Ca²⁺-sensitive phosphoinositide hydrolysis is activated in synovial cells but not in articular chondrocytes

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Cell-to-cell diffusion of second messengers across intercellular channels allows tissues to co-ordinate responses to extracellular stimuli. Intercellular diffusion of inositol 1,4,5-trisphosphate, locally produced by focal stimulations, sustains the propagation of intercellular Ca²⁺ waves, by stimulating the release of intracellular Ca²⁺ in neighbouring cells. We previously demonstrated that in cultured articular chondrocytes and HIG-82 synovial cells, studied with digital fluorescence video imaging, mechanical stimulation of a single cell induced intercellular Ca²⁺ waves dependent on the presence of gap junctions. In the absence of extracellular Ca²⁺ the propagating distance of the wave decreased significantly in HIG-82 cells, but appeared unaffected in chondrocytes. We now show that both cells types express connexin 43 and a similar functional coupling, thus suggesting that the different Ca²⁺ sensitivity of intercellular waves is not due to major differences in gap junction constituent proteins. In HIG-82 synoviocytes, but not in chondrocytes, the Ca²⁺ ionophore ionomycin stimulated phosphoinositide hydrolysis in a con-

centration-dependent manner, an effect strictly dependent on the presence of extracellular Ca²⁺, suggesting the expression, in these cells, of a Ca²⁺-sensitive phospholipase C activity. Such an activity could be stimulated also by Ca²⁺ influx induced by P_{2Y} receptor activation and considerably amplifies ATP-induced inositol phosphate (InsP) production. In contrast, Ca²⁺ influx did not affect considerably the response of chondrocytes to ATP stimulation. In HIG-82 cells, the combined application of ionomycin and ATP maximally stimulated InsP synthesis, suggesting the involvement of two independent mechanisms in inositol phosphate generation. These results suggest that in HIG-82 synovial cells the recruitment of a Ca²⁺-sensitive phospholipase C activity could amplify the cell response to a focally applied extracellular stimulus, thus providing a positive feedback mechanism for intercellular wave propagation.

Key words: phospholipase C, articular tissues, calcium signalling, gap junctions.

INTRODUCTION

A large number of extracellular signals stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] by inositol lipid-specific phospholipase C (PLC). Activation of this enzyme generates two second messengers, inositol 1,4,5-trisphosphate (InsP₃) and diacylglycerol, which, in turn, stimulate the release of Ca²⁺ ions from intracellular stores and activate protein kinase C isoenzymes [1]. The cell response to increased InsP₃ levels often develops in complex spatial and temporal variations of the cytosolic Ca²⁺ concentrations which are sustained by the interplay of InsP₃ and Ca²⁺ ions [1]. These elaborated patterns of signal transduction have been proposed to play an important role in determining the extent and targeting of cellular Ca²⁺ responses [1].

In multicellular organisms intercellular channels clustered in gap junctions allow the bi-directional transfer of ions and small molecules between the cells [2]. Co-ordination of Ca²⁺ signalling by intercellular Ca²⁺ waves occurs both in cultured cells of different origins [3–5] and in more integrated systems, such as tissue slices, in which the native interactions are preserved [6–8]. The mechanism of Ca²⁺ wave propagation depends on the cell type, possibly reflecting differences in the expression of gap junction constituent proteins [9] or in the transduction machinery. Gap junction permeability to both Ca²⁺ ions and InsP₃ [10] suggests that either second messenger could cross the intercellular channel, thus triggering a non-decremental, intercellular propagation of the signal [1]. The limited cytosolic diffusional properties

of Ca²⁺ ions [11], however, favours InsP₃ as the putative diffusing second messenger. This hypothesis had been corroborated by experimental evidence obtained in several cell types. In airway epithelial cells, intercellular Ca²⁺ waves can be induced by injection of InsP₃ [3] or by mechanical stimulation of a single cell [3]; in the latter case, the waves can be blocked by intracellular heparin [12] and by the pharmacological inhibition of PLC [13], demonstrating that the intercellular propagation of Ca²⁺ waves is mediated by InsP₃. Similar results have been obtained in other cells types following mechanical stimulation or focal application of extracellular agonists [5,14], suggesting that InsP₃, synthesized as a consequence of cell stimulation, diffuses across intercellular channels, thereby promoting a propagating Ca²⁺ release.

We recently demonstrated that mechanical stimulation induced, in articular chondrocytes and in HIG-82 synovial cells, intercellular Ca²⁺ waves dependent on cell-to-cell coupling via gap junctions [15]. In both cell types, wave propagation depended on both PLC activation and intracellular Ca²⁺ release, suggesting that InsP₃, synthesized in both type of cells as a consequence of mechanical stimulation, diffuses across gap junctions, thereby promoting intracellular Ca²⁺ release. However, while the number of chondrocytes recruited by the Ca²⁺ wave did not depend on the presence of extracellular Ca²⁺, the number of HIG-82 cells decreased significantly when the stimulation was applied in Ca²⁺-free medium [15].

In the present study we show that both cell types express connexin 43 (Cx43), the most widely distributed gap junction constituent protein, and allow intercellular transfer of Lucifer

Abbreviations used: PLC, phospholipase C; Cx43, connexin 43; fura-2 AM, fura-2 acetoxymethyl ester.

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Yellow dye, suggesting that the differences in wave propagation are not due to major differences in connexin expression. Moreover, we demonstrate the presence, only in HIG-82 cells, of a Ca^{2+} -sensitive PLC activity which can be recruited independently on receptor stimulation and contributes to overall cell response to extracellular stimuli. The possible role of Ca^{2+} -sensitive PLC in potentiating intra- and intercellular Ca^{2+} signalling is discussed.

MATERIALS AND METHODS

Chondrocyte and HIG-82 cultures

Articular cartilage was obtained from the ends of long bones of rabbits weighing 2–4 kg. The tissue was rinsed in PBS (in mM: 137 NaCl, 2.7 KCl, 8.1 Na_2HPO_4 , 1.5 KH_2PO_4 , 0.9 CaCl_2 , 0.49 MgCl_2 ; pH 7.4) and finely chopped in the same medium. Tissue fragments were digested with collagenase (125 units/ml, CLC type II, Worthington, U.S.A.) for 4–5 h at 37 °C under continuous mild agitation. Chondrocytes were plated at a density of 3×10^5 cells/cm² either on to Cell-Tak (Becton Dickinson, U.S.A.) coated coverslips (for fluorimetric measurements) or, at the same density, on to 35 mm diam. Petri dishes coated with polyornithine (10 µg/ml) in Ham's F-12 medium supplemented with 10% (v/v) foetal calf serum and 2 mM L-glutamine and cultured at 37 °C in a humidified atmosphere containing 5% CO_2 until confluent monolayers were observed. HIG-82 synovocytes (A.T.C.C., CRL-1832) were cultured in Ham's F-12 medium supplemented with 10% (v/v) foetal calf serum and 2 mM L-glutamine, subcultured at 1:10 ratio either on coverslips (for fluorimetric measurements) or on to 35 mm diam. Petri dishes and cultured until confluence was attained.

Protein immunoblot analysis

Articular chondrocytes or HIG-82 cells grown to confluence, were solubilized in SDS loading buffer containing a cocktail of protease inhibitors (Sigma). Aliquots (6–8 µg) of total proteins were separated on a SDS 13%-PAGE gel and then transferred to nitrocellulose. The filters were blocked with 1% (w/v) BSA in TPBS (in mM: 137 NaCl, 2.7 KCl, 8.1 Na_2HPO_4 , 1.5 KH_2PO_4 , pH 7.4, 0.1% Tween 20) and incubated with the primary antibody overnight under continuous agitation. The primary antibodies employed were the polyclonal anti-Cx43, anti-Cx32 and anti-Cx26 (Zymed). After washing with TPBS, blots were incubated with peroxidase-conjugated anti-(rabbit Ig) (Jackson Immunoresearch), washed again, and processed for ECL visualization (Super Signal, Pierce).

Dye transfer

Glass capillaries prepared with a dual-step puller (Narishige, Tokyo, Japan) were filled with a 5% (w/v) solution of Lucifer Yellow dissolved in 0.33 M LiCl. Individual cells in confluent monolayers were pressure-injected with a pneumatic PLI-100 pico-injector (Medical Systems Corp., New York, U.S.A.). Cells were fixed, 8 min after injection, with 4% formaldehyde in PBS for 15–20 min and viewed through a Leica DMLS fluorescence microscope (at 430 nm excitation and 530 nm emission).

Inositol phosphate measurements

Cells were labelled with 4 µCi/ml *myo*-[2-³H]inositol for 48 h. Before the experiment the cells were incubated for 1 h in serum-free medium and 10 min with 20 mM LiCl in Ham's F-12. Cells were then stimulated with the chosen agonist for 45 min at 37 °C.

Incubations were stopped with 750 µl of ice-cold 20 mM formic acid and the extraction was allowed to proceed for 30 min at 4 °C. Inositol phosphates (750 µl), diluted with 3.25 ml of 50 mM NH_4OH were loaded on to Dowex 1-X8 anion exchange columns equilibrated with 50 mM NH_4OH . The eluate of this step, containing [³H]inositol and [³H]glycerophosphoinositol incorporated by the cells, was collected into scintillation vials containing 10 ml of liquid scintillation cocktail (Ultima Flo-Packard). Columns were then washed with 8 ml of wash buffer (40 mM COONH_4 /0.1 M HCOOH). The elution of *InsP* (containing *InsP*₁, *InsP*₂ and *InsP*₃ plus *InsP*₄) was obtained with 4 ml of 2 M COONH_4 /0.1 M HCOOH. Fractions were collected into scintillation vials containing 10 ml of liquid scintillation cocktail. Radioactivity in all fractions was determined by liquid scintillation counting. Typical c.p.m. values under basal conditions were 1685 ± 373 in HIG-82 cells ($n = 45$) and 1006 ± 81 in chondrocytes ($n = 54$). ATP stimulation (100 µM) increased c.p.m. to 2111 ± 276 in HIG-82 cells ($n = 7$) and to 3095 ± 116 in chondrocytes ($n = 7$). Values for each *InsP* eluted fraction were normalized to [³H]inositol incorporated. Data are given as percentage over the basal.

Ca^{2+} imaging

Chondrocytes were loaded at room temperature with fura-2 acetoxymethyl ester (fura-2 AM) (3 µM), dissolved in 20% Pluronic (Molecular Probes) (w/v) (fura-2 AM/pluronic, 1:2, v/v) and added to the culture medium. HIG-82 cells were loaded at room temperature with fura-2 AM (10 µM), dissolved in 20% Pluronic (Molecular Probes) (1:1). After 60 min the loading solution was removed and the cells washed three times with a solution containing (mM): NaCl 125, KCl 5, MgSO_4 1, KH_2PO_4 0.7, CaCl_2 2, glucose 6, HEPES-NaOH buffer 25, pH 7.4. Video-microscopy and Ca^{2+} measurements were carried out at room temperature. The digital fluorescence-imaging microscopy system is built around a Zeiss inverted Axiovert 100 TV microscope. Cells were excited at 340 nm and 380 nm by a modified JASCO CAM-230 dual-wavelength microfluorimeter. Fluorescence images collected through a Zeiss oil immersion 40×1.8 NA objective, were captured by a low-light-level CCD camera (Hamamatsu Photonics) and fed into a digital image processor developed in the laboratory. Video frames were then digitized, integrated and processed off-line to convert fluorescence data in Ca^{2+} maps (340/380 nm excitation wavelength ratio method). Calibration of fura-2 fluorescence in terms of Ca^{2+} was calculated from the ratio of 340/380 nm fluorescence values (after subtraction of background fluorescence) according to Grynkiewicz et al. [16] utilizing $K_d = 224$ nM. At the end of each experiment ionomycin (5 µM) and extracellular solutions of high Ca^{2+} (10 mM) and low Ca^{2+} (10 mM EGTA-Tris, pH 8) were applied to ascertain the fluorescence ratios in the presence of saturating Ca^{2+} (R_{max} : 1780 for chondrocytes and 1760 for HIG-82), in the absence of Ca^{2+} (R_{min} : 124 for chondrocytes and 120 for HIG-82) and the $S_{2/3} = B$ value ($865/240 = 3.6$ for chondrocytes and $1159/331 = 3.5$ for HIG-82). Mean values in discrete areas of interest were calculated from sequence of images. Quantitative temporal analysis in spatially restricted areas were thus obtained throughout the experiment.

Mechanical stimulation

Mechanical stimulation of single cells in subconfluent monolayers was performed by briefly deforming the cell surface with a micropipette. The cells were placed in a round chamber containing 1 ml of extracellular solution. A fire-polished glass micropipette (approx. 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. Experiments were rejected when the cell membrane was damaged, as revealed by a leak of the fluorescent probe from the cell. To rule out the possible role of paracrine stimulation on Ca²⁺ signalling, stimulations were performed under a continuous fluid flow of 3 ml/min. The inflow pipette was placed in the proximity (100–300 μ m) of the stimulated cell (same focal plane) and an outflow port was positioned at 1 mm from the stimulated cell, along the direction of the flow.

RESULTS

Chondrocytes from rabbit articular cartilage and HIG-82 synovial fibroblast, grown in culture for 9–11 and 5–7 days respectively, form confluent layers of 40–60 cells; complete confluence is normally attained 2–3 days later for both cell types. Under resting conditions, the intracellular Ca²⁺ concentration was 92 ± 11 nM in chondrocytes ($n = 368$) and 66 ± 8 nM ($n = 387$) in HIG-82 synovial cells. As demonstrated in a previous study [15], in both cell types mechanical stimulation of a single

cell, obtained by briefly distorting the plasma membrane with a fire-polished glass micropipette, induced a rapid increase of the cytosolic Ca²⁺ concentration which was followed by a subsequent decline to resting levels. When mechanically stimulated, both cell types respond with intercellular Ca²⁺ waves that depended on PLC activation and intracellular Ca²⁺ stores mobilized by InsP₃ [15]. In both cell types, on the other hand, caffeine (up to 40 mM) and ryanodine (up to 20 μ M) failed to affect wave propagation, ruling out the involvement of a ryanodine-dependent Ca²⁺-induced Ca²⁺ release in intercellular Ca²⁺ signalling (results not shown). Mechanical stimulation increased the cytosolic calcium to 1957 ± 270 nM in chondrocytes and to 642 ± 132 nM in HIG-82 cells (Figure 1). Besides inducing a Ca²⁺ rise in the stimulated cell, mechanical stimulation gave rise to an intercellular Ca²⁺ wave which propagated radially from the point of stimulation and involved 14 ± 5 chondrocytes ($n = 57$) and 21 ± 9 HIG-82 cells ($n = 39$) (Figure 1). In both cell types, repetitive stimulations of the same cell, at 5 min intervals, induced propagated waves involving the same number of cells (15 ± 2 chondrocytes, $n = 8$, and 21 ± 6 HIG-82, $n = 9$, not shown). We recently proposed that intercellular communication in these cells is mediated by gap

Figure 1 Intercellular calcium waves induced by mechanical stimulation in articular chondrocytes and in HIG-82 synovial cells

Changes in intracellular Ca²⁺ were monitored by calcium imaging in cells loaded with fura-2 by incubation with fura-2 AM. Single-cell stimulations were achieved by slightly distorting the plasma membrane with a micropipette (asterisk). Sequential pseudocolour representations of intracellular Ca²⁺ are expressed as the ratio of fura-2 excitations (340/380), recorded at 510 nm emission. Upper panel: Images from a confluent layer of rabbit articular chondrocytes. (0 s) cells at rest, (1–10 s) images of the same field of cells at sequential time points followed mechanical stimulation performed either in 2 mM extracellular Ca²⁺ or in cells preincubated (1 min) and maintained in Ca²⁺-free, EGTA-containing medium (1–11 s). Intensities of fluorescent ratios are indicated according to the pseudocolour scale. Lower panel: images from a confluent layer of HIG-82 synovial cells. (0 s) resting cells, (1–11 s) images collected at sequential time points following mechanical stimulation performed either in 2 mM extracellular Ca²⁺ or in Ca²⁺-free, EGTA-containing medium. In the monochrome panels (corresponding to the same field of cells excited at 380 nm) cell-to-cell boundaries are outlined (white). Bar: 40 μ m.

Figure 2 Cx43 expression and intercellular communication are similar in articular chondrocytes and HIG-82 synovial cells

(A) Western blotting of cell extracts from confluent cultures. Aliquots (6 μ g) of total protein were resolved on a SDS 13% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was probed with anti-Cx43 antibody and horseradish peroxidase-conjugated anti-(mouse Ig), as described in the Materials and methods section. Lane 1, total mouse brain, positive control for Cx43; lane 2, cell lysate from articular chondrocytes; lane 3, cell lysate from HIG-82 synovial cells. (B) Gap junction-dependent intercellular dye transfer measured in chondrocytes and in HIG-82 cells by microinjection of Lucifer Yellow. Bar = 40 μ m.

junctions, given that 18 α -glycyrrhetic acid, a specific inhibitor of gap-junction-mediated communication, consistently inhibited the intercellular Ca^{2+} spreading [15].

The requirement of extracellular Ca^{2+} for mechanically induced intercellular wave spreading had been investigated in cells bathed in Ca^{2+} -free, EGTA-containing medium (0.5 mM EGTA) [15]. In all the experiments performed, cells were incubated in Ca^{2+} -free solution for 1 min before stimulation. Under these conditions, the intracellular Ca^{2+} stores were not depleted of their content, since both cell types consistently responded to ionomycin stimulation (see below). In the absence of extracellular Ca^{2+} the stimulated chondrocyte responded with a lower Ca^{2+} increase with respect to control experiments, but the spreading of the intercellular Ca^{2+} wave did not differ, involving 14 ± 6 cells ($n = 32$). In contrast, mechanical stimulation of a single HIG-82 cell in Ca^{2+} -free medium, induced a Ca^{2+} response similar to controls, but the Ca^{2+} wave spread only to a limited number of cells around the stimulated one (10 ± 6 , $n = 29$). In Figure 1 are reported the videoimaging recordings of typical intercellular waves induced in chondrocytes and in HIG-82 cells under the two experimental conditions: the interval between the two stimulations was 5 min. The images reported were collected at the times indicated. The decreased number of responding HIG-82 cells in Ca^{2+} -free medium was consistently observed, independent of the order of stimulation.

The diverse sensitivity of the two cell types to the extracellular Ca^{2+} could derive either from the expression of different connexins, the protein constituents of gap junctions, or from differences in the signalling pathways sustaining intracellular calcium rises. Among the most widely expressed connexins, articular chondrocytes synthesize Cx43, but not Cx32 or Cx26 [17]. We therefore compared the pattern of the connexin expressed by the two cell types by immunoblot analysis (Figure 2A). Total cell lysates were run on SDS/PAGE gels, the proteins blotted on to nitrocellulose filters and probed with specific anti-connexin

antibodies (see Materials and methods section). Both chondrocytes and HIG-82 cells express nearly the same amount of a protein of 43 kDa recognized by a polyclonal anti-Cx43 antibody, while both cell types gave negative results when probed with either anti-Cx32 or anti-Cx26 polyclonal antibodies (results not shown).

Assessment of gap junctional communication in confluent cell layers was obtained by microinjecting individual cells with Lucifer Yellow and measuring the intercellular dye transfer. Both cell types displayed a similar extent of cell-to-cell coupling (18 ± 9 chondrocytes, $n = 9$, and 22 ± 8 HIG-82, $n = 7$; Figure 2B), suggesting that, although more subtle differences in the level of other, less represented, connexins cannot be ruled out, the diversity in intercellular signal propagation are not due to major differences in the functional expression of connexins.

Among the steps sustaining the intracellular Ca^{2+} signalling pathway, the activation of PLC is a Ca^{2+} -sensitive process. The Ca^{2+} ion dependence of PLC activity towards inositol lipids *in vitro* has been clearly established for all PLC isoenzymes [18]. However, the Ca^{2+} ion activation of PLC *in vivo* proved to be a more complex issue. Although in many cell types PLC activation occurs at resting Ca^{2+} concentrations (10–100 nM), in some systems an increase in cellular Ca^{2+} results in the stimulation of PLC activity [19,20]. Recent studies have elucidated the role played by PLC isoenzymes in this process, demonstrating that it is only the δ isoform which is activated by physiological Ca^{2+} concentrations (0.1–10 μ M) and might be recruited, independently on receptor activation, by increased Ca^{2+} levels [21,22]. The β and γ isoforms, on the other hand, do not display Ca^{2+} -dependent activation *in vivo* [23].

We investigated the possibility that the different Ca^{2+} sensitivity of intercellular waves in the two cell types could derive from the presence, in HIG-82 cells, of a Ca^{2+} -sensitive PLC activity, responsible for potentiating InsP_3 production under physiological conditions. To induce increases of intracellular Ca^{2+} , independent of receptor stimulation, in a first set of experiments we employed the Ca^{2+} ionophore ionomycin. In parallel experiments we measured the ionomycin-induced increase of intracellular Ca^{2+} concentration and the amount of inositol phosphates ($[\text{H}^3]\text{InsP}$) produced in the two cell types (Figure 3). As expected from its mechanism of action, ionomycin induced, in both cell types, a slow increase of cytosolic calcium. The final level attained depended on both ionomycin concentration and the presence of extracellular Ca^{2+} . In the presence of physiological extracellular Ca^{2+} , the increase was maximal, for both cell types, at 7.5 μ M ionomycin (6219 ± 220 nM for HIG-82, $n = 74$, and 7456 ± 220 nM for chondrocytes, $n = 61$). This value, however, could be artifactually underestimated due to saturation of fura-2, not suitable for Ca^{2+} measurements in the micromolar range (≥ 2 –3 μ M). For our purposes, therefore, we will consider the Ca^{2+} values obtained with 5 μ M ionomycin as the maximal levels with reliability (2563 ± 214 nM in HIG-82 cells, $n = 62$ and 1312 ± 133 nM in chondrocytes, $n = 97$; Figures 3A and 3B). In the absence of extracellular Ca^{2+} , the response to ionomycin addition was much lower (295 ± 20 nM and 216 ± 5 nM, in HIG-82 cells and chondrocytes, respectively, $n = 60$ and $n = 68$; Figures 3A and 3B); under these conditions, the transient Ca^{2+} rise was most likely due to the emptying of intracellular Ca^{2+} stores. While the intracellular Ca^{2+} changes induced by the ionophore were comparable between the two cell types, the amount of $[\text{H}^3]\text{InsP}$ produced by ionomycin stimulation was strikingly different. In HIG-82 cells ionomycin stimulated dose-dependently the production of $[\text{H}^3]\text{InsP}$, while in chondrocytes the ionophore did not induce any appreciable increase in $[\text{H}^3]\text{InsP}$ levels (Figures 3E and 3F). In HIG-82 cells the effect of ionomycin

Figure 3 Effect of ionomycin on intracellular Ca²⁺ concentration and InsP formation

Cytosolic Ca²⁺ was monitored in fura-2-loaded individual cells, as described in the Materials and methods section. Ionomycin (5 μM) increased intracellular Ca²⁺ in both HIG-82 cells (A) and articular chondrocytes (B). The response was measured either in the presence of extracellular Ca²⁺ or in Ca²⁺-free, EGTA-containing medium. (C, D) Intracellular Ca²⁺ increase as a function of ionomycin concentration. In both HIG-82 cells (C), and chondrocytes (D) bathed in 2 mM extracellular Ca²⁺, the level of intracellular Ca²⁺ increased with ionomycin concentration. The results are expressed as means ± S.E.M. (E, F) Cells were labelled with *myo*-inositol as described in the Materials and methods section. Inositol phosphates were determined by anion-exchange chromatography on Dowex 1-X8 formate columns. The results represent the means ± S.E.M. of five (HIG-82, E) and eight (chondrocytes, F) independent experiments done in triplicate. Results are expressed as percentages of the values obtained in control cells, defining InsP formation in buffer (1585 ± 173 c.p.m. for HIG-82 and 1006 ± 137 c.p.m. for chondrocytes) as 100%.

was entirely dependent on the presence of extracellular Ca²⁺, and was already evident (2-fold the basal level, Figure 3E) at ionomycin concentrations (2.5 μM) that increased intracellular Ca²⁺ to physiological levels (206 ± 7 nM, *n* = 71, Figure 3C). The maximal [³H]InsP production (4.5-fold the basal level) was induced by 10 μM ionomycin which, in both cell types, increased the intracellular Ca²⁺ to over 6 μM; increased ionomycin concentrations proved to be toxic for both cell types.

The evidence presented so far suggested that HIG-82 synoviocytes selectively express a Ca²⁺-sensitive PLC activity. In a further set of experiments we explored the possibility that this

activity might be involved in the cellular response to physiological stimuli. In articular chondrocytes, extracellular ATP induces oscillatory increases of cytosolic Ca²⁺ due to the rhythmic discharge of intracellular Ca²⁺ stores [24] secondary to the activation of P_{2Y} receptors [25]. Receptor activation stimulates the G_q/PLCβ pathway, thus leading to the synthesis of InsP₃ and diacylglycerol.

As demonstrated in previous studies [15,26], extracellular ATP was effective in increasing the cytosolic Ca²⁺ concentration in HIG-82 synoviocytes also, suggesting that purinoreceptors are functionally expressed in these cells as well. We therefore

Figure 4 Cytosolic Ca^{2+} increases induced by ATP in HIG-82 cells and articular chondrocytes

(A, B) Cytosolic Ca^{2+} was monitored in fura-2-loaded individual cells. Traces for HIG-82 cells (A) and chondrocytes (B) were obtained by stimulating with $100 \mu\text{M}$ ATP the cells bathed either in 2 mM extracellular Ca^{2+} or in Ca^{2+} -free medium. (C, D) Cytosolic Ca^{2+} level measured in HIG-82 cells (C) and in chondrocytes (D) at the peak of the response induced by different ATP concentrations. The results are expressed as means $\pm$ S.E.M. (E, F) The Ca^{2+} plateau is measured in HIG-82 cells (E) and in chondrocytes (F) as the difference (measured for each cell) between the level reached 5 min after ATP addition and the pre-existing basal value.

compared the ATP-induced Ca^{2+} responses and InsP production in the two cell types.

In the presence of extracellular Ca^{2+} , ATP ($100 \mu\text{M}$) induced a biphasic increase of cytosolic Ca^{2+} in both cell types (Figures 4A and 4B); the initial rapid rise, that reached $345 \pm 8 \text{ nM}$ in HIG-82 cells ($n = 90$) and $385 \pm 11 \text{ nM}$ in articular chondrocytes ($n = 82$), was followed by a slow decline to a plateau, consistently higher than the basal resting level ($40 \pm 1 \text{ nM}$ over the basal in HIG-82 cells, and $48 \pm 2 \text{ nM}$ in chondrocytes). In the absence of extracellular Ca^{2+} the response consisted of a prompt increase ($306 \pm 11 \text{ nM}$ in HIG-82 cells, $n = 81$ and $322 \pm 12 \text{ nM}$ in chondrocytes, $n = 67$; Figures 4A and 4B) followed by a decline towards the pre-existing basal level. These results indicated that,

in both cell types, ATP stimulation induced a rapid intracellular Ca^{2+} release, followed by a slower, long-lasting influx from the extracellular medium.

A series of dose-response experiments was then performed in order to evaluate the influence of agonist concentration on the different phases of the ATP-induced Ca^{2+} rise. Compared with articular chondrocytes, HIG-82 cells revealed sensitivity to lower ATP concentrations: 97% of the cells increased their intracellular Ca^{2+} when challenged with $0.5 \mu\text{M}$ ATP ($n = 86$), a concentration inducing a response only in 12% of chondrocytes ($n = 58$). In HIG-82 cells, the Ca^{2+} concentration at the peak of the response increased consistently with the agonist dose ($n = 171$, Figure 4C), a trend observed also in the absence of extracellular Ca^{2+} ,

Figure 5 Stimulation of inositol phosphate production by ATP in HIG-82 cells and articular chondrocytes

Cells were labelled with *myo*-[2-³H]inositol as described in the Materials and methods section. Inositol phosphates were determined by anion-exchange chromatography on Dowex 1-X8 formate columns. The results (means $\pm$ S.E.M. of seven independent experiments done in triplicate) are given as percentages of the values obtained in control cells. (A) Comparison of ATP-induced inositol phosphate production in HIG-82 cells and in chondrocytes bathed in 2 mM extracellular Ca²⁺. (B) Influence of extracellular Ca²⁺ on the ATP-induced InsP production in HIG-82 cells. (C) Influence of extracellular Ca²⁺ on the ATP-induced InsP production in articular chondrocytes.

although shifted towards higher agonist concentrations (Figure 4C). In chondrocytes, the influence of ATP concentration on the peak Ca²⁺ level was less evident: a reduced peak level was, in fact, observed only in the portion of cells responding to 0.5 μM ATP ($n = 334$, Figure 4D). Similar results were obtained analysing the long-lasting plateau: in HIG-82 cells it increased dose-dependently with ATP concentrations (Figure 4E), while, in chondrocytes, it appeared less influenced by agonist doses (Figure 4F).

The graded Ca²⁺ responses observed in HIG-82 cells, compared with the all-or-none behaviour of articular chondrocytes, suggested that the two cells might differ either in the type of purinoreceptors expressed or in one or more steps of the transduction pathways leading to intracellular Ca²⁺ release. We, therefore, compared the ATP-stimulated phosphoinositide hydrolysis in the two cell types. In the presence of extracellular Ca²⁺, ATP stimulated dose-dependently [³H]InsP production in both cell types (Figure 5A). However, while, in HIG-82 cells, maximal ATP concentrations (100 μM) stimulated a 12-fold increase in [³H]InsP levels, in chondrocytes, maximal stimulation (500 μM ATP) induced only a 3-fold increase (Figure 5A). The response of HIG-82 cells was deeply influenced by the presence of extracellular Ca²⁺ (Figure 5B): in EGTA-containing medium, the maximal [³H]InsP production did not exceed 3.5-fold over the basal, the same order of magnitude measured in chondrocytes. In these latter cells, [³H]InsP production was not affected by extracellular Ca²⁺ at lower ATP concentrations, and was only slightly stimulated (0.55-fold over Ca²⁺-free) for ATP above 50 μM (Figure 5C). In both cell types, the level of [³H]InsP

Figure 6 Potentiation of ATP-stimulated inositol phosphate production in HIG-82 synoviocytes

Cells were labelled with *myo*-inositol as described in the Materials and methods section. Inositol phosphates were determined by anion-exchange chromatography on Dowex 1-X8 formate columns. The results (means $\pm$ S.E.M. of three independent experiments done in triplicate) are given as percentages of the values obtained in control cells. Stimuli were applied to cells bathed in 2 mM extracellular Ca²⁺.

production in Ca²⁺-free medium could not be further decreased after depletion of intracellular Ca²⁺ stores with thapsigargin (2 μM, results not shown), suggesting that [³H]InsP production in Ca²⁺-free medium results mainly from G_q/PLC β activation, without any major contribution from Ca²⁺-sensitive pathways.

In order to test whether maximal stimulation of Ca²⁺-sensitive PLC activity could further increase [³H]InsP production in HIG-82 cells, we performed an ATP dose-response experiment in the presence of 5 μM ionomycin (Figure 6). The addition of the ionophore increased [³H]InsP production at any ATP concentration tested, but was particularly effective at low doses (0.5–1 μM ATP), at which the production more than doubled. In the presence of ionomycin, 5 μM ATP was sufficient to elicit the maximal [³H]InsP production (15-fold over the basal level).

DISCUSSION

The aim of this study was to identify the mechanism responsible for the differences observed in intercellular Ca²⁺ wave propagation between articular chondrocytes and HIG-82 synovial cells [15]. When mechanically stimulated, both cell types respond with intercellular Ca²⁺ waves that depended on PLC activation and intracellular Ca²⁺ stores [15]. However, in the two cell types, the extent of wave propagation appeared to be differently affected by extracellular Ca²⁺: while the number of responding chondrocytes did not vary when the stimulation was performed in Ca²⁺-free medium, the number of HIG-82 cells was significantly reduced (Figure 1, [15]).

Besides being mediated by gap junctions, intercellular Ca²⁺ waves could also arise due to paracrine stimulation, secondary to the release of extracellular signalling molecules (see, among others, [27,28]). We recently demonstrated that in co-cultures of articular chondrocytes and HIG-82 synoviocytes, mimicking *in vitro* the cell-to-cell interactions occurring in the synovial pannus, paracrine stimulation of P_{2Y} receptors contributes to intercellular communication [26]. Although our imaging experiments were performed under continuous perfusion, the possibility that paracrine stimulation could also be involved in cell-to-cell signalling in homotypic cultures could not be ruled out. The effect of extracellular Ca²⁺ on the extent of Ca²⁺ wave propagation, however, tends to exclude the possibility that paracrine

signalling plays a major role in both chondrocytes and HIG-82 synovial cells. It has been demonstrated, that paracrine stimulation of P_{2Y} receptors increases considerably in the absence of extracellular Ca^{2+} , due to stimulation of ATP release [29]. In our system, on the contrary, the extent of the intercellular wave was either unmodified (in chondrocytes) or even significantly reduced (in HIG-82 synoviocytes). Moreover, wave propagation, in both cell types, could be inhibited by 18 α -glycyrrhetic acid [15], an inhibitor of gap junction-dependent communication, suggesting that cell-to-cell signalling followed a direct route through intercellular channels.

The differences we observed in the properties of Ca^{2+} wave propagation in the two cell types could possibly reflect major differences in the type of connexin expressed [9]. Here we demonstrated that both chondrocytes and HIG-82 cells express Cx43 and that intercellular dye transfer is similar in the two cell types, suggesting that the diversity in intercellular signal propagation cannot be ascribed to major differences in the functional expression of gap junction constituent proteins.

Mechanical stimulation is known to induce $InsP_3$ synthesis in several cell types [30–32], although little is known regarding the mechanism of enzyme activation. The hydrolysis of $PtdIns(4,5)P_2$ is stringently controlled by many PLC isoenzymes, differently linked to the various receptors [19]. The PLC β isoenzymes are activated by either the α or the $\beta\gamma$ subunits of the G_q family of heterotrimeric G-proteins. The isoenzymes of the PLC γ family are activated by the direct interaction with and phosphorylation by receptor and non-receptor tyrosine kinases, thus linking the Ca^{2+} /protein kinase C signalling to the transduction pathways activated by mitogenic stimuli. The PLC δ isoenzymes are the most widely distributed, being expressed from mammals [33] to yeast [34], where they represent the only type of PLC expressed. In spite of their wide distribution, the mechanism of activation of PLC δ isoenzymes has been only partially elucidated. Recent studies agree in identifying in PLC δ the enzyme(s) responsible for the Ca^{2+} -dependent $PtdIns(4,5)P_2$ hydrolysis [21,22] occurring, in several cell types, independently on receptor activation [20].

The evidence that in HIG-82 synoviocytes, but not in chondrocytes, ionomycin stimulation induced the hydrolysis of $PtdIns(4,5)P_2$, which was strictly dependent on the presence of extracellular Ca^{2+} , indicates that HIG-82 cells express a Ca^{2+} -sensitive PLC activity. In these cells, $PtdIns(4,5)P_2$ hydrolysis was already stimulated at 200 nM cytosolic Ca^{2+} (induced by 2.5 μ M ionomycin), suggesting that the Ca^{2+} -sensitive activity may act as a positive feedback system for potentiating intracellular signalling following small increases of cytosolic Ca^{2+} .

This Ca^{2+} -sensitive PLC activity contributed, in HIG-82 cells, to $PtdIns(4,5)P_2$ hydrolysis following ATP stimulation: similarly to ionomycin, the effect was dependent on extracellular Ca^{2+} . Since the Ca^{2+} levels at the peak of the response did not vary considerably in the presence or in the absence of extracellular Ca^{2+} , the activation of Ca^{2+} -sensitive $PtdIns(4,5)P_2$ hydrolysis is presumably due to an ATP-stimulated Ca^{2+} entry pathway, abolished by removal of extracellular Ca^{2+} . This evidence suggests slower kinetics for the activation of Ca^{2+} -sensitive PLC, compared with that of the other PLC isoenzymes. Interestingly, in PLC δ -transfected CHO cells, vasopressin-induced [3H]InsP production, which was strictly dependent on extracellular Ca^{2+} , displayed a different time-course compared with control cells: while vector-transfected cells reached a plateau (2-fold the basal level) 5 min after stimulation, PLC δ -transfected cells showed a progressive increase of [3H]InsP production reaching a plateau (6.5-fold the basal level) 30 min after stimulation [21].

The sensitivity to extracellular Ca^{2+} for maximal PLC activation in HIG-82 cells was highlighted in experiments em-

ploying both ionomycin and ATP to stimulate [3H]InsP production: the evidence that the ionophore could potentiate $PtdIns(4,5)P_2$ hydrolysis at any ATP concentration tested, further suggested the involvement of two independent mechanisms in [3H]InsP generation.

The cellular response to ATP stimulation, studied with Ca^{2+} imaging, showed further differences between the two cell types. In HIG-82 cells, the level of the Ca^{2+} plateau tended to increase with ATP concentration, and followed a parallel dose-dependent increase of the peak response. The close link between the two events suggests a likely candidate for Ca^{2+} influx in the capacitative entry pathway through the I_{CRAC} channels [35] activated by intracellular Ca^{2+} release [36]. The graded Ca^{2+} and InsP responses of HIG-82 cells to ATP stimulation might reflect either the progressive increase in the number of activated P_{2Y} receptors or a heterogeneous sensitivity of intracellular Ca^{2+} stores. Unlike HIG-82 cells, articular chondrocytes displayed a dose-dependent increase in InsP production, but a nearly all-or-nothing Ca^{2+} response, suggesting that, in these cells, ATP-induced signalling might involve other second messengers besides $InsP_3$. The complexity of the signal transduction pathways activated by ATP in articular chondrocytes is presently under investigation.

It had been proposed that in some systems, the stimulation of PLC δ by Ca^{2+} ions could follow the stimulation of PLC β or PLC γ , thus leading to a potentiation of the Ca^{2+} response [20]. Our Ca^{2+} imaging results rule out a major role of Ca^{2+} -sensitive PLC activity in ATP-induced Ca^{2+} responses in HIG-82 cells: at the higher ATP concentration tested, the amplitude of calcium rise did not increase, suggesting that the level of $InsP_3$ produced was sufficient for saturating the intracellular binding sites.

Ca^{2+} -dependent $PtdIns(4,5)P_2$ hydrolysis, however, could be crucial in intercellular Ca^{2+} signalling by potentiating the effect of a locally applied extracellular stimulus: an increased amount of $InsP_3$ produced in the stimulated cell would, in fact, support a higher diffusion distance across intercellular channels [14]. Moreover, the regenerative formation of $InsP_3$ in neighbouring cells, stimulated by the intracellular Ca^{2+} release, might further amplify the response [37], ultimately leading to a long-range propagated Ca^{2+} wave. Present data would, therefore provide an explanation for the evidence that in HIG-82 cells, at difference with articular chondrocytes, extracellular Ca^{2+} is required for long-range propagation of mechanically induced intercellular Ca^{2+} waves. According to this hypothesis, in HIG-82 cells, the elevated Ca^{2+} levels attained in the stimulated cell due to both Ca^{2+} influx and intracellular Ca^{2+} release [15] would activate a Ca^{2+} -sensitive PLC, thus potentiating, by a parallel pathway, the mechanically induced PLC activation.

In articular cartilage *in vivo* chondrocytes are often organized in clusters of cells surrounded by extracellular matrix, usually called 'chondrons' [38]; in osteoarthritis chondrocyte proliferation and abnormal chondron formation is usually observed [39]. In cartilage physiology and pathology, therefore, cell-to-cell interactions are limited to cells enclosed in chondrons, and intercellular communication is therefore expected to co-ordinate the activity of a limited number of cells. Synoviocytes, on the other hand, form a continuous layer at the synovial membrane, which lines the inner surface of the joint capsule and covers all intra-articular tissues [40]. Synoviocytes synthesize and secrete synovial fluid, required for lubrication and nutrition of the joint tissues. Under these circumstances, the extent of intercellular signalling could be critically involved in integration and/or amplification of overall tissue responsiveness to extracellular stimuli: mechanisms providing long-range signal propagation, therefore, might play a fundamental role in synovial tissue homeostasis.

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