Journal of Cellular Biochemistry 86:290–301 (2002)

Effects of Cell Swelling on Intracellular Calcium and Membrane Currents in Bovine Articular Chondrocytes

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Abstract Chondrocytes experience a dynamic extracellular osmotic environment during normal joint loading when fluid is forced from the matrix, increasing the local proteoglycan concentration and therefore the ionic strength and osmolarity. To exist in such a challenging environment, chondrocytes must possess mechanisms by which cell volume can be regulated. In this study, we investigated the ability of bovine articular chondrocytes (BAC) to regulate cell volume during a hypo-osmotic challenge. We also examined the effect of hypo-osmotic stress on early signaling events including $[Ca^{2+}]_i$ and membrane currents. Changes in cell volume were measured by monitoring the fluorescence of calcein-loaded cells. $[Ca^{2+}]_i$ was quantified using fura-2, and membrane currents were recorded using patch clamp. BAC exhibited regulated volume decrease (RVD) when exposed to hypo-osmotic saline which was inhibited by Gd^{3+}. Swelling stimulated $[Ca^{2+}]_i$ transients in BAC which were dependent on swelling magnitude. Gd^{3+}, zero $[Ca^{2+}]_o$, and thapsigargin all attenuated the $[Ca^{2+}]_i$ response, suggesting roles for Ca^{2+} influx through stretch activated channels, and Ca^{2+} release from intracellular stores. Inward and outward membrane currents significantly increased during cell swelling and were inhibited by Gd^{3+}. These results indicate that RVD in BAC may involve $[Ca^{2+}]_i$ and ion channel activation, both of which play pivotal roles in RVD in other cell types. These signaling pathways are also similar to those activated in chondrocytes subjected to other biophysical signals. It is possible, then, that these signaling events may also be involved in a mechanism by which mechanical loads are transduced into appropriate cellular responses by chondrocytes. J. Cell. Biochem. 86: 290–301, 2002.

Key words: mechanotransduction; ion channel; gadolinium; osmolarity; regulatory volume decrease

In most cell types, mechanisms exist to maintain cell volume within tightly controlled levels in order to respond to changes in internal osmolarity, for example, a change in the concentration of internal metabolites [O'Neill, 1999].

In chondrocytes, however, a further threat to cell volume is change in extracellular osmolarity. Chondrocytes exist within a unique osmotic environment that is dictated by the nature of the cartilage matrix and influenced by mechanical loading. Due to the concentration of negatively-charged proteoglycans, the fixed charge density (FCD) of cartilage is high. Since the FCD attracts cations into the matrix, the resulting ionic strength and tissue osmolarity are also high. During mechanical loading, this osmotic environment is perturbed as fluid is forced from the matrix, increasing the local proteoglycan concentrations and therefore the ionic strength and osmolarity [Urban, 1994; Urban and Hall, 1994; Mobasheri et al., 1998]. Conversely, as the load is removed fluid moves back into the matrix, reversing changes in ionic strength and osmolarity. As a result of normal joint motion, therefore, chondrocytes experience a

Grant sponsor: National Institute of Arthritis and Musculoskeletal and Skin Diseases; Grant number: 1R03 AR47154-01; Grant sponsor: National Institutes on Aging; Grant number: AG-15107; Grant sponsor: Wellcome Trust Research Fellowship.

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Received 13 February 2002; Accepted 11 April 2002
DOI 10.1002/jcb.10217

dynamic extracellular osmotic environment. To exist in such a challenging environment, chondrocytes must possess mechanisms by which cell volume can be regulated. Indeed it has been demonstrated that chondrocytes possess both regulatory volume increase (RVI) [Errington and Hall, 1995; Errington et al., 1997; Erickson et al., 2001] and regulatory volume decrease (RVD) [Errington et al., 1997; Bush and Hall, 2001; Hall and Bush, 2001] mechanisms in order to regain cell volume following a hyper-osmotic (shrinking) or hypo-osmotic (swelling) stimulus, respectively.

While many of the membrane transport and intracellular signaling mechanisms activated in response to hyper-osmotic stress have been identified in chondrocytes [Mobasheri et al., 1998], little is known about those activated by hypo-osmotic stress. These may be particularly significant in disease states such as osteoarthritis in which chondrocytes are subject to extended periods of hypo-osmolarity caused by the loss of proteoglycans from the matrix. In other cell types, ion extrusion pathways, typically K^+ and Cl^- channels, are activated by cell swelling, resulting in a loss of ions, water, and recovery of initial cell volume [Hoffman and Dunham, 1995; O'Neill, 1999]. In many cell types, Ca^{2+} also plays a pivotal role in this volume-regulatory process [McCarty and O'Neil, 1992]. Cell swelling commonly results in an increase in $[Ca^{2+}]_i$ [McCarty and O'Neil, 1991a; Ross and Cahalan, 1995; Chen et al., 1996] which if inhibited can attenuate RVD in some cells [McCarty and O'Neil, 1991b; Altamirano et al., 1998]. The mode of action of Ca^{2+} is unclear, but there is evidence that Ca^{2+} may be required to activate K^+ and Cl^- ion channels and thereby, effect the extrusion of ions from the cell [Rothstein and Mack, 1990, 1992; Kawahara et al., 1991; Kotera and Brown, 1993; Yu and Sokabe, 1997; Weskamp et al., 2000].

Comparatively little is known about the effects of cell swelling on $[Ca^{2+}]_i$ and membrane currents in bovine articular chondrocytes (BAC). Such effects may also have the potential to play a role in mechanotransduction in BAC, since changes in $[Ca^{2+}]_i$ and activation of membrane ion channels have also been demonstrated in chondrocytes subjected to a variety of other physical stimuli including direct membrane deformation, fluid flow, and hydrostatic pressure [Wright et al., 1996; Yellowley et al., 1997, 1999; Guilak et al., 1999; Edlich et al., 2001]. Indeed, it has been proposed that chondrocytes use load-induced changes in their osmotic environment as a stimulus to maintain the integrity of the matrix [Urban, 1994; Urban and Hall, 1994]. Changing external osmolarity has been shown to have profound effects on matrix protein synthesis in BAC [Urban and Bayliss, 1989; Urban et al., 1993; Urban and Hall, 1994]. It is possible then that signaling pathways that are actively involved in the mechanisms of volume regulation may also be involved in the mechanism by which mechanical signals are sensed and ultimately transduced into cellular-metabolic responses.

The aim of this study was to investigate the effects of hypo-osmotic swelling on intracellular Ca^{2+} in BAC, to determine the effects of swelling magnitude, and identify mechanisms underlying the $[Ca^{2+}]_i$ response. In parallel, we also studied the ability of BAC to regulate cell volume in response to hypo-osmotic swelling and a role for $[Ca^{2+}]_i$ in this process. Finally, using the patch clamp technique, we looked at the effect of hypo-osmotic swelling on whole cell ionic current activation in BAC.

MATERIALS AND METHODS

Chondrocyte Isolation and Culture

BAC were isolated as previously described [Yellowley et al., 1997]. Briefly, slices of articular cartilage were dissected from bovine-hock joints, diced and digested for 2–4 h in a mixture of 0.15 mg/ml deoxyribonuclease, 2 mg/ml collagenase, and 0.1 mg/ml hyaluronidase at 37°C. The supernatant was removed, filtered through a 120 μM Nytex filter (Tetko, Briarcliff Manor, NY), and centrifuged at 200*g* for 5 min. The resulting cell pellet was washed three times in Hank's balanced salt solution and resuspended in RPMI-1640 medium supplemented with 20% fetal bovine serum and 1% penicillin and streptomycin (all Gibco-BRL, Grand Island, NY). The cells were plated in 25 cm^2 culture flasks (Corning) and grown to confluency (10–14 days). The cells were subcultured with a 0.25% sterile trypsin solution and plated onto 24×60 mm, 1.6 mm thick quartz slides (Friedrich and Dimmock, Inc., Millville, NJ) for the $[Ca^{2+}]_i$ experiments, and onto 25-mm diameter glass coverslips for cell volume and patch clamp experiments. Only passage 1 cells were used for experiments. We have previously shown that chondrocytes isolated and cultured

in this manner express characteristics of the chondrocyte phenotype, including a chondrocytic morphology and expression of type II collagen as detected by indirect immunofluorescence [Yellowley et al., 1997].

Cell Swelling and Pharmacological Agents

Cells were perfused with Tyrode's solution containing in millimoles; 140 NaCl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 5 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid, and 10 glucose titrated to pH 7.4. For cell swelling external NaCl content was reduced to 100, 80, and 60 mM, reducing osmolarity from 295 mOsm (normal) to 220 (−75), 185 (−110), and 145 mOsm (−150), respectively. Three pharmacological agents were used to assess the contribution of intracellular stores and membrane ion channels to the swelling-induced $[Ca^{2+}]_i$ response in BAC. These included, 1) thapsigargin (50 nM) which inhibits the ATP-dependent Ca^{2+} pump of intracellular stores and causes Ca^{2+} discharge [Thastrup et al., 1990]; 2) nifedipine (20 μM), an inhibitor of L-type voltage gated calcium channels; 3) gadolinium chloride (10 μM), an agent that has been widely used in the study of stretch activated responses [Yellowley et al., 1997; Guilak et al., 1999] and which inhibits stretch activated channels [Yang and Sachs, 1989]. Cells were exposed to each drug 5–30 min prior to experimentation.

Ca^{2+} Imaging

Preconfluent cells were washed with Tyrodes solution at 37°C. Cells were then incubated with the dual excitation Ca^{2+}-probe Fura-2 (acetoxymethyl (AM) ester 1 μM, Molecular Probes, Eugene, OR) in Tyrode's solution for 30 min at 37°C. The cells were then washed with fresh Tyrode's solution at 37°C, and the slide mounted on a parallel plate flow chamber, which allowed us to superfuse the cells with solutions of varying osmolarity. We have described this chamber previously [Yellowley et al., 1997]. The chamber was placed on an inverted fluorescence microscope (Nikon Diaphot 300) and left undisturbed for 30 min.

The cells were illuminated as described previously [Yellowley et al., 1997]. A Metafluor imaging system (Universal Imaging, West Chester, PA) was used to sample and record the emitted light from the cells in the field of view once every 2.5 s (emission wavelength 510 nm) and Metafluor imaging software was used to subtract the background fluorescence from each image and to outline and calculate the 340:380 ratio of light emitted in response to excitation at 340 and 380 nm for each cell in the field of view, as this ratio reflects $[Ca^{2+}]_i$. A calibration curve was constructed by acquiring 340:380 values (background subtracted) for a series of solutions of known free Ca^{2+} concentration (0–39.8 μM, Molecular Probes) and 1 μM fura2 pentapotassium salt (Molecular Probes). This calibration curve was used to convert ratio values from individual cells into $[Ca^{2+}]_i$.

Cell Volume Measurements

Cell volume was determined indirectly using the fluorescent probe calcein [Crowe et al., 1995; Sonnentag et al., 2000; Hall and Bush, 2001]. Chondrocytes were detached from the culture flask using a cell scraper, transferred to 25 mm diameter glass coverslips and allowed to adhere for 45 min. This settling time resulted in cells which were rounded and firmly attached to the coverslip. A rounded cell morphology was required to allow for uniform cell swelling. After attachment, cells were loaded with calcein dye (2 μM) for 10 min washed with fresh Tyrode's solution and transferred to the epifluorescent microscope. A 40 ×/1.3, oil immersion fluor objective was used to visualize dye loaded chondrocytes. Calcein was excited at 450–490 nm and the emitted light collected at 515 nm. Calcein fluorescence was recorded from a small region of the chondrocyte by outlining digitally an area of the cell approximately 3–5% of the total cell area as described by Crowe et al. [1995]. Calcein fluorescence was monitored for 2–3 min prior to swelling to establish a steady baseline. The bathing solution was then replaced with hypo-osmotic Tyrode's solution (−150 mOsm) and fluorescence recorded for a total of 35 min.

Electrical Recording

For recording, cells were placed on the stage of an inverted microscope (Nikon Diaphot) and superfused at a rate of 1.25 ml/min with Tyrode's solution. Two miniature solenoid valves allowed for switching between solutions perfusing the preparation. Cell membrane ionic currents were recorded using the perforated whole cell configuration of the patch clamp technique [Korn et al., 1991; Rae et al., 1991]. Patch pipettes were made from unfilamented borosilicate Kovar sealing glass (Corning #7052; A.M.

systems Inc.) pulled in two stages on a vertical puller (Narishige). Electrode tips were then polished using a microforge (Narishige) to a final resistance of between 3 and 5 MΩ. The internal solution contained in millimoles: 130 KCl; 10 NaCl; 2 $MgCl_2$; 10 HEPES; 5 glucose; and was titrated to a final pH of 7.2 with 10 mM KOH. The tip of each patch electrode was filled with internal solution by dipping the tip in solution for 1 s. Electrodes were then backfilled with internal solution containing amphotericin B. Amphotericin B was added to the internal solution to a final concentration of 250 μM (240 μg/ml). Once a seal was achieved, typically greater than 5 GΩ, the access resistance (R_a) was monitored using pClamp 7 software (Axon Instruments, CA). An R_a of less than 10 MΩ was achieved prior to starting the experimental recording. Data from whole-cell voltage clamp experiments were recorded and analyzed using an Axopatch 200 amplifier (Axon instruments, CA) and a CV-201 headstage. The voltage command was produced by pClamp7 (Axon Instruments) patch and voltage clamp software in conjunction with a digidata A/D interface (Axon Instruments, CA).

Data Analysis

To identify Ca^{2+} transients, we used a numerical procedure adapted from mechanical fatigue analysis, known as Rainflow Cycle Counting [Downing and Socie, 1982]. This simple algorithm reliably and automatically identifies and determines the amplitudes of spikes and transients in time history data even when superimposed over each other or in the presence of background noise which otherwise might make amplitude determination challenging [Jacobs et al., 2000]. We have previously used this algorithm to identify transients in $[Ca^{2+}]_i$ in chondrocytes and bone cells [Jacobs et al., 1998; Yellowley et al., 1999; Edlich et al., 2001]. We defined a response as a transient increase in $[Ca^{2+}]_i$ of 20 nM or greater. Data were collected for 1 min at the start of each experiment prior to swelling, and then for a period of 7 min during swelling.

For cell volume measurements, calcein fluorescence in hypo-osmotic saline was expressed as a percentage of initial fluorescence in control Tyrode's solution for each cell. Data are expressed as mean ± SEM. To compare observations from different groups with unequal sample size, a two sample Student's *t*-test was used in which sample variance was not assumed to be equal. To compare observations from more than two groups, a one-way analysis of variance was used followed by a Student–Newman Keuls multiple comparisons post test (Instat, GraphPad Software Inc.). $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Cell Swelling on $[Ca^{2+}]_i$ in BAC

Initial experiments aimed to determine the $[Ca]_i$ response to swelling in BAC. Approximately 1 min following application of hypo-osmotic Tyrode's solution (−150 mOsm) a number of BAC displayed transient increases in $[Ca^{2+}]_i$, Figures 1 and 2. Most cells responded within the first 3–4 min of swelling. To analyze our data statistically we determined the percentage of cells responding to hypo-osmotic Tyrode's as a function of the strength of the hypo-osmotic solution. Consistent with what we have observed previously [Yellowley et al., 1997, 1999], 6.0 ± 2.2% of control cells showed spontaneous $[Ca^{2+}]_i$ transients in the absence of swelling; 28.7 ± 3.6% of cells responded to a decrease in osmolarity of −75 mOsm; 43.9 ± 10.7% to −110 mOsm and 73.2 ± 5.5% to −150 mOsm, Figure 3. Responses to −110 and −150 mOsm were significantly greater than control. There was no significant difference between experimental groups with regard to mean $[Ca^{2+}]_i$ response amplitudes. Mean $[Ca^{2+}]_i$ response amplitudes for those cells showing a response greater than 20 nM were 42.7 ± 1.9, 41.9 ± 4.1, and 64.3 ± 10.2 nM for −75, −110, and −150, respectively, Figure 3. The mean amplitude of spontaneous transients in BAC in isotonic saline was 30.5 ± 3.1 nM and was not significantly different from that of transients observed in BAC exposed to hypo-osmotic Tyrode's. Hence the principal effect of the hypo-osmotic swelling stimulus was to increase the proportion of cells that exhibited a Ca^{2+} response, without significantly altering response amplitude.

Experiments were then performed to assess the contribution of extracellular and intracellular Ca^{2+} to the swelling-induced $[Ca^{2+}]_i$ response, and to identify possible Ca^{2+} entry pathways. BAC were exposed to −150 mOsm hypo-osmotic Tyrode's, which was found to elicit the maximum percent response in the experiments described above, in the presence or

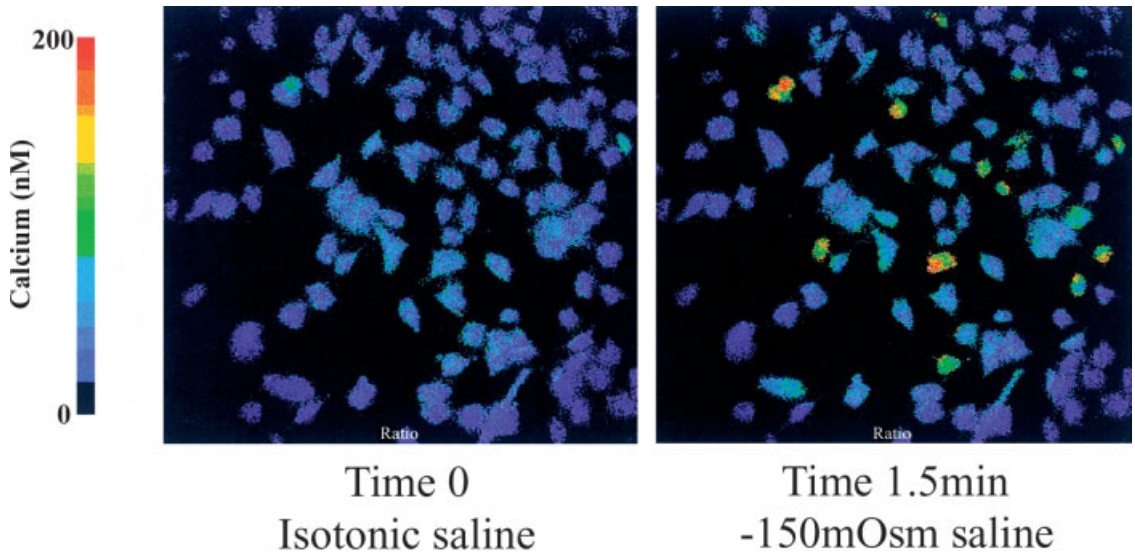

Fig. 1. Pseudocolor image of BAC loaded with Fura-2-AM before (time 0) and 1.5 min after exposure to hypo-osmotic Tyrode's. An increase in fluorescence which corresponds to an increase in $[Ca^{2+}]_i$, is indicated by the warmer red and yellow tones. During exposure to hypo-osmotic Tyrode's a number of BAC display $[Ca^{2+}]_i$ increases.

absence of nifedipine, gadolinium chloride, thapsigargin and zero extracellular Ca^{2+}, Figure 4. A total of $73.2 \pm 5.5\%$ of cells responded to -150 mOsm saline. In the presence of 20 μM nifedipine, the percentage of cells responding was $67.5 \pm 11.2\%$ which was not significantly different from control (-150). However, in the presence of 10 μM gadolinium chloride, 50 nM thapsigargin or zero extracellular Ca^{2+}, the percentage of cells responding to a drop in osmolarity of -150 mOsm was significantly decreased compared to control (-150), to 7.2 ± 2.5, 8.1 ± 3.0 and $27.1 \pm 8.8\%$, respectively (Fig. 4). There was no significant difference between experimental groups with respect to mean $[Ca^{2+}]_i$ response amplitude. Mean $[Ca^{2+}]_i$ response amplitudes for those cells showing a response greater than 20 nM were 64.3 ± 10.2, 42.6 ± 2.3, 37.2 ± 9.8, 25.9 ± 2.7, and 29.5 ± 6.6 nM for control (-150), nifedipine, gadolinium, thapsigargin and 0 external Ca^{2+}, respectively. There was no significant effect of any inhibitor treatment on baseline calcium levels.

Cell Volume Regulation in BAC

BAC exposed to hypo-osmotic Tyrode's (-150 mOsm) swelled in size to a peak at approximately 5 min, as indicated by a decrease in

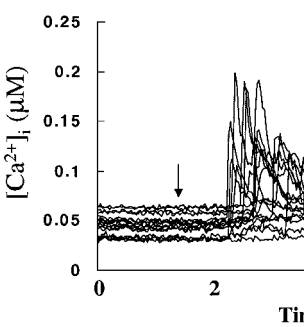

Fig. 2. Effect of hypo-osmotic swelling on $[Ca^{2+}]_i$ in BAC. Each line represents the $[Ca^{2+}]_i$ signal from a single cell. Cells were exposed to -150 mOsm Tyrode's solution for 7 min. ↓ Indicates the onset of exposure.

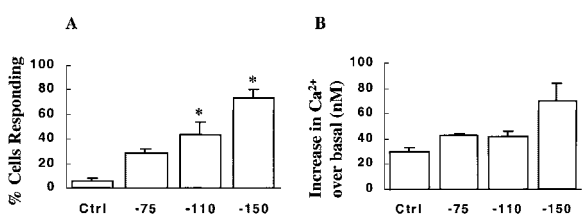

Fig. 3. **A**: Mean percentage of cells showing a spontaneous $[Ca^{2+}]_i$ transient in normal (Ctrl) saline and a $[Ca^{2+}]_i$ response in the presence of hypo-osmotic Tyrode's -75, -110, and -150 mOsm. **B**: Mean increase in $[Ca^{2+}]_i$ in cells showing spontaneous transients (Ctrl) and a response in the presence of hypo-osmotic Tyrode's -75, -110, and -150 mOsm. Each bar represents the mean ± SEM and each experiment was repeated on 3, 5, 7, and 14 separate slides for Ctrl, -75, -110, and -150, respectively. Approximately 60 cells are imaged per slide. *Statistically significant difference from control.

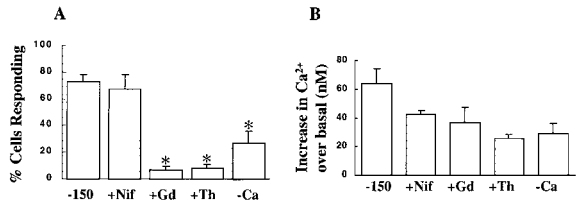

Fig. 4. **A**: Mean percent of cells showing a $[Ca^{2+}]_i$ response to hypo-osmotic Tyrode's (−150 mOsm) in the presence or absence of nifedipine (Nif), gadolinium (Gd), thapsigargin (Th) or zero external Ca^{2+} (−Ca). **B**: Mean increase in $[Ca^{2+}]_i$ in cells showing a response to hypo-osmotic Tyrode's (−150 mOsm) in the presence or absence of nifedipine (Nif), gadolinium (Gd), thapsigargin (Th) or zero external Ca^{2+} (−Ca). Each bar represents the mean ± SEM and each experiment was repeated on 14, 3, 4, 3, 7 separate slides for −150, Nif, Gd, Th and 0 Ca, respectively. *Statistically significant difference from −150.

calcein fluorescence, and were able to gradually regulate their size back towards normal over the following 30 min, Figure 5. In similar experiments, we have obtained photomicrographic images of BAC before and during the swelling period which were digitized and cell area determined at 30 s intervals. These data (not shown) confirmed that peak cell size was attained 5 min after application of hypo-osmotic Tyrode's solution. In the presence of gadolinium, which significantly inhibited the swelling-induced Ca^{2+} transients as described above, volume regulation appeared to be inhibited and cell size continued to increase over the entire time period. Calcein dye fluorescent intensity was maximally reduced to 87.4 ± 1.1% of baseline fluorescence in cells exposed for 30 min to −150 mOsm Tyrode's solution. In the presence of gadolinium, the calcein dye fluorescence was reduced significantly further to 76.6 ± 4.0% of baseline, Figure 6A. Gadolinium significantly inhibited volume recovery in BAC. Calcein dye fluorescent intensity at the end of the 30 min hypo-osmotic (−150 mOsm) exposure period was 105.8 ± 1.3% of baseline in the absence of gadolinium and 77.7 ± 4.2% in the presence of gadolinium.

Effects of Cell Swelling on Whole Cell Membrane Currents in BAC

The foregoing data indicate that BAC responded to a hypo-osmotic stimulus with generation of transient increases of $[Ca^{2+}]_i$ and increases in cell size. Both responses were sensitive to external application of Gd^{3+}. To investigate this phenomenon further, we measured whole cell ionic current from BAC and tested effects of swelling and external application of Gd^{3+} on this. Figure 7A (upper panel) shows representative raw current traces elicited from a cell by a voltage ramp protocol shown in the lower panel of Figure 7A. In control, comparatively little current was elicited at negative membrane potentials, and current became progressively more outward at more positive potentials. External osmolarity was then decreased by

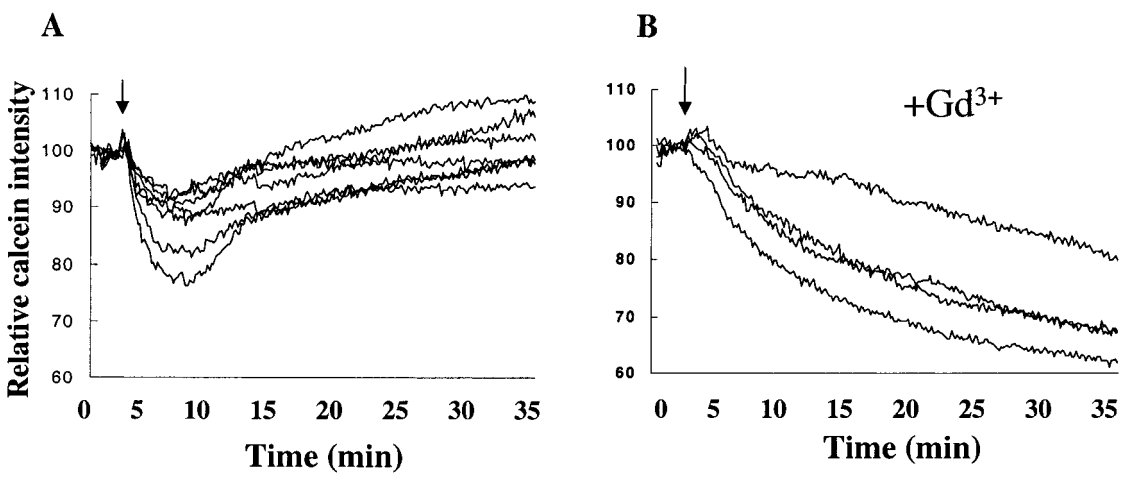

Fig. 5. Calcein dye fluorescence intensity as a percentage of baseline values in cells exposed to −150 mOsm Tyrode's solution for 30 min in the absence (**A**) and presence (**B**) of gadolinium chloride (10 μM). Each line represents the fluorescent calcein signal from a single cell and ↓ indicates the application of hypo-osmotic Tyrode's.

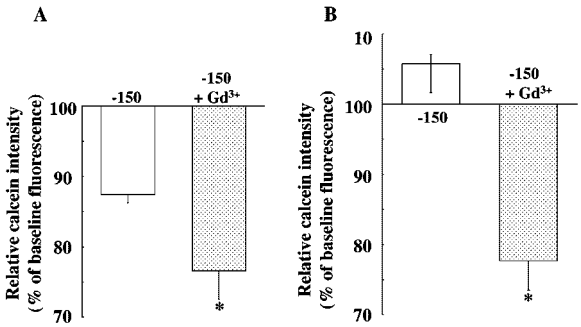

Fig. 6. **A**: Maximum decrease in calcein dye fluorescent intensity relative to baseline in cells exposed to −150 mOsm Tyrode's solution for 30 min in the absence and presence of gadolinium chloride (10 μM). **B**: Mean calcein dye fluorescent intensity relative to baseline measured at the end of the 30 min hypo-osmotic (−150 mOsm) exposure period in the absence and presence of gadolinium chloride (10 μM). Each bar represents the mean ± SEM and each experiment was repeated on 17 or 8 separate cells for −150 or $-150 + Gd^{3+}$, respectively. *Statistically significant difference from control (−150).

−150 mOsm. During a 6 min hypo-osmotic swelling period there was a marked increase in both inward and outward whole cell current, which was partially reversible on returning to isotonic saline. The "swelling-activated" current was determined by subtracting the current in control from that in −150-mOsm solution (Fig. 7B). This current was outwardly rectifying and reversed negative to 0 mV (the mean reversal potential from four cells was −10.6 ± 3.2 mV). Figure 7C shows the mean current–voltage (I–V) relationship for swelling activated current (n = 4). For each cell data were normalized to cell capacitance (which reflects cell surface area), and the resulting current density values were pooled. The mean I–V relationship showed a weakly outwardly rectifying profile.

The effects of Gd^{3+} are shown in Figure 8. In the presence of gadolinium, baseline current was attenuated and the current response to the hypo-osmotic challenge was largely abolished (Fig. 8A). Figure 8B,C contain histograms showing mean current density plots at +80 mV (Fig. 8B) and −80 mV (Fig. 8C) for absolute current under the different experimental conditions. At both potentials, swelling produced a significant increase in current in the absence, but not in the presence of Gd^{3+}.

DISCUSSION

A swelling-induced increase in $[Ca^{2+}]_i$ has been identified in many cells [McCarty and

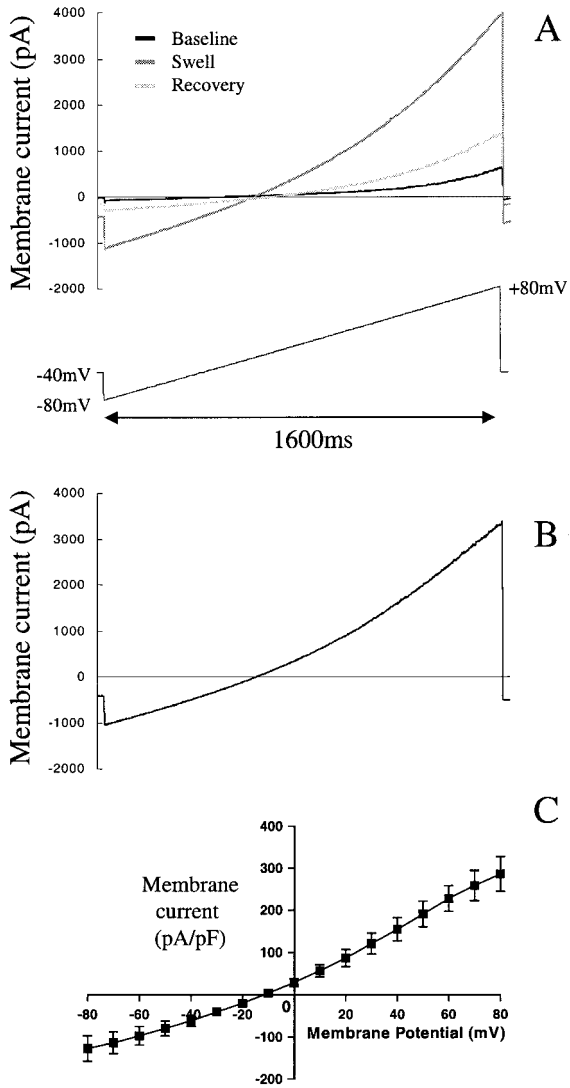

Fig. 7. Effects of hypo-osmotic saline on whole cell membrane current in BAC. **A**: Membrane potential was held at −40 mV and stepped from −80 to +80 mV in a continuous ramp over 1,600 ms (ramp was applied every 2 s).''Baseline'' membrane currents were recorded in normal Tyrode's. Cells were then superfused with hypo-osmotic Tyrode's (−150 mOsm) for 6 min. ''Swell'' was the maximum membrane current recorded during the swelling period. After 6 min, cells were returned to normal Tyrode's and recovery of whole cell current was monitored for up to 20 min. ''Recovery'' was membrane current recorded at the end of the recovery period. This trace is typical of four similar recordings. **B**: Swelling sensitive current generated by subtracting ''baseline'' whole cell current from ''swell'' current. **C**: Mean I/V relationship for swelling sensitive currents. Current amplitudes were measured in response to a voltage ramp as described above in normal and hypo-osmotic Tyrode's. Swelling sensitive currents were obtained by subtraction and normalized to cell capacitance. The mean ± SEM was plotted for four cells.

O' Neil, 1992] and has been described as transient [Ross and Cahalan, 1995], sustained [Chen et al., 1996], or a combination of transient and sustained [McCarty and O'Neil, 1991a]. In BAC, the $[Ca^{2+}]_i$ response was transient and in addition was activated after a delay of at least 60 s. A delay prior to $[Ca^{2+}]_i$ increase has been noted in other cells in response to hypo-osmotic swelling, for example, Ca^{2+} influx in mouse thymocytes was activated after a delay of 70 s which corresponded with maximum volume during the swelling period [Ross and Cahalan, 1995]. One possibility is that the chondrocytes possess an excess of cell membrane, for example in membrane folds or ruffles that must be pulled out during the first minute of swelling, before the membrane is truly stretched. This hypothesis is supported by data from Guilak and Ting-Beal [1999] who demonstrated that isolated chondrocytes do possess many ruffles in isosmotic media, which smooth out during hypo-osmotic swelling. In BAC, peak volume was attained after approximately 4 min suggesting that the cell membrane did not have to be maximally stretched for the $[Ca^{2+}]_i$ response to occur. In experiments by Hall and Bush [2001], using almost identical fluorescent techniques, chondrocyte peak volume was attained after approximately 1 min in hypo-osmotic medium (−160 mOsm). This discrepancy in the time to peak is most likely explained by the fact that our experiments were performed at room temperature while Bush and Hall performed experiments at 37°C. It has been shown that temperature significantly affects RVD such that as the temperature is decreased, the time to attain maximum volume is increased [Kanli and Norderhus, 1998].

In addition, our experiments show that there was an inverse relationship between the percentage of cells displaying a $[Ca^{2+}]_i$ transient and external osmolarity. That is, as the external osmolarity decreased the percentage of cells responding increased. We have reported a similar relationship between $[Ca^{2+}]_i$ transients in BAC activated in response to fluid flow, and

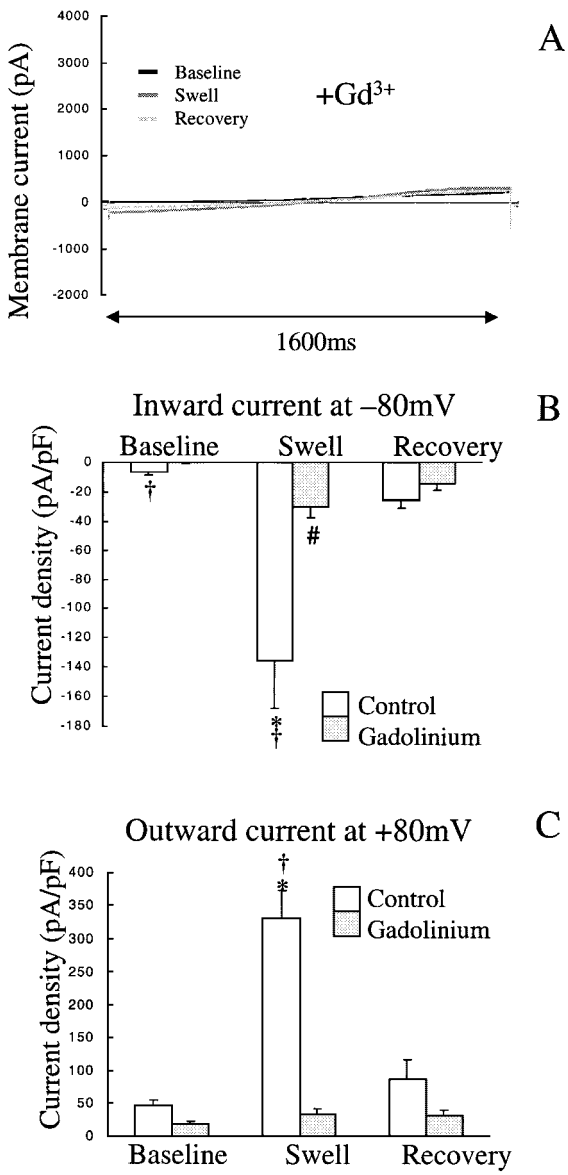

Fig. 8. Effects of gadolinium (Gd^{3+}) on swelling currents generated in the presence of hypo-osmotic saline in BAC. **A**: The cell was held at −40 mV and stepped from −80 to + 80 mV in a continuous ramp over 1,600 ms as described in Figure 7. All currents were recorded in the presence of 10 μM Gd^{3+}. ''Baseline'' membrane currents were recorded in normal Tyrode's solution. Cells were then superfused with hypo-osmotic Tyrode's (−150 mOsm) for 6 min. ''Swell'' was the maximum membrane current recorded during the swelling period. After 6 min, cells were returned to normal saline and recovery of whole cell current was monitored for up to 20 min. ''Recovery'' was membrane current recorded at the end of the recovery period. This trace is typical of three similar recordings. **B**: Maximum inward current measured at −80 mV during the baseline, swell or recovery period in the presence and absence of Gd^{3+}. **C**: Maximum outward current measured at +80 mV during the baseline, swell or recovery period in the presence and absence of Gd^{3+}. Each bar represents the mean ± SEM and each experiment was repeated on four and three cells for control and Gd^{3+}, respectively. †Statistically significant difference from Gd^{3+} within an experimental pair. *Statistically significant difference from baseline control. #Statistically significant difference from baseline gadolinium.

flow rate [Yellowley et al., 1997]. We have speculated that a possible explanation for the stimulus magnitude dependence of the $[Ca^{2+}]_i$ response might be that individual cells express varying levels of a "mechano (flow)-receptor" (as yet undefined), depending on their differentiation state or stage in the cell cycle [Yellowley et al., 1997]. In this scenario, cells expressing low levels of the receptor would display a $[Ca^{2+}]_i$ transient only at the lowest external osmolarity or rather the greatest swelling stimulus. Interestingly, there was no effect of decreasing external osmolarity on the amplitude of the $[Ca^{2+}]_i$ signal suggestive of an all-or-nothing type response.

The source of Ca^{2+} for swelling induced $[Ca^{2+}]_i$ increase in other cell types is varied and has been shown to be extracellular, extracellular supplemented by intracellular, or solely intracellular [McCarty and O' Neil, 1992]. Our data suggest that the source of Ca^{2+} in BAC may be comprised of both extracellular and intracellular components, since removing extracellular calcium or applying thapsigargin each significantly inhibited, but did not completely abolish the response. While the pathway for extracellular Ca^{2+} influx is unclear, an attractive hypothesis is that swelling, and the accompanying membrane stretch, activates stretch activated Ca^{2+} permeable ion channels and effects $[Ca^{2+}]_i$ increases [Christensen, 1987]. Such a scenario has been proposed in a number of cell types where swelling-induced extracellular Ca^{2+} influx is inhibited by gadolinium, a putative stretch activated channel blocker [McCarty and O' Neil, 1992; Chen et al., 1996; Tsuzuki et al., 2000]. Indeed, in this study gadolinium inhibited the swelling-induced Ca^{2+} transients in BAC. In general, data obtained with Gd^{3+} should be interpreted with caution, because although gadolinium has been widely used as a stretch activated channel blocker [Yang and Sachs, 1989], it has also been shown to block other Ca^{2+} currents including L-type Ca^{2+} current and Na^+Ca^{2+} exchange current [Biagi and Enyeart, 1990; Lacampagne et al., 1994; Zhang and Hancox, 2000]. While Ca^{2+} influx through L-type Ca^{2+} channels has been implicated as the Ca^{2+} influx pathway in a number of cell types in response to cell swelling, this does not seem to be the case in BAC since nifedipine, an L-type channel inhibitor, had no effect on the swelling-induced Ca^{2+} increase, and the reversal potential of the swelling-activated current was not commensurate with its identity as a pure I_{Ca}. Thapsigargin, which inhibits the ATP-dependent Ca^{2+} pump on intracellular stores and causes discharge significantly inhibited swelling induced Ca^{2+} transients in BAC. Since both removal of extracellular Ca^{2+} and thapsigargin reduced the percentage of cells responding by almost equal magnitude, it is possible that both are linked in the same pathway. Considering our data collectively, it is possible that swelling activates a Ca^{2+} influx across the cell membrane which triggers Ca^{2+} release from intracellular stores via a Ca^{2+} induced-Ca^{2+} release mechanism as has been suggested in renal A6 cells [Urbach et al., 1999].

It is of interest to note that in recent experiments performed by Erickson et al. [2001] chondrocytes exposed to hyper-osmotic stress also display calcium transients with very similar pharmacology to those described here. Specifically, shrinking elicited transients in $[Ca^{2+}]_i$ that were significantly inhibited by gadolinium, removal of external calcium and blockers of intracellular Ca^{2+} store release [Erickson et al., 2001]. This raises the possibility that both swelling and shrinking may activate the same signaling pathways in chondrocytes via a common mechanism.

In response to a hypo-osmotic challenge, cell swelling activates ion extrusion pathways such as membrane K^+ and Cl^- channels, resulting in a net loss of osmolytes, water and cell shrinkage back to baseline volume in a process known as RVD [Hoffman and Dunham, 1995; O'Neill, 1999]. Our data using calcein to follow cell volume changes suggest that BAC possess an active volume regulatory mechanism. This is in agreement with recent studies by Bush and Hall [2001] and Hall and Bush [2001] who looked at RVD in both isolated and in situ chondrocytes. Interestingly, RVD was significantly inhibited in the presence of gadolinium which we show also inhibits the swelling-induced Ca^{2+} transients in BAC, suggesting a possible role for Ca^{2+} in the RVD mechanism. Ca^{2+} has been shown to play a major role in RVD in many cell types (for review see McCarty and O' Neil [1992]). However, the mechanism by which Ca^{2+} acts is both unclear and controversial. A direct action of Ca^{2+} on ion efflux has been proposed as a result of experiments which demonstrate activation of Ca^{2+} dependent K^+ and Cl^- channels in response to cell swelling [Rothstein and Mack, 1990, 1992; Kawahara et al., 1991; Kotera

and Brown, 1993; Yu and Sokabe, 1997; Weskamp et al., 2000]. However, others have suggested that the mechanism of action of Ca^{2+} may be indirect, for example via activation of a volume sensitive Cl^- current via stimulation of a G protein-coupled Ca^{2+} sensing receptor [Shimizu et al., 2000; Okada et al., 2001]. To complicate the matter further, RVD in other cell types is thought to be calcium independent [Grinstein and Smith, 1990; Medrano and Gruenstein, 1993; Moran et al., 1997]. Several mechanisms by which ion efflux might occur independent of Ca^{2+} have been proposed including activation of ion channels via swelling-induced disruption of the actin cytoskeleton [Schwiebert et al., 1994] and swelling-activation of G-protein signaling [Voets et al., 1998; Estevez et al., 2001]. Therefore, the evidence suggests that the role of Ca^{2+} in RVD is complex, may occur via a number of distinct pathways and is cell-type dependent. With regards to BAC, our collective data may support a mechanism by which RVD is calcium dependent and that the Ca^{2+} entry pathway may be dependent on activation of stretch-activated channels.

As mentioned above, the cellular mechanism of RVD frequently involves activation of K^+ and/or Cl^- channels which effect efflux of ions and, therefore, cell shrinkage. In BAC, we observed an outwardly rectifying swelling current, which reversed at around -10 mV. Using the Nernst equation for a K^+ or Cl^- selective membrane: $E_k = 2.303\ RT/zF\ \log_{10}\ [K]_o/[K]_i$; $E_{Cl} = 2.303\ RT/zF\ \log_{10}\ [Cl]_i/[Cl]_o$, where gas constant $R = 8.315$ $JK^{-1}\ mol^{-1}$, Faraday's constant $F = 9.648 \times 10^4\ Cmol^{-1}$, absolute temperature $T(K) = 273.16 + T°C$ (24°C), z represents the ionic valence, with 4 mM external K and 130 mM internal K and 144 mM internal Cl and 90 mM external Cl^-, E_k, and E_{Cl} were predicted to be -88.7 and $+12$ mV, respectively, during the swelling period. Since the swelling current reverses around -10 mV, this might suggest that the majority of the current is carried by Cl^- ions and partially by K^+. The swelling current was significantly inhibited by gadolinium ions as has been shown in other cell types [Ackerman et al., 1994; Hall et al., 1997; Piao et al., 2001]. As alluded to above, the mechanism of action of gadolinium on membrane current is likely to be an indirect effect on swelling-induced Ca^{2+} influx. Our data suggest that swelling-induced membrane currents in BAC may be modulated by internal Ca^{2+} concentration. Further experiments are underway to confirm the ionic and molecular nature of the swelling activated current in BAC and its cellular regulation.

In summary, we have demonstrated that hypo-osmotic swelling activates Ca^{2+} transients, membrane ion channel currents, and regulated volume decrease in BAC. We propose that a transient rise in intracellular calcium mediated by Ca^{2+} influx through stretch sensitive channels and supplemented by Ca^{2+} release from intracellular stores, may activate Cl^- and K^+ currents in BAC, either directly or indirectly, and therefore effect ion efflux as part of an RVD mechanism. It is likely that ion extrusion via Cl^- and K^+ channels is just one component of the mechanism of RVD in BAC. For example, studies by Hall et al. have shown that chondrocyte swelling activates an osmolyte channel which is capable of transporting both taurine and K^+ out of the cells and which may contribute to RVD [Hall, 1995; Hall et al., 1996; Hall and Bush, 2001].

The ability to regulate cell volume is critical for all cells, but especially for chondrocytes where external osmolarity is dictated by local proteoglycan concentrations which are influenced by cartilage tissue hydration and mechanical load. Interestingly, it has been suggested that chondrocytes may sense load-induced changes in their physical environment, including osmotic change, and interpret them as a stimulus to maintain the integrity of the matrix [Urban, 1994; Urban and Hall, 1994; Smith et al., 1996; Guilak et al., 1999; Hung et al., 2000; Edlich et al., 2001]. Indeed, it has been shown that changes in, for example, extracellular osmolarity do have profound effects on cell matrix metabolism [Urban and Bayliss, 1989; Urban et al., 1993; Urban and Hall, 1994]. Further investigations will be required, however, to determine whether signaling events which occur as a result of RVD, such as Ca^{2+} transients and ion channel activation, are also part of a mechanism by which mechanical loads are transduced by BAC into appropriate metabolic responses.

ACKNOWLEDGMENTS

The authors acknowledge the following funding sources: the National Institute of Arthritis and Musculoskeletal and Skin Diseases (to C.E.Y.), the National Institutes on Aging

(to H.J.D.), and a Wellcome Trust Research Fellowship (J.C.H.).

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