Mechanisms Involved in the Increase in Intracellular Calcium Following Hypotonic Shock in Bovine Articular Chondrocytes

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Abstract. The extracellular osmotic environment of chondrocytes fluctuates during joint loading as fluid is expressed from and reimbibed by the extracellular matrix. Matrix synthesis by chondrocytes is modulated by joint loading, possibly mediated by variations in intracellular composition. The present study has employed the Ca^{2+}-sensitive fluoroprobe Fura-2 to determine the effects of hypotonic shock (HTS) on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and to characterise the mechanisms involved in the response for isolated bovine articular chondrocytes.

In cells subjected to a 50% dilution, $[Ca^{2+}]_i$ rapidly increased by approximately 250%, a sustained plateau being achieved within 300 s. The effect was inhibited by thapsigargin or by removal of extracellular Ca^{2+}, indicating that the rise in $[Ca^{2+}]_i$ reflects both influx from the extracellular medium and release from intracellular stores. Inhibition of the response by neomycin implicates activation of PLC and IP_3 synthesis in the mobilisation of Ca^{2+} from intracellular stores.

The rise was insensitive to inhibitors of L-type voltage-activated Ca^{2+} channels (LVACC) or reverse mode Na^+/Ca^{2+} exchange (NCE) but could be significantly attenuated by ruthenium red, an inhibitor of transient receptor potential vanilloid (TRPV) channels and by Gd^{3+}, a blocker of stretch-activated cation (SAC) channels. The HTS-induced rise in $[Ca^{2+}]_i$ was almost completely absent in cells treated with Ni^{2+}, a non-specific inhibitor of Ca^{2+} entry pathways. We conclude that in response to HTS the opening of SACC and a member of TRPV channel family leads to Ca^{2+} influx, simultaneously with the release from intracellular stores.

Key words: Hypoosmotic shock — Chondrocytes — Calcium — TRPV channels — SAC channels

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Introduction

Articular cartilage provides a mechanically resilient surface to articulating bones which minimises friction and facilitates joint movements (Hall et al. 1996; Huber et al. 2000). During joint movement it is subjected to fluctuations in mechanical load, which initiate variations in the physicochemical environment of chondrocytes. These cells are solely responsible for the upkeep of the extracellular matrix (Muir 1995) and their synthesis of matrix macromolecules is principally regulated by loading patterns (Urban 1994; Wilkins et al. 2000a,b). A number of studies have implicated altered intracellular composition as a transduction mechanism by which load can modulate chondrocyte metabolism (Gray et al. 1988; Behrens et al. 1989; Hall et al. 1991; Urban et al. 1993).

The intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) is one factor which has been shown to be a determinant of matrix synthesis rates. Moreover, as for other intracellular ions, $[Ca^{2+}]_i$ is sensitive to components of mechanical stress (Mobasheri et al. 1998; MacLeod and Hamilton 1999a; D'Andrea et al. 2000; Weskamp et al. 2000; Yellowley et al. 2002). One component which has pronounced effects on $[Ca^{2+}]_i$ in other cells is osmotic changes. Matrix osmolarity is subject to cyclical fluctuations as water is expressed and reimbibed upon onset and release of static load (Urban 1993; Wilkins et al. 2000a). Studies in a range of cells, including chondrocytes (Ross and Cahalan 1995; Chen et al. 1996; MacLeod and Hamilton 1999b; Yellowley et al. 2002), have shown that hypoosmolarity can increase $[Ca^{2+}]_i$. In other cells, the $[Ca^{2+}]_i$ rise has been found to be one of a number of signals which induce activation of membrane transport mechanisms in order to mobilize solutes and water during regulatory volume increase (RVI) (McCarthy and O'Neil 1992; Dascalu et al. 1996; Weskamp et al. 2000; Pasantes-Morales and Morales 2000; Ruwhof et al. 2001) although there is some evidence that activation of RVI is not dependent on $[Ca^{2+}]_i$ in chondrocytes (Kerrigan and Hall 2000). Given, however, the sensitivity of matrix synthesis to variations in $[Ca^{2+}]_i$, understanding the mechanisms underlying the changes in $[Ca^{2+}]_i$ in response to hypoosmotic shocks is an important goal.

As for all cells, chondrocyte $[Ca^{2+}]_i$ is tightly regulated, its level determined by the balance of influx and efflux across the cell membrane and uptake and release from intracellular stores (Busa 1996). A number of potential pathways for these fluxes could operate in the cell membrane, including channels (leak channels or channels activated by voltage, ligands or stretch) and active transporters, a Ca^{2+}-ATPase and a Na^+/Ca^{2+} exchanger (NCE). There is evidence that all of these pathways contribute to $[Ca^{2+}]_i$ regulation in chondrocytes (Mobasheri et al. 1998; Browning and Wilkins 2002). In addition, release of Ca^{2+} from intracellular stores by phospholipase C activation and IP_3 generation has been demonstrated in chondrocytes following exposure to histamine, parathyroid hormone or intracellular alkalinisation (Iannotti et al. 1990; Horwitz et al. 1996; Yellowley et al. 1997; Browning and Wilkins 2002).

In the present study, we have characterised the increase in $[Ca^{2+}]_i$ in bovine

articular chondrocytes, which results following hypotonic shock (HTS). In addition to replicating the findings of earlier studies, we present evidence for the involvement of a transient receptor potential vanilloid (TRPV) channel in the response to hypoosmotic challenge in articular chondrocytes.

Materials and Methods

Media and chemicals

All chemicals and solutions were obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated. Cartilage slices and isolated chondrocytes were incubated in Dulbecco's modified Eagle's medium (DMEM), supplemented with glutamine (2 mmol/l) and antibiotics (Penicillin 100 μmol/l and Streptomicin 100 μmol/l).

The standard experimental solution (HBS) contained (mmol/l): NaCl 145, KCl 5, $CaCl_2$ 2, HEPES 15, Glucose 10, with pH adjusted to 7.4 using concentrated NaOH. In some experiments, solutions lacking Ca^{2+} and supplemented with ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA, 1 mmol/l) to chelate trace Ca^{2+} were employed.

HTS, 290 to 145 mOsm/kg H_2O, was induced by 50 % dilution of the solution with distilled water (DW). The response recorded was not noticeably different using this protocol than when an equivalent osmotic shock was produced by addition of an appropriate volume of buffered solution lacking only NaCl.

Stock solutions of Fura-2 (1 mmol/l), neomycin (1 mol/l), ruthenium red (10 mmol/l), verapamil (10 mmol/l), thapsigargin (100 μmol/l) and ionomicin (3 μmol/l) were made up in dimethylsulphoxide (DMSO). Nigericin (300 μmol/l) was dissolved in ethanol. Trypan blue (5 mmol/l), EGTA (100 mmol/l), $NiCl_2$ (100 mmol/l), $ZnCl_2$ (10 mmol/l), $GdCl_3$ (1 mmol/l) were dissolved in distilled water.

In all cases the stock solutions were made up so that the final concentrations of all the solvents in the cell suspensions or media were not greater than 1 % and often considerably less.

Isolation of chondrocytes

Cells were isolated using a method modified from that described for porcine chondrocytes (Zanetti et al. 1985). Forefeet from 18 to 36 month-old cattle were obtained at slaughter at local abattoir and kept chilled from within 2 h of slaughter until they were used. All feet were used within 72 h of slaughter. The feet were cleaned, skinned and the hooves removed and the metacarpophalangeal joints opened under aseptic conditions.

Cartilage shavings were cut from the articular surfaces and placed in DMEM supplemented with collagenase type I (2000 $U \cdot ml^{-1}$). They were incubated for 18 hours, at 37 °C. At the end of the incubation period, undigested matrix was removed by filtration through a 20–μm Nitex filter cloth (R. Cadish and Sons, London, UK).

The resulting filtrate was centrifuged, the supernatant discarded and the cells resuspended in fresh DMEM. This procedure was repeated twice before cell number was assessed using a Neubauer haemocytometer under light microscopy (Leica

StrataLab) and normalised by resuspension at a concentration of 1×10^6 cells ml^{-1}. Chondrocyte viability was assessed by Trypan blue (0.5 %) exclusion after isolation and during experimental procedures and was greater than 95 % in all the experiments described here.

Measurement of intracellular calcium concentration

$[Ca^{2+}]_i$ was measured for Fura-2-loaded cells in cuvette suspension for periods of 300 s with HTS initiated after 30 s. Isolated cells were loaded with dye by incubation in DMEM containing Fura-2-AM (5 μmol/l) for 30 min at 20 ℃ followed by 15 min at 37 ℃ (Negulescu and Machen 1990). The cell suspension was then centrifuged and the cells resuspended in the appropriate experimental medium, before being transferred to a cuvette. Measurements were made in a fluorimeter (F-2000 spectrophotometer, Hitachi, Tokyo, Japan), thermostatically regulated at 37 ℃ and equipped with a magnetic stirrer.

The dye was alternately excited at 380 nm and 340 nm, with fluorescence emission measured at 510 nm. For each experiment, calibration of the 380 nm/340 nm signal ratio was performed, using the method of Grynkiewicz et al. (1985). The fluorescence ratio was measured in HBS lacking $CaCl_2$ and supplemented with EGTA (1 mmol/l) and also in a 2 mmol/l Ca^{2+} HBS supplemented with ionomycin (300 nmol/l), a concentration of Ca^{2+} at which Fura-2 is saturated. Maximal and minimal ratios (R_{max} and R_{min}) were obtained under these two conditions and $[Ca^{2+}]_i$ values derived using the equation:

$$[Ca^{2+}]_i = K_d(R - R_{min}/R_{max} - R)(S_{f2}/S_{b2})$$

where K_d is the dissociation constant for Fura-2. In the present study, a K_d value of 224 nmol/l was employed. K_d values for Fura-2 are highly variable and dependent on a number of factors including temperature. The value employed here was obtained by Grynkiewicz et al. (1985) under experimental conditions which approximate to those employed in the present study. In the equation, R is the experimental ratio, S_{f2} is the fluorescence measured at 380 nm in Ca^{2+}-free conditions and S_{b2} is the fluorescence measured at 380 nm with saturating Ca^{2+} (2 mmol/l).

Statistics

Results are presented as mean +/− standard error of the mean (SEM), where n is the number of isolation batches. Each isolation batch represents the digestion of cartilage obtained from one joint. For each batch, each experiment was performed in triplicate. Significant differences were determined by Student's unpaired t-test.

Results

The effects of HTS on $[Ca^{2+}]_i$ were recorded over periods of 300 s and the difference from the steady-state $[Ca^{2+}]_i$ at the end of the recording period was calculated.

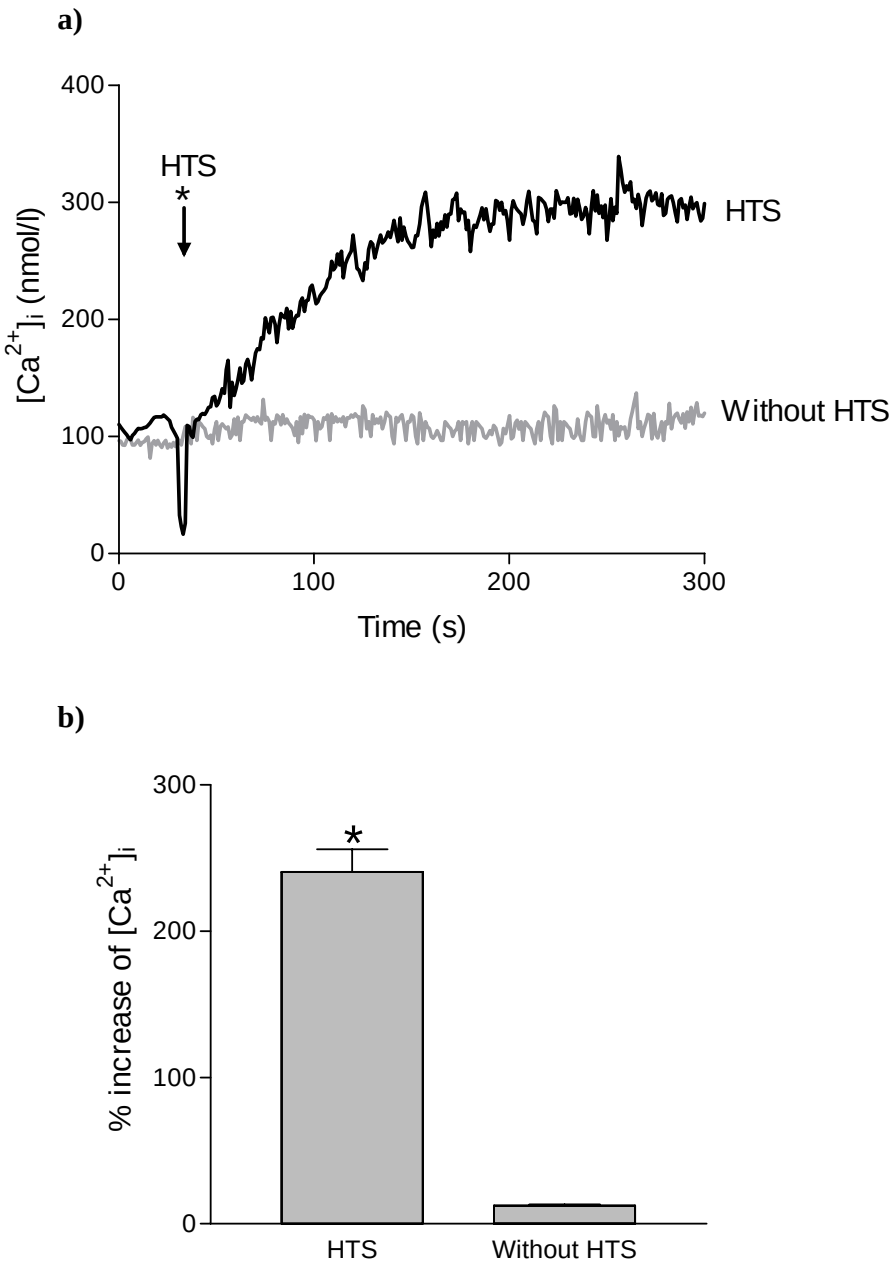

Figure 1. a) Representative recordings of $[Ca^{2+}]_i$ in Fura-2-loaded cells in steady-state conditions and following HTS. Cells were resuspended in HBS and fluorescence was recorded for 300 s. HTS (290 to 145 mOsm/kg H_2O) was produced by dilution with distilled water. **b)** Mean percentage increase in $[Ca^{2+}]_i$ in steady-state conditions ($n = 8$) and following HTS ($n = 5$). * $p < 0.05$.

$[Ca^{2+}]_i$ following HTS was significantly increased (Fig. 1), reaching a sustained plateau within 5 min.

To establish the origin of this increase in $[Ca^{2+}]_i$, chondrocytes were treated with thapsigargin (1 μmol/l, 30 min preincubation in Ca^{2+}-free HBS) to deplete intracellular stores or resuspended in Ca^{2+}-free HBS. In both cases, there was a partial but significant attenuation of the rise following HTS (Fig. 2). Furthermore, there was almost total inhibition of the rise in $[Ca^{2+}]$ following HTS in cells both pretreated with thapsigargin and resuspended in Ca^{2+}-free media. Neomycin (10 mmol/l), a phospholipase C (PLC) inhibitor, also significantly reduced the response (Fig. 3).

To identify membrane transport pathways mediating Ca^{2+} influx following HTS, $[Ca^{2+}]_i$ was measured in chondrocytes which had been treated with a range of inhibitors of potential pathways for Ca^{2+} entry. $NiCl_2$ (2 mmol/l), a non-specific inhibitor of Ca^{2+} channels, attenuated the response, as did $GdCl_3$ (10 μmol/l), an inhibitor of stretch-activated cation (SAC) channels, although to a lesser extent. Ruthenium red (10 μmol/l), a potent inhibitor of TRPV channels (Gunthorpe et al. 2002; Hoenderop et al. 2002; Watanabe et al. 2002), also attenuated the rise. In contrast, verapamil (100 μmol/l), an inhibitor of L-type voltage-activated Ca^{2+} channels (LVACC), and KBR7943 (50 μmol/l), a specific inhibitor of NCE reverse mode, had no effects (Fig. 3).

Discussion

In the present study, we have investigated the response of chondrocyte $[Ca^{2+}]_i$ to HTS and the involvement of the different transport mechanisms in this effect. We have found evidence for the osmotic activation of a TRPV channel in bovine articular chondrocytes.

As previously described in a number of cell types including chondrocytes, exposure to HTS produced an sustained increase in $[Ca^{2+}]_i$ (MacLeod and Hamilton 1999a,b; Souza et al. 2000; Weskamp et al. 2000; Yellowley et al. 2002), which our results indicate originates from both influx from the extracellular solution and release of Ca^{2+} from intracellular stores in roughly similar proportion. A study using fluorescent imaging of Fura-2-loaded chondrocytes by Yellowley et al. (2002) found a similar dependence on extracellular and intracellular sources of Ca^{2+} in the response to hypoosmolarity.

In the present study, we found that Ca^{2+} enters the cell through both SAC and a TRPV channels. $NiCl_2$, $GdCl_3$ and ruthenium red all attenuated the increase of $[Ca^{2+}]_i$ following HTS. While the inhibition induced by $NiCl_2$ was nearly complete, $GdCl_3$ and ruthenium red only partially inhibited the rise, although in each case the effect was significant. In contrast, experiments in which we inhibited the activity of other potential pathways for Ca^{2+} influx failed to show any role for LVACC or the reverse mode operation of NCE. The pronounced inhibition by Ni^{2+} presumably reflects the non-specific effects of this ion against a variety of other Ca^{2+} transport

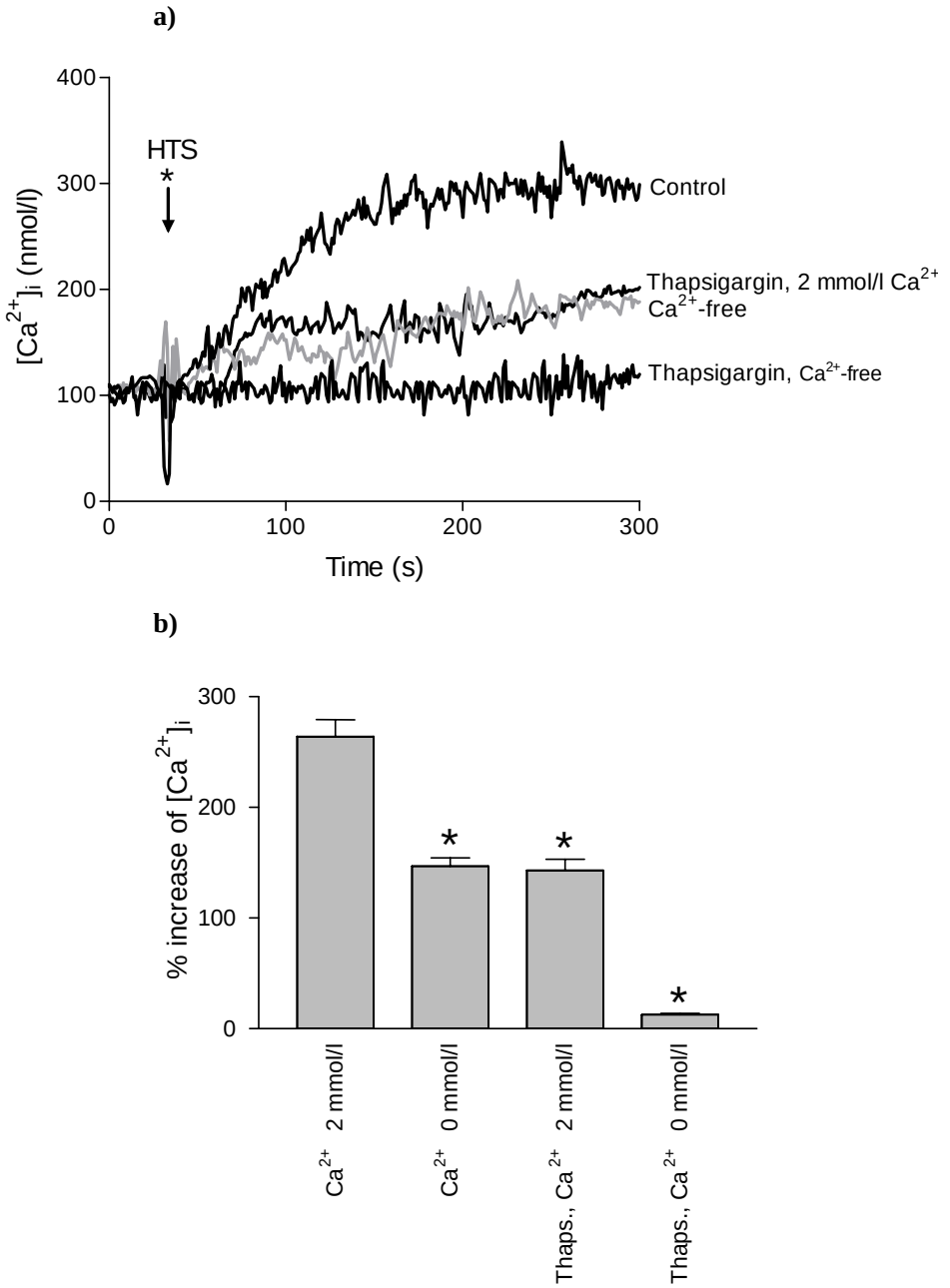

Figure 2. a) Representative recordings of $[Ca^{2+}]_i$ in Fura-2-loaded cells resuspended in HBS (control), HBS supplemented with thapsigargin (1 μmol/l), free-Ca^{2+} medium and free-Ca^{2+} medium supplemented with thapsigargin (1 μmol/l) following HTS. **b)** Mean % increase in Fura-2-loaded cells in the same conditions ($n = 4$ in each case). * $p < 0.05$.

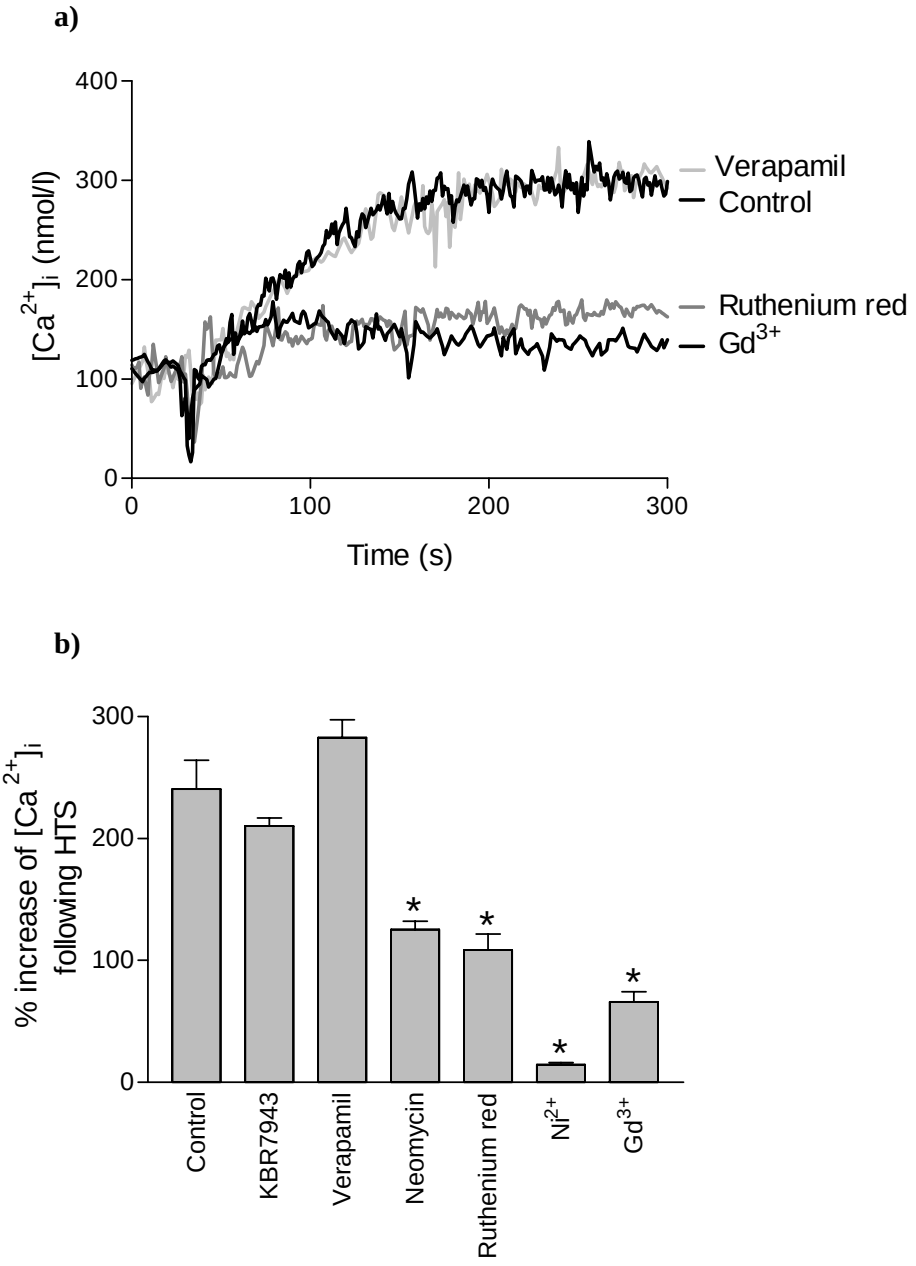

Figure 3. a) Representative recordings of $[Ca^{2+}]_i$ following HTS to Fura-2-loaded cells resuspended in HBS (control) or HBS supplemented with verapamil (100 μmol/l), ruthenium red (100 μmol/l) or $GdCl_3$ (10 μmol/l). **b)** Mean % increase in Fura-2-loaded cells treated with a diversity of pharmacological agents, concentrations indicated in the text. * $p < 0.05$.

pathways (Shibuya and Douglas 1992; Peng et al. 1999; Hobai et al. 2000; Viana et al. 2001).

The sensitivity to Gd^{3+} and ruthenium red reflects participation of SAC and TRPV channels respectively. The role of Gd^{3+}-sensitive SAC channel (Sackin 1995) as a pathway for influx of Ca^{2+} following membrane stretch has been confirmed in a number of studies of diverse cell types, including chondrocytes (Christensen 1987; Filipovic and Sackin 1991; Wright et al. 1996; Yellowley et al. 1997; Guilak et al. 1999).

It should be noted that ruthenium red also inhibits ryanodine receptors (RyR) (Xu et al. 1999), which are characteristic of excitable cells (Fill and Copello 2002) but have also been described in non-excitable cells (Larini et al. 1995). However, with the exception of osteoclasts (Moonga et al. 2000), RyR are always expressed on intracellular membranes, to which the highly charged ruthenium red molecule will not have access. For this reason, sensitivity to extracellular ruthenium red is best interpreted as evidence for the function of a TRPV channel.

TRPV channels are a subfamily of channels named TRP proteins, that share common features: amino-terminal ankyrin repeats; six transmembrane domains; a pore loop between transmembrane segment 5 and 6; permeability for monovalent cations and Ca^{2+}; inhibition by ruthenium red and osmotic sensitivity (Gunthorpe et al. 2002; Montell et al. 2002). They play important roles in many processes ranging from sensory physiology to vasorelaxation and the epithelial transport of Ca^{2+}. Since identification of the first member, the vallinoid receptor (TRPV1), a family comprising at least six members has emerged, the most studied of which have been the epithelial Ca^{2+} channels, ECaC 1 and 2 (TRPV5 and 6 respectively).

Originally characterized in epithelial cells (Suzuki et al. 2000; Hoenderop et al. 2001), ECaC expression has also been demonstrated in a range of non-epithelial cells (Müller et al. 2000), although its role in chondrocytes had not been previously assessed. However, since chondrocytes possess vitamin D receptors (Suda et al. 1985; Tetlow and Woolley 2001), a characteristic of cells expressing ECaC (Hoenderop et al. 1999, 2002; Vennekens et al. 2001a) and vitamin D has been shown to modulate matrix synthesis by these cells (Plachot et al. 1982; Dickson and Maher 1985), the ruthenium red sensitivity we observe may reflect operation of ECaC. Alternatively, TRPV4 may be the variant responsible, since the expression of this channel has recently been described in bovine and human chondrocytes (Votta et al. 2003) and its sensitivity to osmolarity has been shown in chondrocytes (Alford et al. 2003) and other cell types (Liedtke et al. 2000; Strotmann et al. 2000).

The concentration of ruthenium red required to inhibit ECaC and TRPV4 are of approximately similar magnitude (1–10 μmol/l) (Gunthorpe et al. 2002), which precludes the use of this inhibitor to distinguish the roles of the different channels in the hypoosmotic induced increase in $[Ca^{2+}]_i$ which we have recorded.

The mechanisms by which the channels respond to HTS are not clear, although it is possible that other events, initiated in response to HTS, may be involved. Hence, there is evidence that TRPV proteins may be sensitive to $[Ca^{2+}]_i$ itself (Hoenderop et al. 2001, 2002; Nilius et al. 2001; Gunthorpe et al. 2002) and to

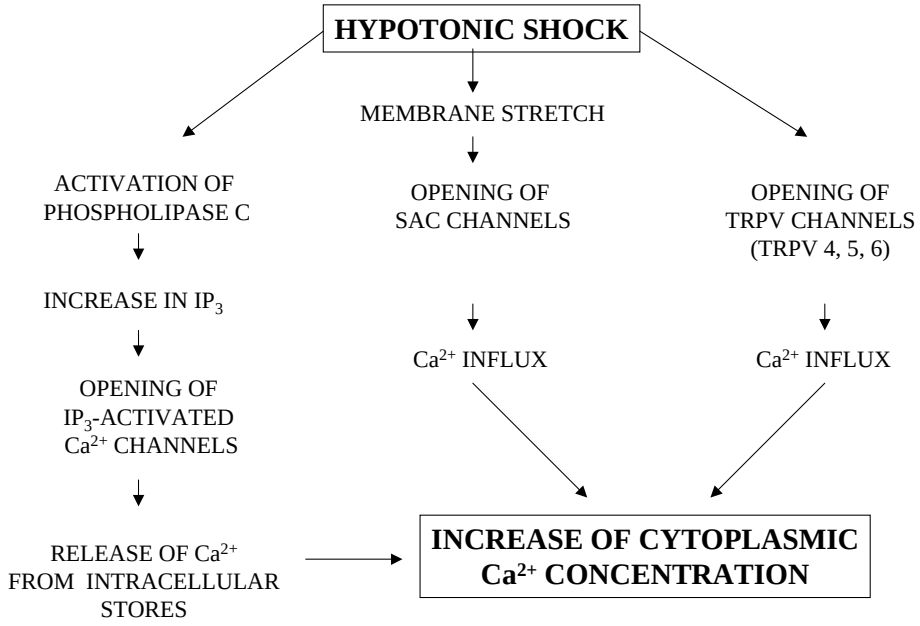

Figure 4. Summary of the possible mechanisms of increase in $[Ca^{2+}]_i$ following HTS in articular bovine chondrocytes.

pH (Vennekens et al. 2001b; Gunthorpe et al. 2002), another factor which we have found to be altered in response to osmotic challenges (Yamazaki et al. 2000).

Our findings suggest that the increase in $[Ca^{2+}]_i$ which follows HTS also involves the release of Ca^{2+} from intracellular stores. This process requires the activation of PLC since neomycin significantly attenuated this effect. PLC activation presumably increases production of IP_3, which opens ligand-gated channels on intracellular membranes. This common mechanism has been described in a wide range of cells in response to a variety of triggers and has been described in several studies of chondrocytes (Iannotti et al. 1990; Horwitz et al. 1996; Yellowley et al. 1997; Browning and Wilkins 2002). At present, the mechanism of activation of PLC is unclear, although activation by membrane stretching is a plausible possibility (Tanaka et al. 1994; Ruwhof et al. 2001). The mobilisation of Ca^{2+} from intracellular stores may also result from IP_3-independent processes such as prior entry of Ca^{2+} through SAC channel, with subsequent Ca^{2+}-induced Ca^{2+} release or by a direct action of membrane stretch on the store Ca^{2+} channel (Mohanty and Li 2002).

In summary, our results confirm that $[Ca^{2+}]_i$ increases in response to HTS in bovine articular chondrocytes as a consequence of activation of SAC and TRPV channels, which allow influx of Ca^{2+} from extracellular solution and also by activation of PLC, which initiates the release of Ca^{2+} from intracellular stores (Fig. 4).

The identity of the TRPV family member remains uncertain; while other studies implicate TRPV4 (Alford et al. 2003), it is currently not possible to exclude the additional involvement of ECaC in the osmotic response. These $[Ca^{2+}]_i$ changes in response to HTS may have a role to play in the regulation of matrix turnover by mechanical challenges associated with joint movements.

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