FEBS Letters 400 (1997) 58–64 FEBS 17962

Propagation of intercellular Ca^{2+} waves in mechanically stimulated articular chondrocytes

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Received 25 October 1996

Abstract Intercellular Ca^{2+} signalling in primary cultures of articular chondrocytes was investigated with digital fluorescence video imaging. Mechanical stimulation of a single cell induced a wave of increased Ca^{2+} that was communicated to surrounding cells. Intercellular Ca^{2+} spreading was inhibited by 18α-glycyrrhetinic acid, demonstrating the involvement of gap junctions in signal propagation. In the absence of extracellular Ca^{2+} mechanical stimulation failed to induce Ca^{2+} responses and communicated Ca^{2+} waves. Under these conditions Ca^{2+} microinjection induced intercellular waves involving the cells immediately surrounding the stimulated one. Mechanical stress induced Ca^{2+} influx in the stimulated, but not in the adjacent cells, as assessed by the Mn^{2+} quenching technique. Cell treatment with thapsigargin failed to block mechanically induced signal propagation, but significantly reduced the number of cells involved in the communicated Ca^{2+} wave. Similar results were obtained with the phospholipase C inhibitor U73122, which is known to prevent $InsP_3$ generation. These results provide evidence that mechanical stimulation induces a cytosolic Ca^{2+} increase that may permeate gap junctions, thus acting as an intercellular messenger mediating cell-to-cell communication in articular chondrocytes.

Key words: Calcium signalling; Gap junction; Mechanical stimulation

1. Introduction

Cell-to-cell communication through gap junctions gives tissues the ability to respond uniformly to localized stimuli [1]. Mechanical stimulation of a single cell, obtained by slightly distorting the plasma membrane, can, in cells coupled via gap junctions, give rise to communicated Ca^{2+} waves that propagate radially from the origin of stimulus and involve several adjacent cells [2–5]. The nature of the signals and second messengers that permeate gap junctions is not fully established: both Ca^{2+} and $InsP_3$ (as well as other second messengers) can cross the gap junctional channel, thus inducing a communicated Ca^{2+} wave.

In most of the systems studied so far, intercellular Ca^{2+} propagation could be observed in Ca^{2+}-free media [2,3], but was totally prevented by thapsigargin [5], an inhibitor of intracellular Ca^{2+} pumps [6]. Moreover, in airway epithelial cells, one of the best characterized systems, signal spreading could be induced by iontophoretic $InsP_3$ injection [2], and intercellular waves could be blocked by intracellular heparin [4], thus demonstrating a crucial involvement of $InsP_3$-mediated intracellular Ca^{2+} discharge.

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Chondrocytes from articular cartilage, maintained in primary culture, respond to several extracellular agonists by rhythmically increasing their cytosolic Ca^{2+} concentration [7,8]. Gap junctions, expressed in these cultured cells [9], mediate the intercellular spreading of Ca^{2+} waves induced by both ATP and mechanical stimulation [10].

In the present video imaging study, performed on Fura-2-loaded semiconfluent chondrocyte cultures, evidence is presented for a mechanism of spreading that requires the cooperation between Ca^{2+} and $InsP_3$, via the intercellular diffusion of Ca^{2+} through gap junctions.

Mechanically induced Ca^{2+} rising and spreading could be observed only in the presence of extracellular Ca^{2+}, while in Ca^{2+}-free experiments, communicated Ca^{2+} waves could be induced only upon Ca^{2+} microinjection. Mechanical stimulation induced Ca^{2+} influx in the stimulated, but not in the adjacent cells, thus ruling out a direct role of plasma membrane Ca^{2+} channels in signal propagation. Finally, cell treatment with thapsigargin, or with the phospholipase C inhibitor U73122 [11], failed to inhibit signal coordination, giving rise to intercellular Ca^{2+} waves involving, however, a restricted number of cells. Given the role of mechanical stress in joint movement, the possible relevance of these findings for articular cartilage physiology and pathology is discussed.

2. Materials and methods

2.1. Cell cultures

Articular cartilage was obtained from the scapula-humerus joints of 150–200 kg pigs killed in an abattoir. The tissue was rinsed in phosphate buffered saline (PBS; in mM: 137 NaCl, 2.7 KCl, 8.1 Na_2PO_4, 1.5 KH_2PO_4, 0.9 $CaCl_2$, 0.49 $MgCl_2$; pH 7.4) and collagenase treated as described [8]. Chondrocytes were plated at a density of 5×10^5 cells/cm^2 onto Cell-Tak (Becton Dickinson, USA) coated coverslips in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and 2 mM L-glutamine and cultured at 37°C in a humidified atmosphere containing 5% CO_2 for 5–7 days, until semiconfluent monolayers of at least 20–30 cells were observed.

2.2. Ca^{2+} imaging

Articular chondrocytes were loaded at room temperature with Fura-2 (3M), dissolved in Pluronic (Molecular Probes) 20% (1:2) and added to a solution containing (in mmol/l): NaCl 125, KCl 5, $MgSO_4$ 1, KH_2PO_4 0.7, $CaCl_2$ 2, glucose 6, HEPES-NaOH buffer 25, pH 7.4 and bovine serum albumin (fraction V) 1%. After 40 min the loading solution was removed and the cells washed three times with the same solution (without Fura-2 and BSA). Videomicroscopy and Ca^{2+} measurements were carried out at 37°C as described [8]. Drugs were dissolved in the incubation medium and directly perfused in the proximity (50–100 μm) of the cells. Calibration of Fura-2 fluorescence in terms of $[Ca^{2+}]_i$ was calculated from the ratio of 340/380 nm fluorescence values (after subtraction of background fluorescence) according to Grynkiewicz et al. [12].

2.3. Mechanical stimulation

Mechanical stimulation of single cells in subconfluent monolayers

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PII S0014-5793(96)01356-7

was performed by briefly deforming the cell surface with a micropipette. A fire-polished glass micropipette (approximately 1 μm tip diameter) was positioned over a single cell by a micromanipulator (Narishige, Japan). The pipette was briefly deflected downward manually, to transiently distort the membrane. A fluid flow of 1 ml/min was constantly applied by placing a second micropipette in the proximity of the cells (50–100 μm), to prevent the possible response of adjacent cells to secretion of intracellular constituents. For injection experiments the pipettes (0.3 μm tip diameter, not polished) were filled with an intracellular solution containing KCl 140 mM, $MgCl_2$ 1 mM, HEPES-KOH 20 mM, pH 7.2, $CaCl_2$ 10 μM (Fura-2 pentasodium salt 20 μM was added to prevent the possible loss of the fluorescence signal).

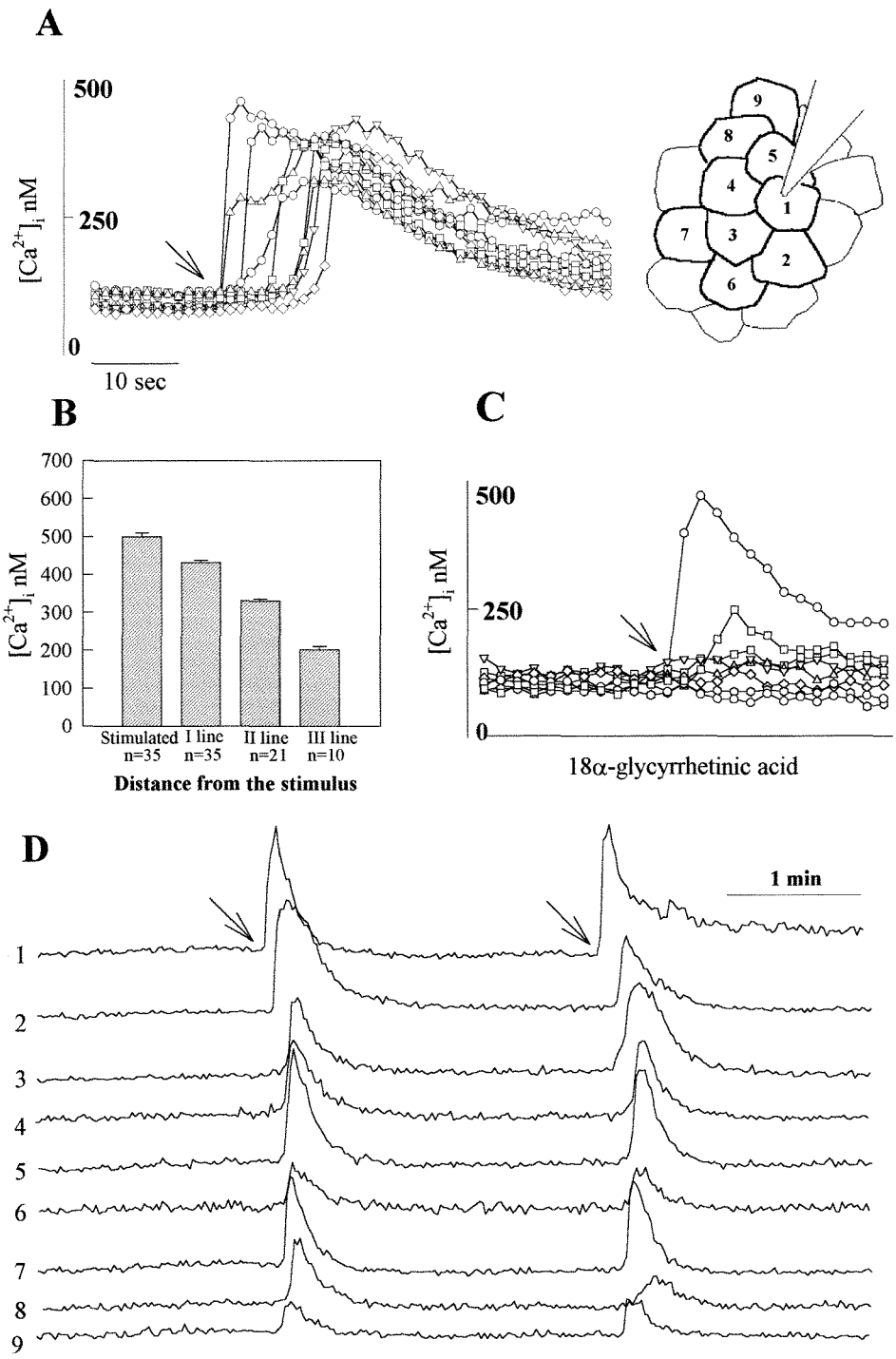

Fig. 1. Properties of communicated Ca^{2+} waves in response to mechanical stimulation. A: Sequential activation of a cell monolayer upon mechanical stimulation of a single cell. In the drawing the position of the cells involved by mechanical stimulation and Ca^{2+} spreading (bold line) is outlined. B: Peak magnitude of cytosolic Ca^{2+} as a function of distance from point of stimulus. Values represent the average cytosolic Ca^{2+} concentration at the peak of the response ± S.D. C: Intercellular Ca^{2+} spreading is inhibited by 18α-glycyrrhetinic acid. Cells were incubated with 18α-glycyrrhetinic acid (20 μM) for 10 min and were then mechanically stimulated in the presence of the inhibitor. D: Two subsequent stimulations (arrows) of the same cell (2.5 min apart) gave analogous Ca^{2+} responses in the nine cells involved by the intercellular Ca^{2+} wave.

3. Results

Chondrocytes from articular cartilage, grown in culture for 5–7 days, form semiconfluent monolayers of 20–40 cells. Mechanical stimulation of a single cell, obtained by briefly distorting the cell membrane with a fire-polished glass micropipette, resulted in a rapid (<0.5 s) increase of cytosolic Ca^{2+} in the stimulated cell (498 ± 30 nM from a basal level of 80 ± 9 nM, S.D., $n=35$) that was followed by a subsequent decline to resting levels. Besides inducing an intracellular Ca^{2+} rise in the stimulated cell, mechanical stimulation gave rise to an intercellular Ca^{2+} wave involving several neighboring cells (Fig. 1A). The number of cells recruited in each experiment was variable (10 ± 3, $n=35$) and was not apparently related to any appreciable cell boundary in the monolayer. The intercellular delay for Ca^{2+} rise was variable (3.2 ± 2.2 s, $n=35$), and the lag time for Ca^{2+} rise was proportional to the distance from the stimulated cell. The rising phase of the Ca^{2+} response was steeper in the stimulated cells (210 ± 20 nM/s, $n=35$) compared to adjacent cells (10 548 nM/s, $n=312$), while the amplitude of the Ca^{2+} signal tended to decrease with the distance from the stimulated cell (Fig. 1B). As previously reported [10], the gap junction inhibitor 18α-glycyrrhetinic acid (20 μM) [13] prevented intercellular spreading (Fig. 1C, Fig. 6), thus demonstrating the involvement of gap junctions in intercellular communication.

Mechanical stimulation produced by a glass micropipette could induce cell injury, loss of intracellular constituents, and exposure of intracellular sites to external environment. In these cases a dramatic loss of Ca^{2+} homeostasis is expected, as well as the inability of the cells to respond to further stimulation. To assess whether mechanical stimulation induced cell damage in our system, we repeated the stimulation after an interval of a few minutes. As shown in Fig. 1D, both the stimulated and the neighboring cells (9 ± 1, $n=5$) undergo nearly the same Ca^{2+} changes upon repetitive stimulation, thus ruling out major cell damage.

The requirement of extracellular Ca^{2+} for mechanically induced Ca^{2+} response and spreading was investigated in experiments in which the stimulation was applied to cells bathed

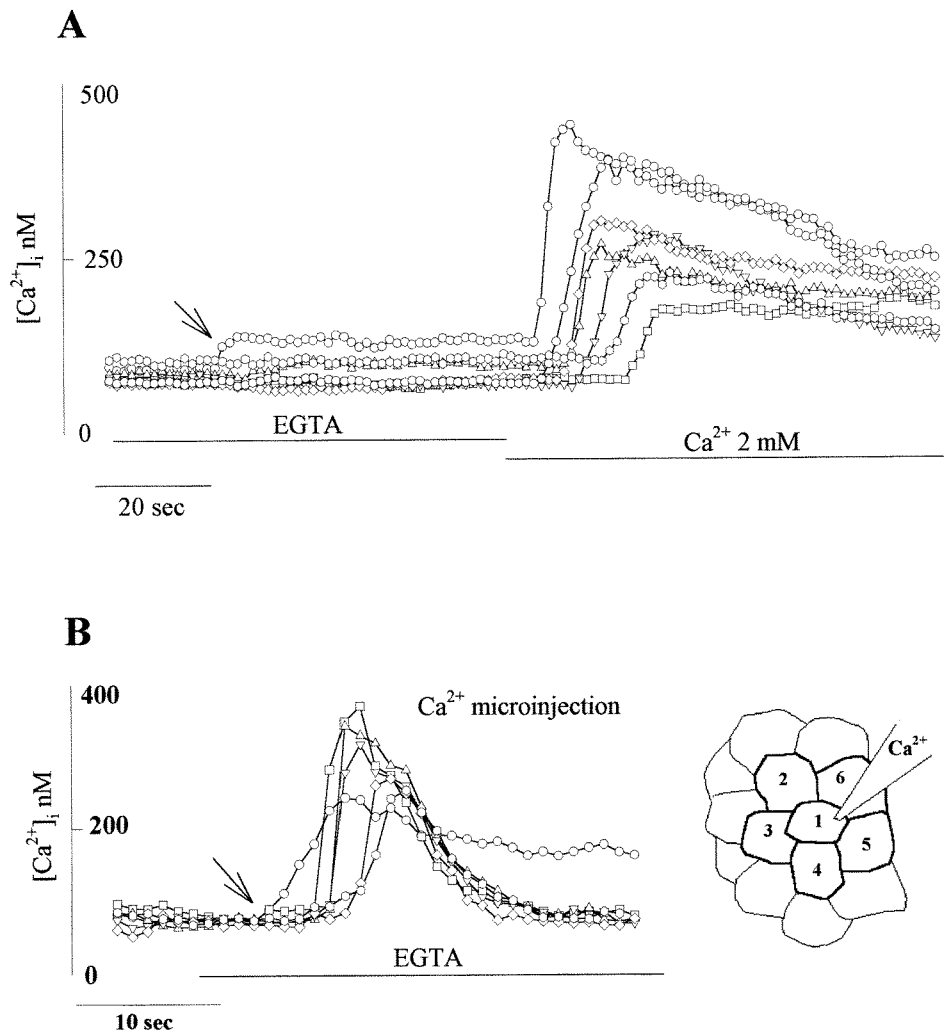

Fig. 2. Effect of mechanical stimulation in the absence of extracellular Ca^{2+}. A: For cells bathed in Ca^{2+}-free, EGTA-containing medium (0.5 mM EGTA) mechanical stimulation (arrow) slightly increases Ca^{2+} in the stimulated cell, but fails to induce Ca^{2+} responses in the adjacent cells. Upon Ca^{2+} addition (2 mM) a rapid rise of cytosolic Ca^{2+} is observed both in the stimulated cell and in seven neighboring cells. B: Ca^{2+} microinjection into cells bathed in Ca^{2+}-free, EGTA-containing medium (EGTA 0.5 mM). A narrow tip pipette filled with a Ca^{2+} containing (10 μM) intracellular solution was used to impale one of the cells (arrow), thus causing an increase of Ca^{2+}. After 2–4 s a Ca^{2+} rise was observed in five adjacent cells. The drawing shows the position of the cells recruited by the Ca^{2+} wave (bold line).

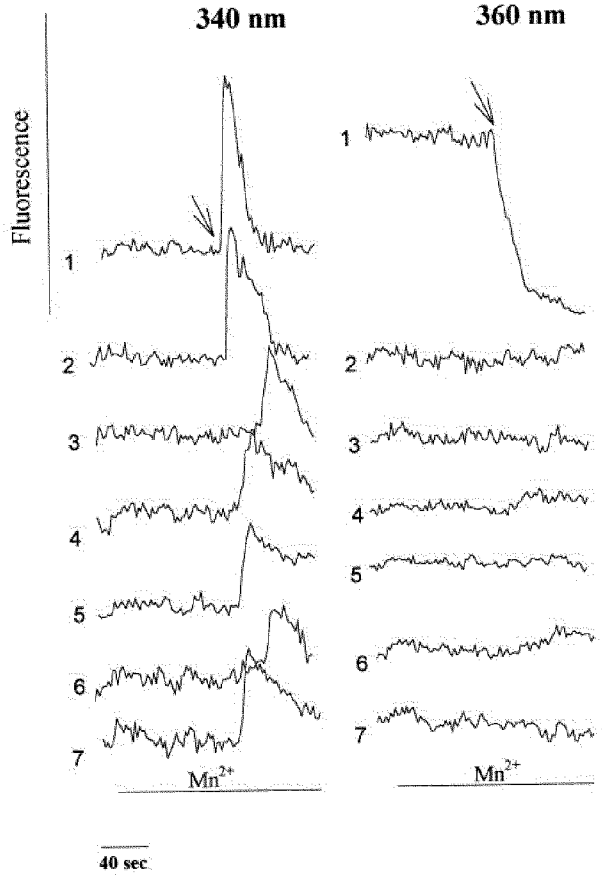

Fig. 3. Mechanical stimulation induces Mn^{2+} influx in the stimulated cell, but not in the adjacent cells. The manganese quenching experiment illustrates the effect of mechanical stimulation on Ca^{2+} influx in a confluent cell layer. The 340-nm tracings show the Ca^{2+} response to mechanical stimulation of cell 1 (arrow), subsequently involving six neighboring cells. The 360-nm tracing shows the rate of fluorescence quenching induced by Mn^{2+} in the same cells.

in Ca^{2+}-free, EGTA-containing medium (EGTA 0.5 mM). Fig. 2A shows the time course of a typical experiment. At the beginning of the experiment the solution perfusing the cells was switched to Ca^{2+}-free, 0.5 mM EGTA buffer; under these conditions the mechanical distortion of the cell membrane (arrow) slightly increased Ca^{2+} in the stimulated cell (110 ± 6 nM, $n = 12$), but failed to induce any Ca^{2+} signal in the adjacent cells. A relevant Ca^{2+} increase (485 ± 85 nM, $n = 12$), followed by a communicated Ca^{2+} wave involving up to 12 cells (9 ± 3 cells, $n = 12$) could be observed only when the extracellular medium was replaced by a solution containing a physiological Ca^{2+} concentration (2 mM). These results suggested that a diffusible second messenger permeating the gap junctions was responsible for intercellular spreading; given the lack of response of cells bathed in Ca^{2+}-free medium, however, a major role for $InsP_3$ seemed unlikely. Since gap junctions are known to permeate Ca^{2+} [14,15], the possibility was evaluated that intercellular spreading could derive from diffusion of Ca^{2+}. In a further series of experiments, we employed cells bathed in Ca^{2+}-free, EGTA-containing medium. Instead of inducing a simple distortion of the cell membrane, we penetrated the cell with a narrow tip micropipette filled with an intracellular solution containing Ca^{2+} (10 μM, see Section 2). Cell viability was estimated at the end of each experiment by visual inspection. Results are shown in Fig. 2B and summarized in Fig. 6. The injection resulting from cell impalement was effective in inducing a Ca^{2+} wave that was propagated to the cells immediately surrounding the injected cell (4 ± 1, $n = 8$). In these cases, however, a different kinetics of Ca^{2+} recovery was observed: after the rapid onset, the stimulated cell showed a persistent Ca^{2+} rise, while adjacent cells rapidly recovered to basal values (compare Ca^{2+} recovery in Figs. 1A and 2B). Although we cannot rule out the possibility that membrane damage could occur in such experiments, the spreading of a Ca^{2+} wave to neighboring cells, and their rapid kinetics of Ca^{2+} recovery, makes massive cell damage unlikely.

Intracellular Ca^{2+} rises can derive either from Ca^{2+} influx through plasma membrane channels, or from release from intracellular stores; the possibility that the propagated Ca^{2+} increase could derive from Ca^{2+} influx in each cell, as a possible consequence of slow electrical coupling, was evaluated with the Mn^{2+} quench technique [16]. Manganese is known to penetrate into the cells through voltage-independent Ca^{2+} influx pathways; once inside the cells it binds Fura-2 with high affinity, thus quenching the fluorescence signal. In cells like chondrocytes, which do not express voltage-gated Ca^{2+} channels (as testified by the lack of response when depolarized with isotonic 50 mM K^+, not shown), it is possible to relate the Ca^{2+} increase to Ca^{2+} influx by simultaneously recording the Ca^{2+} signal (at 340 nm) and the Fura-2 quenching rate (at 360 nm).

Mn^{2+} (100 μM) was added to the extracellular solution at the beginning of the experiment, to estimate the rate of basal quenching; subsequently the mechanical stimulus was applied (Fig. 3, arrow). While a Ca^{2+} increase was clearly observed both in the stimulated cell and in six adjacent cells (340 nm tracings, 7 ± 1 cells, $n = 15$), significant quenching of Fura-2 fluorescence was recorded only in the stimulated cell (360 nm tracing), thus suggesting that mechanical stimulus did induce Ca^{2+} influx in the stimulated cell, but failed to evoke Ca^{2+} influx in the adjacent cells.

The role of intracellular Ca^{2+} stores in mechanically induced Ca^{2+} rise and spreading was evaluated in a final series of experiments. Thapsigargin, a well known inhibitor of intracellular Ca^{2+} pumps [6], prevents intracellular store refilling, thus leading to store depletion. In articular chondrocytes thapsigargin-sensitive Ca^{2+} stores account for the entire intracellular Ca^{2+} pool that is mobilized upon agonist stimulation [8,17], while other drugs affecting intracellular Ca^{2+} storage, such as caffeine (5–40 mM) and ryanodine (5–20 μM), failed to evoke any response (not shown). One of the most potent agonists activating Ca^{2+} discharge from thapsigargin-sensitive intracellular stores in chondrocytes is extracellular ATP (10–200 μM) [8], acting on P2y purinoreceptors [18]. When administered to ATP-responsive cells (Fig. 4, ATP 100 μM), thapsigargin (2 μM), besides leading to a transient intracellular Ca^{2+} rise, totally prevented a further response to ATP stimulation, thus demonstrating its efficacy in depleting intracellular Ca^{2+} stores. Under these conditions, mechanical stimulation (arrow) could still induce a propagated signal, leading to an intercellular Ca^{2+} wave involving 6 ± 2 cells ($n = 8$). Similar results were obtained when the phospholipase C inhibitor U73122 (10 μM) [11] was employed (Fig. 5): although effective in preventing ATP-evoked Ca^{2+} rise, the inhibitor failed

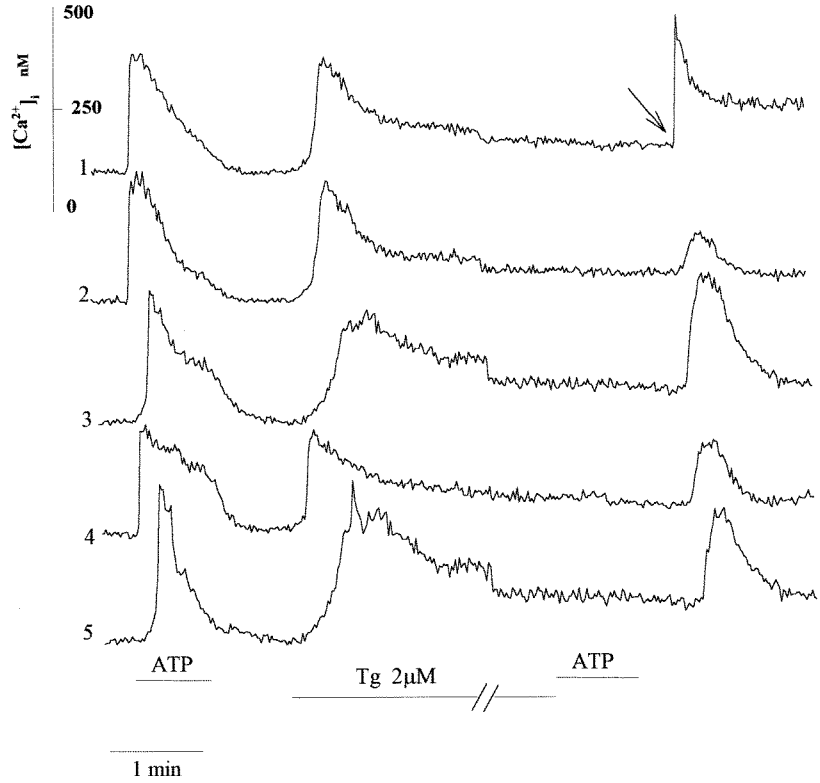

Fig. 4. Intracellular store depletion does not prevent mechanically induced intercellular Ca^{2+} spreading. Cell treatment with thapsigargin (Tg, 2 μM) inhibited ATP-mediated Ca^{2+} response in ATP-responsive cells (ATP 100 μM), but failed to block the propagated Ca^{2+} wave induced in four adjacent cells by mechanical stimulation of cell 1 (arrow). After Tg administration, the recording was interrupted for 5 min to allow the complete depletion of intracellular stores.

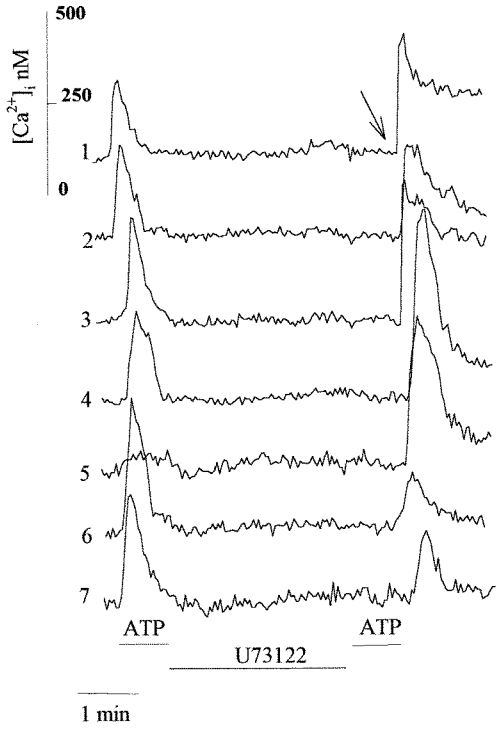

Fig. 5. Mechanically induced intercellular Ca^{2+} waves are not prevented by PLC inhibition. The phospholipase C inhibitor U73122 (10 μM), effective in preventing the ATP-evoked Ca^{2+} rise in ATP-responsive cells (ATP 100 μM), failed to inhibit the communicated Ca^{2+} rise induced in six neighboring cells by mechanical stimulation of cell 1 (arrow).

to prevent Ca^{2+} wave propagation (6 ± 1 cells, $n = 13$) induced by mechanical stimulus (Fig. 5, arrow).

Fig. 6 summarizes the effects of the various treatments on intercellular Ca^{2+} spreading: although the spreading distance decreased with treatments affecting either intracellular Ca^{2+} storage or $InsP_3$ formation, neither $InsP_3$ nor intracellular Ca^{2+} release seems to represent an absolute requirement for intercellular propagation of Ca^{2+} waves.

4. Discussion

Complex patterns of Ca^{2+} signalling can be induced in articular chondrocytes maintained in primary culture: agonists linked to polyphosphoinositide hydrolysis, like PDGF, endothelin, bradykinin, or ATP, evoke Ca^{2+} transients and Ca^{2+} oscillations [7,8,17]. When induced in subconfluent cell layers, Ca^{2+} oscillations can be propagated, through gap junctions, to several cells in the form of intercellular Ca^{2+} waves [10]. Similarly to $InsP_3$-linked agonists, mechanical stimulation of a single cell induces communicated waves of increased cytosolic Ca^{2+}. In contrast to agonist-induced Ca^{2+} transients and oscillations, however, mechanically induced Ca^{2+} waves did not occur in the absence of extracellular Ca^{2+}, thus demonstrating that Ca^{2+} influx is an absolute requirement for the process to be initiated. Ca^{2+} influx, on the other hand, apparently occurs only in stimulated cells, presumably through stretch-activated pathways [19–21]; in communicating cells, a direct involvement of Ca^{2+} influx appears unlikely, as suggested by the lack of Mn^{2+} entry.

In airway epithelial cells, propagated Ca^{2+} waves are

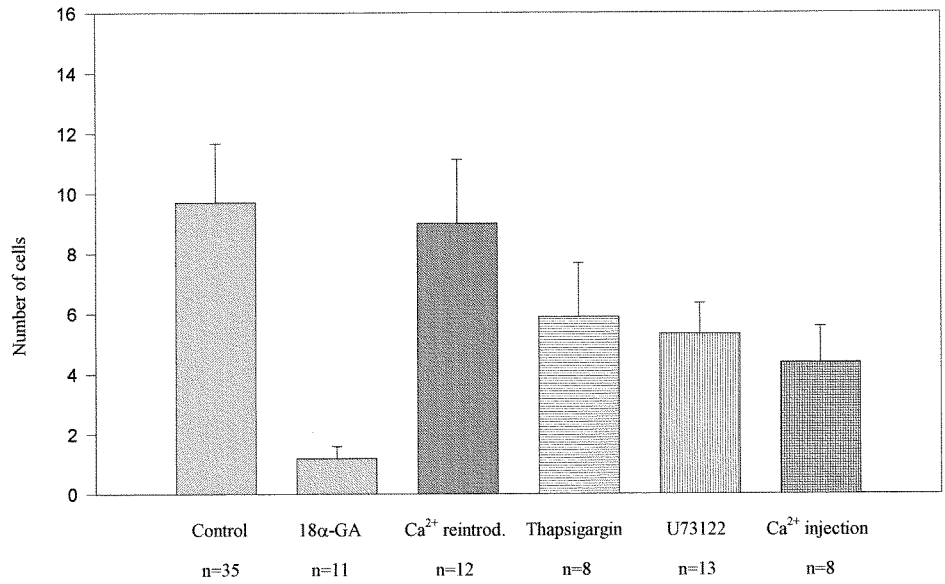

Fig. 6. Summary of the effects produced by different treatments on intercellular Ca^{2+} spreading. Values represent the average number of cells ± S.D. involved in the intercellular Ca^{2+} wave. Control: Mechanical stimulation initiated a Ca^{2+} response involving 10 ± 2 cells. Ca^{2+} spreading was consistently inhibited by 18α-glycyrrhetinic acid (20 μM). Reintroducing a physiological Ca^{2+} concentration in the extracellular medium after the cells had been stimulated in Ca^{2+}-free medium gave rise to an intercellular wave similar to controls (9 ± 3 cells). Pretreatment with thapsigargin (2 μM) failed to prevent intercellular spreading, but significantly decreased the number of the cells recruited (6 ± 2 cells, $P < 0.0001$). Similarly, the phospholipase C inhibitor U73122 (10 μM) significantly reduced the number of cells involved (5 ± 1 cells, $P < 0.00001$). Ca^{2+} injection into cells bathed in Ca^{2+}-free medium induced a Ca^{2+} rise involving 4 ± 1 cells.

known to result from $InsP_3$-dependent Ca^{2+} release: in these cells $InsP_3$, generated by mechanical stimulation, reaches neighboring cells by crossing gap junctions [2,4], thereby inducing intracellular Ca^{2+} release. If this were the mechanism in articular chondrocytes, inhibition of $InsP_3$ formation, and/or depletion of intracellular stores responsible for $InsP_3$-mediated Ca^{2+} release is expected to prevent signal spreading. This was not the case, since both thapsigargin and U73122, although decreasing the number of cells recruited in the spreading, failed to block intercellular communication, resulting in Ca^{2+} waves propagated to 6–8 cells. These results suggest a role for Ca^{2+} diffusion through gap junctions. Consistent with this hypothesis is the finding that only Ca^{2+} microinjection could give rise to propagated waves in cells maintained in Ca^{2+}-free media. The rapid decay of the propagated Ca^{2+} wave observed under these circumstances and the evidence that treatments affecting intracellular Ca^{2+} storage and handling decreased the number of cells involved in signal spreading suggest, however, that Ca^{2+} diffusion could not entirely account for the response observed.

Based on present results, a mechanism for mechanically induced Ca^{2+} rise and spreading in articular chondrocytes can be proposed. In this model, the initial trigger would be Ca^{2+} influx in the stimulated cell due to the opening of stretch-activated channels. Ca^{2+}-conducting, stretch-activated channels have been identified in a variety of cells by patch-clamp techniques (see, among others, [19–21]). Although the evidence for stretch-activated Ca^{2+} channels in these cells is still lacking, stretch-activated K^+ channels have been recently recorded (M. Martina, personal communication): their activation is expected to hyperpolarize the plasma membrane, thus increasing the driving force for Ca^{2+} influx. Following Ca^{2+} influx into the stimulated cell, Ca^{2+} would permeate gap junctions, thus reaching neighboring cells, similarly to what has been described in the corpora smooth muscle cells [15]. In the presence of basal $InsP_3$ concentrations, the increased Ca^{2+} level could promote $InsP_3$-dependent intracellular Ca^{2+} release [22], thus potentiating the Ca^{2+} response in the first line of adjacent cells. The recruitment of further cells, at increased distances from the stimulus, will follow the same pathway; however, given the poor diffusion properties of Ca^{2+} [23], a decrease in signal amplitude is expected, until Ca^{2+} levels eventually drop below the values needed for triggering the feed-forward mechanism for Ca^{2+} discharge. In this model, therefore, $InsP_3$-sensitive stores would play a role in optimizing intercellular transmission within large cell clusters, while the sole Ca^{2+} diffusion could account for signal spreading among a limited cell number.

Articular cartilage is a tissue designed to withstand compressive forces during joint movement and is subjected to a wide range of mechanical loading forces in vivo. Previous studies have demonstrated that in cultured articular chondrocytes, mechanical loading induced cytoskeletal changes [24], prostaglandin E_2 formation and proteoglycan synthesis [25], thus demonstrating that mechanosensitivity contributes to chondrocyte metabolism and cartilage homeostasis.

The present results demonstrate that articular chondrocytes can transduce the signal of a localized pressure to a cluster of communicating cells, via gap junctional permeability to intracellular Ca^{2+}.

In articular cartilage in vivo, chondrocytes are often organized in clusters of cells surrounded by extracellular matrix, usually called chondrons [26]: in osteoarthritis chondrocyte proliferation and abnormal chondron formation is usually observed [27]. Under these circumstances intercellular communication could be critically important in integration and/or amplification of tissue response to extracellular signals, thus allowing coordination of metabolic activity. Mechanisms re-

sponsible for intercellular communication are likely to have important consequences for cartilage physiology and pathology.

Acknowledgements: We would like to thank Ms. Valentina Paschini and Ms. Alessandra Calabrese for primary cultures of articular chondrocytes, and the Foundation 'Carlo e Dirce Callerio' for providing laboratory facilities. The work was supported by M.U.R.S.T., Università di Trieste, CNR, Italy.

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