication in the CNS. Sci STKE 2006;2006:pe42.
48 Maertens C, Wei L, Voets T, Droogmans G, Nilius B: Block by fluoxetine of volume-regulated anion channels. Br J Pharmacol 1999;126:508-514.
49 Fan HT, Morishima S, Kida H, Okada Y: Phloretin differentially inhibits volume-sensitive and cyclic AMP-activated, but not Ca-activated, Cl^- channels. Br J Pharmacol 2001;133:1096-1106.
50 Decher N, Lang HJ, Nilius B, Brüggemann A, Busch AE, Steinmeyer K: DCPIB is a novel selective blocker of I(Cl,swell) and prevents swelling-induced shortening of guinea-pig atrial action potential duration. Br J Pharmacol 2001;134:1467-1479.
51 Valverde MA, Mintenig GM, Sepúlveda FV: Differential effects of tamoxifen and I- on three distinguishable chloride currents activated in T84 intestinal cells. Pflugers Arch 1993;425:552–554.
52 Sahebgharani M, Hardy SP, Lloyd AW, Hunter AC, Allen MC: Volume activated chloride currents in HeLa cells are blocked by tamoxifen but not by a membrane impermeant quaternary analogue. Cell Physiol Biochem 2001;11:99-104.
53 Almaça J, Tian Y, Aldehni F, Ousingsawat J, Kongsuphol P, Rock JR, Harfe BD, Schreiber R, Kunzelmann K: TMEM16 proteins produce volume-regulated chloride currents that are reduced in mice lacking TMEM16A. J Biol Chem 2009;284:28571–28578.
54 Kunzelmann K, Tian Y, Martins JR, Faria D, Kongsuphol P, Ousingsawat J, Thevenod F, Roussa E, Rock J, Schreiber R: Anoctamins. Pflugers Arch 2011;462:195-208.
55 Coca-Prados M, Sánchez-Torres J, Peterson-Yantorno K, Civan MM: Association of ClC-3 channel with Cl^- transport by human nonpigmented ciliary epithelial cells. J Membr Biol 1996;150:197-208.
56 Hermoso M, Satterwhite CM, Andrade YN, Hidalgo J, Wilson SM, Horowitz B, Hume JR: ClC-3 is a fundamental molecular component of volume-sensitive outwardly rectifying Cl^- channels and volume regulation in HeLa cells and Xenopus laevis oocytes. J Biol Chem 2002 Oct 18;277:40066-40074.
57 Youn I, Choi JB, Cao L, Setton LA, Guilak F: Zonal variations in the three-dimensional morphology of the chondron measured in situ using confocal microscopy. Osteoarthritis Cartilage 2006;14:889-897.
58 Franzén A, Inerot S, Hejderup SO, Heinegård D: Variations in the composition of bovine hip articular cartilage with distance from the articular surface. Biochem J 1981;195:535-543.
59 Lipshitz H, Etheredge R 3rd, Glimcher MJ: Changes in the hexosamine content and swelling ratio of articular cartilage as functions of depth from the surface. J Bone Joint Surg Am 1976;58:1149-1153.

60 Stanescu V, Stanescu R, Maroteaux P: [Differences in distribution of type I and type II collagens in the superficial and intermediary zones of articular cartilage]. C R Acad Sci Hebd Seances Acad Sci D 1976;283:279-282.
61 Wong M, Carter DR: Articular cartilage functional histomorphology and mechanobiology: a research perspective. Bone 2003;33:1-13.
62 Bush PG, Parisinos CA, Hall AC: The osmotic sensitivity of rat growth plate chondrocytes in situ; clarifying the mechanisms of hypertrophy. J Cell Physiol 2008;214:621-629.
63 Jentsch TJ, Stein V, Weinreich F, Zdebik AA: Molecular structure and physiological function of chloride channels. Physiol Rev 2002;82:503-568.
64 Nilius B, Droogmans G: Amazing chloride channels: an overview. Acta Physiol Scand 2003;177:119-147.
65 d'Anglemont de Tassigny A, Souktani R, Ghaleb B, Henry P, Berdeaux A : Structure and pharmacology of swelling-sensitive chloride channels, I(Cl,swell). Fundam Clin Pharmacol 2003;17:539–553.
66 Isoya E, Toyoda F, Imai S, Okumura N, Kumagai K, Omatsu-Kanbe M, Kubo M, Matsuura H, Matsusue Y: Swelling-activated $Cl^{(-)}$ current in isolated rabbit articular chondrocytes: inhibition by arachidonic Acid. J Pharmacol Sci 2009;109:293-304.
67 Nilius B, Eggermont J, Voets T, Droogmans G: Volume-activated Cl^- channels. Gen Pharmacol 1996;27:1131-1140.
68 Wang J, Xu H, Sun X, Niu W: Pharmacological and biophysical properties of swelling-activated chloride channel in mouse cardiac myocytes. Chin J Physiol 2006;49:126-131.
69 Mobasheri A, Lewis R, Maxwell JE, Hill C, Womack M, Barrett-Jolley R: Characterization of a stretch-activated potassium channel in chondrocytes. J Cell Physiol 2010;223:511-518.
70 Lewis R, Asplin K, Bruce G, Dart C, Mobasheri A, Barrett-Jolley R: The role of the membrane potential in chondrocyte volume regulation. J Cell Physiol 2011;226:2979-2986.
71 Liu XK, Katchman A, Ebert SN, Woosley RL: The antiestrogen tamoxifen blocks the delayed rectifier potassium current, IKr, in rabbit ventricular myocytes. J Pharmacol Exp Ther 1998;287:877-883.
72 Best L, Yates AP, Decher N, Steinmeyer K, Nilius B: Inhibition of glucose-induced electrical activity in rat pancreatic beta-cells by DCPIB, a selective inhibitor of volume-sensitive anion currents. Eur J Pharmacol 2004;489:13-19.
73 Choi JS, Choi BH, Ahn HS, Kim MJ, Han TH, Rhie DJ, Yoon SH, Jo YH, Kim MS, Hahn SJ: Fluoxetine inhibits A-type potassium currents in primary cultured rat hippocampal neurons. Brain Res 2004;1018:201-207.
74 Kennard LE, Chumbley JR, Ranatunga KM, Armstrong SJ, Veale EL, Mathie A: Inhibition of the human two-pore domain potassium channel, TREK-1, by fluoxetine and its metabolite norfluoxetine. Br J Pharmacol 2005;144:821-829.
75 Banderali U, Roy G: Anion channels for amino acids in MDCK cells. Am J Physiol 1992;263:C1200-1207.
76 Boese SH, Wehner F, Kinne RK: Taurine permeation through swelling-activated anion conductance in rat IMCD in primary culture. Am J Physiol 1996;271:F498-F507.
77 Galietta LJ, Falzoni S, Di Virgilio F, Romeo G, Zegarra-Moran O: Characterization of volume-sensitive taurine-and Cl^- permeable channels. Am J Physiol 1997;273:C57-C66.
78 Wang L, Chen L, Jacob TJ: The role of ClC-3 in volume-activated chloride currents and volume regulation in bovine epithelial cells demonstrated by antisense inhibition. J Physiol 2000;524,63–75.
79 Zhou JG, Ren JL, Qiu QY, He H, Guan YY: Regulation of intracellular Cl^- concentration through volume-regulated ClC-3 chloride channels in A10 vascular smooth muscle cells. J Biol Chem 2005;280:7301-7308.