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SPATIAL AND TEMPORAL Ca^{2+} SIGNALLING IN ARTICULAR CHONDROCYTES

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SUMMARY. Stimulation of pig articular chondrocytes with either bradykinin, fetal calf serum or the Ca^{2+}-ATPase inhibitor thapsigargin induced increases of the cytosolic Ca^{2+} concentration. By computerized videoimaging, the spatial and temporal aspects of the Ca^{2+} signal were revealed at single cell level. The cell response depended on Ca^{2+} release from intracellular stores without significant contribution of Ca^{2+} influx. A great heterogeneity in the cell population was found with respect to the Ca^{2+} storage ability. The Ca^{2+} response initiated in a discrete subcellular region and then spread in a nondecremental fashion to involve the whole cytosol. Such a behaviour was independent of the stimulus applied, thus suggesting a functional heterogeneity of the intracellular Ca^{2+} stores involved. In the region from which the response started, local Ca^{2+} spikes were recorded, revealing a spatially restricted pulsatile activity.

Articular cartilage is a specialized tissue designed to resist to compressive forces during joint movement. Inflammatory joint deseases deeply alter articular cartilage, initially leading to matrix resorption and ultimately resulting in the destruction of the articular tissue (1). Several inflammatory mediators, activating different signalling cascades, are known to initiate these processes in articular chondrocytes (2-4); among them, bradykinin has been shown to stimulate the breakdown of polyphosphoinositides, to increase the intracellular Ca^{2+} concentration and to promote the synthesis of prostaglandin E_2 (2).

This study was performed by Fura-2 videoimaging on primary cultures of pig articular chondrocytes, to investigate the spatial and temporal evolution of the Ca^{2+} signal at single cell level. Evidence for a preminent role of intracellular Ca^{2+} release is presented and profound differences among the cells in the extent the Ca^{2+} stores involved in the process are reported. Most intringuingly, the videoimaging technique allowed to reveal spatial and temporal heterogeneity of the Ca^{2+} response which appeared to start from a discrete subcellular region and subsequently spread to invade the rest of the cell; in such a "starting zone" the signal appeared pulsatile, giving rise to "miniature" oscillations which failed to involve the rest of the cell.

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MATERIALS AND METHODS

Articular chnodrocytes were obtained as previously described (5) and plated onto Cell-Tak (Becton Dikinson, U.S.A.) 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 an humidified atmosphere containing 5% CO_2.

Ca^{2+} imaging. Videomicroscopy and Ca^{2+} measurements were carried out at room temperature on Fura2-AM (1 μM) loaded chondrocytes. The incubation medium employed contained (in mmol/l): NaCl 150, KCl 5, MgSO4 1, CaCl2 2, glucose 10, and HEPES-NaOH buffer 30, pH 7.4. The digital fluorescence-imaging system employed has been described elsewhere (6). Video frames were digitized, integrated in real time and finally processed off-line to convert fluorescence data in Ca^{2+} maps (340/380 nm excitation wavelength ratio method). 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. Intensities were expressed as ratio values (R=340/380). Manganese quenching experiments where performed according to Irvine (7) using two exchitation wavelengths to detect contemporarily the flourescence quenching (360 nm) and the Ca^{2+} signal (380 nm).

RESULTS AND DISCUSSION

Articular chondrocytes responded to bradykinin stimulation (bradykinin 1-100 nM) by transiently increasing the intracellular Ca^{2+} concentration (Fig.1A). In the presence of physiological concentration of extracellular Ca^{2+} (2 mM), the response to bradykinin was preceded by a short latency (5-10 sec), and was charachterized by a steep onset to a maximal Ca^{2+} level (R=2.7±1.2 starting from a basal R=0.71±0.08, n=87), followed by a slower decline to preexisting values . Decreasing the concentration of the extracellular Ca^{2+} by adding the chelator EGTA (5mM, extimated external Ca^{2+} < 500 nM), did not alter neither the latency nor the onset of the response to a second agonist challenge, resulting in a maximal Ca^{2+} level comparable to the previous response ($R_{Ca^{2+}} - R_{EGTA} = 0.11\pm0.03$, n=16); under these conditions, the declining phase appeared sometimes shortened and resulted in final Ca^{2+} values slightly lower than the prestimulation level (R=0.69±0.02, n=16). Finally, reintroduction of extracellular Ca^{2+} (final concentration from 2mM up to 10 mM) failed to increase intracellular Ca^{2+}, thus suggesting that bradykinin does not evoke Ca^{2+} influx in these cells. Direct confirmation of this hypothesis was obtained through the Mn^{2+} quench technique. Mn^{2+} (100μM) was added to the extracellular solution, followed by the addition of either bradykinin (100nM, Fig1B) or ionomycin (500 nM, Fig.1C) employed as a positive control. Manganese is known to penetrate into the cells through the receptor and second messenger-mediated Ca^{2+} influx pathways. Inside the cells it binds to Fura-2 thus leading to a quench of the fluorescence signal (8). While ionomycin consistently increased the rate of fluorescence quenching (measured at the isosbestic wavelength of 360 nm, Fig.1C, n=17), bradykinin failed to alter the rate of basal quench observed after the addition of Mn^{2+} (Fig.1B, n=20). Both the agents, however, increased the cytosolic calcium concentration, as revealed by the steep decrease of fluorescence measured at 380 nm. Taken together these results demonstrated that bradykinin did not evoke appreciable Ca^{2+} influx in articular chondrocytes, and, therefore, the Ca^{2+} rise was primarily due to mobilization of the ion from intracellular pools.

The tumor promoter thapsigargin is known to deplete the intracellular Ca^{2+} pools by irreversibly inhibiting the Ca^{2+}-ATPases responsible for refilling (9). In articular chondrocytes thapsigargin totally inhibited the subsequent bradykinin response (not shown). To evaluate the

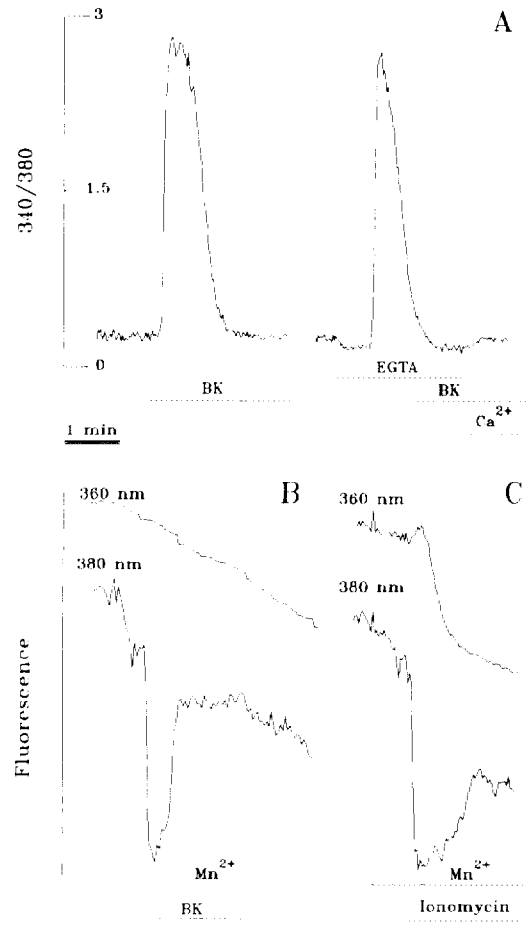

Fig.1. Cytosolic Ca^{2+} response to bradykinin: effect of extracellular Ca^{2+}. A) Representative tracing of Ca^{2+} response to bradykinin (BK, 100 nM) in a cell maintained in physiological external Ca^{2+} (2mM). After removal of the agonist (interruption of the recording), the extracellular Ca^{2+} concentration was lowered by addition of EGTA (5mM).The cell was then challenged with the same concentration of bradykinin (100 nM). Restoring the extracellular Ca^{2+} by addition of $CaCl_2$ (Ca^{2+}, 5 mM) was without effect on the intracellular Ca^{2+} concentration. B) The 380-nm tracing shows the Ca^{2+} response elicited by bradykinin (BK, 100 nM), while the 360-nm tracing shows the rate of fluorescence quenching induced by Mn^{2+}. The basal rate of quenching, determined by extracellular addition of Mn^{2+}, was not altered by the subsequent bradykinin administration. C) Experiment similar to that in B, employing ionomycin (500 nM) to stimulate Ca^{2+} influx.

contribution of the intracellular Ca^{2+} pools to the bradykinin response, we exposed the cells to thapsigargin 1μM and compared the subsequent Ca^{2+} rise to the response previously evoked by maximal bradykinin concentration (100 nM). In Fig.2 are reported the representative traces. The Ca^{2+} level attained in response to bradykinin (that varied greately from cell to cell) was related to the amount of Ca^{2+} subsequently released by thapsigargin (compare Fig.2A, B and C). Since thapsigargin mobilizes Ca^{2+} without activating the PLC - $InsP_3$ cascade (10), this result suggests a heterogeneity involving the extent of the intracellular pools . The thapsigargin treatment prevented a

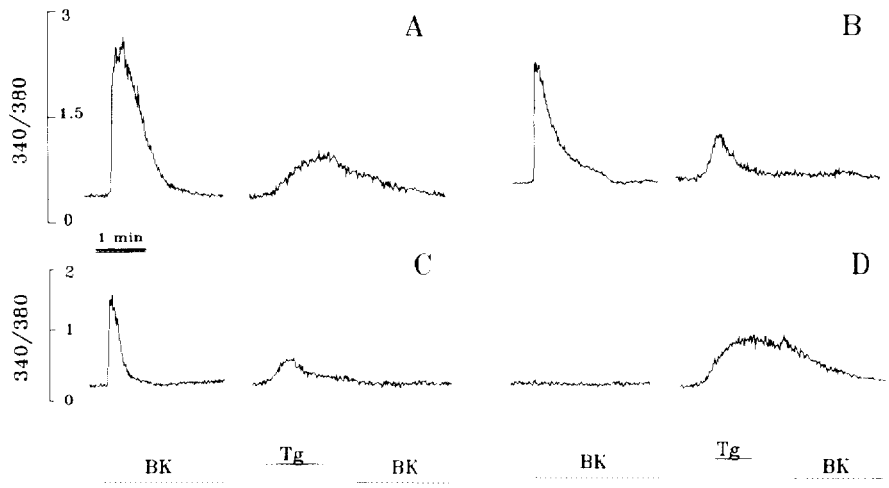

Fig.2. Role of the intracellular Ca^{2+} pools in the response to bradykinin. A-D) Representative tracings from different cells initially stimulated with maximal bradykinin concentration (BK, 100 nM) and then challenged with the Ca^{2+}-ATPase blocker thapsigargin (Tg, 1 μM). Tracings are interrupted in instances of cell washing. A second challenge with bradykinin failed to evoke any signal. D) Tracing representative of a fraction of the cells (10%) that were unresponsive to bradykinin, but retained the ability to respond to thapsigargin (1μM).

subsequent bradykinin challenge to evoke any signal, thus demonstrating the overlap between the Ca^{2+} pools mobilized by the two agents. A minority (10%) of the cells which failed to respond to bradykinin, released Ca^{2+} in response to thapsigargin (Fig. 2D), thus showing a difference either in the degree of receptor expression, or in the transduction machinery.

The videoimaging technique enabled us to reveal, in single cells, the spatial aspects of the Ca^{2+} response. In 47% of the cells cytosolic Ca^{2+} was shown to increase initially in a defined subcellular region, from which it subsequently spread to involve the rest of the cell. In Fig.3 are reported two series of 8 consecutive images collected at 2 frames/sec; the cell was initially stimulated with bradykinin (Fig.3A) and then challenged with thapsigargin (Fig.3B). Agonist stimulation initially led to a localized Ca^{2+}increase (Fig.3A, the "starting zone" is indicated by an arrow), which was followed by the spreading of the signal to the whole cell. The subsequent thapsigargin treatment evoked a prolonged Ca^{2+} rise (Fig.3B), which started from the same region and, in this particular case, remained localized. We next tested the ability of other agonists to induce a localized response, by comparing the temporal and spatial distribution of the Ca^{2+} increase induced by bradykinin and by fetal calf serum in single cells. In Fig.4 are reported three series of images taken from a cell stimulated with bradykinin (100 nM, Fig.4A), fetal calf serum (1%, Fig.4B) and thapsigargin (1μM, Fig. 4C). The arrow indicates the "starting zone" that appears to be the same, indipendently on the stimulus applied, thus ruling out the possibility that the localized signal could derive from a heterogeneous distribution of plasma membrane receptors.

At high temporal resolution (8 frames/sec) the analysis of the bradykinin-evoked Ca^{2+} response recorded in two different regions of the cells (Fig.5, A-D) revealed another interesting

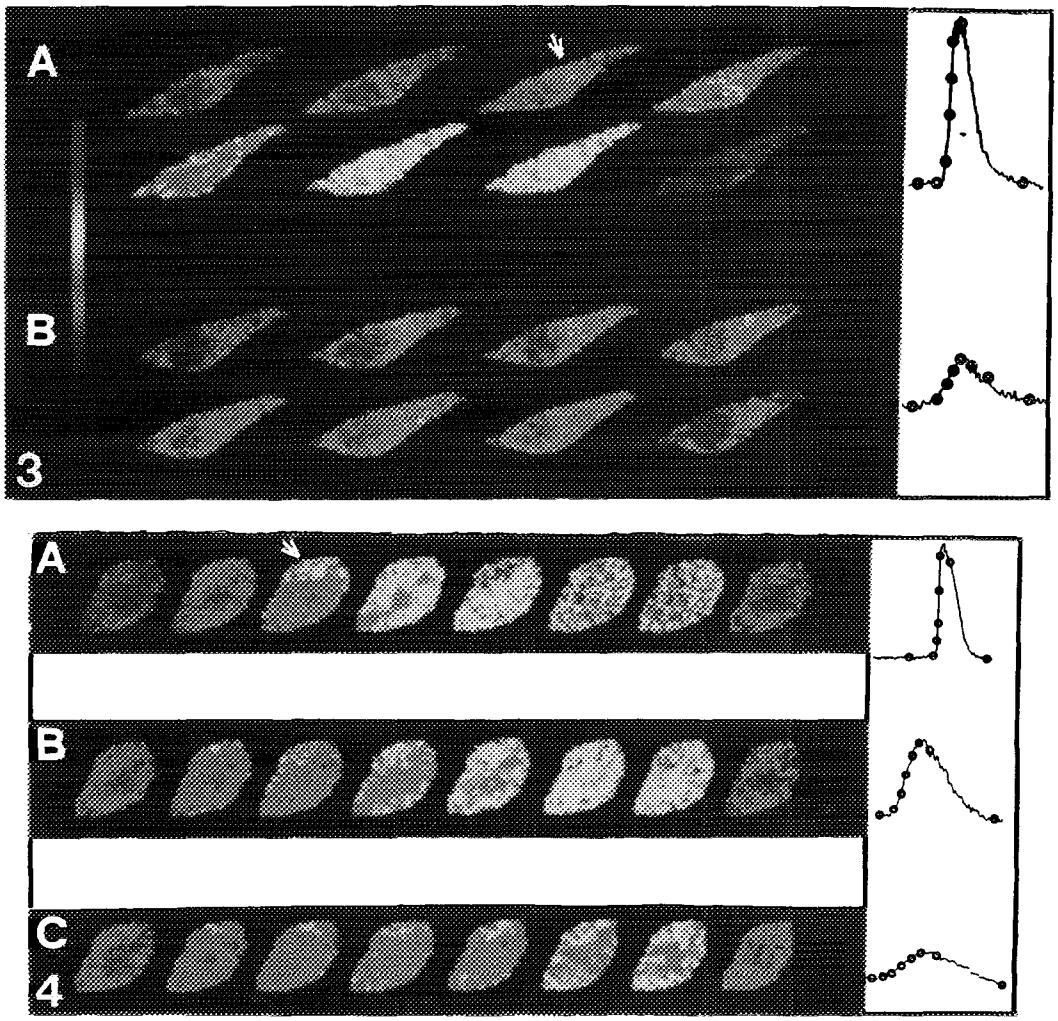

Fig.3. Spatial and temporal Ca^{2+} changes in response to bradykinin and thapsigargin. Two series from the same cell, each composed of eight images, illustrating the map of Ca^{2+} changes during cell responses to bradykinin (100 nM, Fig.3A) and thapsigargin (1μM, Fig.3B). The tracings to the right illustrate the full transients and mark the time corresponding to the image series. A) In the response to bradykinin is evident the "starting zone" (arrow), from which the signal initiated and then spread to the whole cell in a regenerative way. B) The response to thapsigargin appears to start in the same region of the cell and remained localized. Intensity of fluorescence ratio values is represented according to the calibration bar to the left, where the lowest values are coded blue. Acquisition rate was two images/sec.

Fig.4. The spatial localization of the Ca^{2+} changes is independent of the stimulus applied. A-C) three series from the same cell, each composed by eight images. The tracings to the right illustrate the full transients and indicate the points when the images were collected. A) Response to bradykinin (100 nM), showing the "starting zone" (arrow) that was followed by non decremental signal spreading. B) Response to fetal calf serum (1%), although weaker than the previous one, revealed the same spatial and temporal evolution. C) Blockade of Ca^{2+}-ATPase by thapsigargin (1μ M) resulted in a Ca^{2+} release initially localized in the same subcellular area; in this case the signal spread to involve other regions of the cell. Acquisition rate was two images/sec.

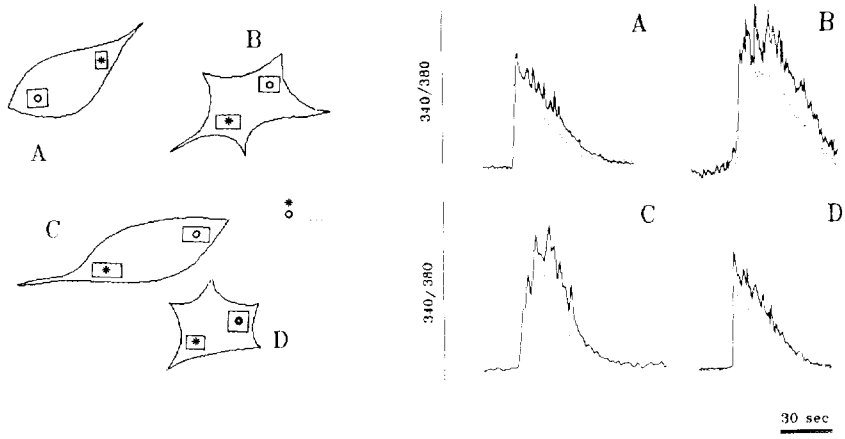

Fig.5.Miniature Ca^{2+} oscillations involving restricted subcellular regions.
A-D) Bradykinin stimulation induced a different evolution of the Ca^{2+} signal in the two distinct areas marked on the cell profiles to the left [starting zone(*) and spreading zone (o)] and represented in the tracings by the solid and the dotted line,respectively. In the first the Ca^{2+} signal appeared oscillatory, giving rise to rhythmic spikes which persisted during most of the falling phase. Such a pulsatile activity failed to involve the rest of the cell where the signal developed more uniformly. Acquisition rate was eight images/sec.

aspect. In the "starting zone" (solid line) the Ca^{2+} release appeared pulsatile, giving rise to local Ca^{2+} spikes. On the contrary, the remaining part of the cell, which was involved later by the Ca^{2+} rise (dotted line), failed to show any pulsatile activity, thus confirming a functional difference between the two regions of the cell. Such a heterogeneity was recorded in 35% of the cells tested. Although we failed to detect global and sustained Ca^{2+} oscillations in articular chondrocytes stimulated by bradykinin, the local Ca^{2+} spikes recorded at the "starting region" suggests the presence of a high sensitive pulsatile pacemaker, similar to that observed in other cell types (6, 11,12). Such Ca^{2+} pacemaker, capable of triggering both the initiation of a Ca^{2+} wave and the rhythmic activity associated to Ca^{2+} oscillations, is believed to be associated to high sensitive $InsP_3$ receptors, the intracellular Ca^{2+} release channels coupling the polyphosphoinositide hydrolisis to the Ca^{2+} release (13,14).

In articular cartilage, chondrocytes are embedded in a matrix acting as a barrier for extracellular stimuli; under this condition they are characterized by very low metabolic activity, cell quiescence and low matrix turnover (5). In pathological situations, on the other hand, cartilage damage increases cell accessibility to agents known to stimulate cell metabolism and/or proliferation (1). Intracellular Ca^{2+} waves and oscillations represent the signalling system activated by some of these agents in articular chodrocytes. Such complex spatial and temporal Ca^{2+} responses are likely to have profound biological significance in transducing the signals to the specific and final targets.

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