Interleukin 1-induced calcium signalling in chondrocytes requires focal adhesions

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The cytokine interleukin 1 (IL-1) is an important mediator of connective-tissue destruction in arthritic joints but the mechanisms by which IL-1 mediates signal transduction in chondrocytes is poorly understood. Previous results have indicated that IL-1 receptors co-localize with focal adhesions [Qwarnstrom, Page, Gillis and Dower (1988) J. Biol. Chem. **263**, 8261–8269], discrete adhesive domains of cells that function in cell attachment and possibly in signal transduction. We have determined whether focal adhesions restrict IL-1-induced Ca^{2+} signalling in primary cultures of bovine chondrocytes. In cells grown for 24 h on fibronectin, the basal intracellular Ca^{2+} ion concentration ($[Ca^{2+}]_i$) was 100 ± 3 nM. Optimal increases of $[Ca^{2+}]_i$ above baseline were induced by 10 nM IL-1 (183 ± 30 nM above baseline). There was no significant difference between cells plated on fibronectin or type II collagen ($P > 0.2$; 233 ± 90 nM above baseline). Ca^{2+} transients were significantly decreased by the inclusion of 0.5 mM EGTA in the bathing buffer (74 ± 11 nM above baseline), and 1 μM thapsigargin completely blocked Ca^{2+} transients. Cells plated on poly-(L-lysine) or suspended cells showed no Ca^{2+} increases, whereas cells grown on fibronectin exhibited IL-1-induced Ca^{2+} responses that corresponded temporally to the time-dependent cell spreading after plating on fibronectin. Cells plated on poly-(L-lysine) and incubated with fibronectin-coated beads exhibited vinculin staining in association with the beads. In identical cell preparations, IL-1 induced a 136 ± 39 nM increase of $[Ca^{2+}]_i$ above baseline in response to 10 nM IL-1β. There were no IL-1-induced Ca^{2+} increases when cells on poly-(L-lysine) were incubated with fibronectin-coated beads for only 15 min at 37 °C, in cells maintained for 3 h at 4 °C, in cells incubated with BSA beads for 3 h at 37 °C, or in cells pretreated with cytochalasin D. Labelling of IL-1 receptors with ^{125}I-IL-1β showed 3-fold more specific labelling of focal adhesion complexes in cells incubated with fibronectin-coated beads compared with cells incubated with BSA-coated beads, indicating that IL-1 receptor binding or the number of IL-1 receptors was increased in focal adhesions. These results indicate that, in chondrocytes, IL-1-induced Ca^{2+} signalling is dependent on focal adhesion formation and that focal adhesions recruit IL-1 receptors by redistribution in the cell membrane.

INTRODUCTION

Irreparable degradation of articular cartilage is a characteristic feature of arthritic diseases. Much of this degradation occurs as a result of increased levels of matrix metalloproteinases such as collagenase and stromelysin [1–4]. Numerous reports have demonstrated that proinflammatory cytokines such as interleukin 1 (IL-1) are elevated in arthritic joints and induce a myriad of chondrocyte responses, including decreased proteoglycan synthesis [5] and increased production of matrix metalloproteinases, prostaglandin E_2 and reactive oxygen species, molecules that are thought to be directly involved in cartilage degradation [6–10]. Although the biological activities of IL-1 are well documented, the molecular mechanisms by which IL-1 activates these responses in chondrocytes are not clear. Virtually all known IL-1 signalling systems have been implicated (e.g. phospholipase C, G-proteins, protein kinase C, cAMP and arachidonic acid). The initial signalling step seems to involve binding to plasma membrane receptors [11,12]. Although a great deal is known about IL-1 receptor heterogeneity and binding, there is little agreement on how the signal transduction pathways are utilized and restricted [13,14].

Evidence from internalization and localization studies in fibroblasts indicates that IL-1 receptors are concentrated at focal adhesions [15], suggesting that IL-1-induced responses might be regulated through interactions of connective-tissue cells with the extracellular matrix, particularly at focal adhesions [16]. Indeed, IL-1 causes a transient increase in phosphorylation and redistribution of the focal adhesion protein talin by rapid post-translational modification [17]. In the context of signalling through cytoskeletal elements, IL-1 induces serine/threonine kinase activity, and some of the phosphorylated target proteins include actin-related or focal adhesion proteins [18,19]. As IL-1-induced increases in intracellular Ca^{2+} are dependent on cell attachment [20], cellular interactions with matrix ligands might regulate the generation of signals after IL-1 binding with its cell-surface receptor.

Alterations in intracellular Ca^{2+} ion concentration ($[Ca^{2+}]_i$) provide a ubiquitous cell signalling system that mediates a variety of cellular processes in a spatially and temporally contained manner [21]. In chondrocytes treated with the Ca^{2+} ionophore A23187, there is strong, dose-dependent up-regulation of gelatinase expression [22,23], indicating that Ca^{2+} is important for IL-1-induced downstream signalling. Important regulatory enzymes controlled by Ca^{2+} through calmodulin include ATPases, Ca^{2+}-dependent phospholipase A_2, protein kinases, protein phosphatases and the cAMP regulators phosphodiesterase and adenylate cyclase [24]. However, there are very few reports on IL-1 regulation of $[Ca^{2+}]_i$. IL-1 does not seem to induce $[Ca^{2+}]_i$ responses in UMR-160 cells, an osteoblastic cell line [25], in human neutrophils [26], in a T-lymphoma cell line [27] or in a pre-B-cell line [28]. IL-1 increases $[Ca^{2+}]_i$ in fibroblasts [20,29,30],

Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} ion concentration; fura 2/AM, fura 2 acetoxymethyl ester; IL, interleukin.

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but the regulation of $[Ca^{2+}]_i$ by IL-1 in chondrocytes is unknown. In this study we report Ca^{2+} signalling in chondrocytes in response to IL-1 and the regulation of signalling by focal adhesions.

MATERIALS AND METHODS

Materials

Recombinant human IL-1β was obtained from R&D Systems (Minneapolis, MN, U.S.A.). The protein was purified by sequential chromatography (more than 97% purity) and the endotoxin level was determined to be not more than 0.1 ng/μg of IL-1. Fura 2 and fura 2 acetoxymethyl ester (fura 2/AM) were obtained from Molecular Probes (Eugene, OR, U.S.A.). Ionomycin was from Calbiochem (La Jolla, CA, U.S.A.). The following reagents were purchased from Sigma (St. Louis, MO, U.S.A.): bovine fibronectin, poly-(L-lysine), collagen type II, BSA, mouse monoclonal antibody to vinculin, α_5-integrin, β-actin, α-talin, FITC-conjugated antibodies, EGTA, thapsigargin and dithiobis-succinimidyl propionate. ^{125}I-rIL-1β was purchased from ICN (specific radioactivity 157 μCi/μg).

Isolation of bovine articular chondrocytes

Bovine chondrocytes were isolated by the procedure previously described by Kuettner et al. [31], with some modifications. Briefly, cartilage fragments were washed three times in Ham's F12 medium, and incubated for 1 h with 15 ml of the same medium containing 0.5% Pronase. The cartilage was washed and then incubated overnight with 0.1% bacterial collagenase in the same volume of medium containing 5% (v/v) fetal bovine serum. The cells were washed and plated on the culture dish in Ham's F12 medium containing 5% (v/v) fetal bovine serum. The cells were allowed to recover for 24 h at 37 °C in a humidified air/CO_2 (19:1) atmosphere.

$[Ca^{2+}]_i$ measurement

Chondrocytes were plated on round glass coverslips 0.1 mm thick and 31 mm in diameter (no. 0; Biophysica Technologies, Sparks, MD, U.S.A.) in 35 mm Petri dishes. The cells were loaded with 3 μM fura 2/AM for 30 min at room temperature to obtain uniform dye loading. The cells were washed twice to remove extracellular dye, transferred to a tissue-culture chamber (Corning, Cambridge, MA, U.S.A.), and finally suspended in the buffer containing 117.2 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 20 mM Hepes and 1.8 mM $CaCl_2$ at pH 7.2. The Ca^{2+} response to IL-1 was studied in cells plated on coverslips coated with fibronectin (10 μg/ml), poly-(L-lysine) (1 mg/ml) or collagen type IV (500 μg/ml), or in suspended cells. In some experiments, fibronectin- or BSA-coated latex beads (4.5 μm in diameter; Polyscience, Warrington, PA, U.S.A.; see [32] for coating method, and see below) were added to the cells grown on poly-(L-lysine)-coated coverslips for a 3 h incubation period. In experiments with Ca^{2+}-free medium, 0.5 mM EGTA was added to the above buffer without Ca^{2+} to chelate free Ca^{2+} ions before adding IL-1β.

The relative fluorescence intensity of cells was estimated by dual-wavelength excitation (346/380 nm; Photon Technology International, London, ON, Canada) with emission at 510 nm. The slit widths were set at 4 nm. A Nikon $\times$40 oil-immersion objective (numerical aperture 1.32) was used. A variable-aperture mask within the optical path was used to restrict measurements to a group of approximately five cells. The emission signal was filtered by a 510 nm long-pass barrier filter, and detected by photon counting. The output of the photon counter was digitized and recorded on the system computer. Signals from the photomultiplier tube (D104, PTI) were recorded at five points per second. Estimates of $[Ca^{2+}]_i$ independent of the precise intracellular concentration of fura 2 were calculated from emitted fluorescence, where $[Ca^{2+}]_i$ (in nM) $= K_d \times (Sf_2/Sb_2) \times [(R-R_{min})/(R_{max}-R)]$. The K_d (224 nM) and Sf_2/Sb_2 ratio were calculated at 380 nM from 11 excitation wavelength scans of 1 μM fura 2 free acid in buffers with $[Ca^{2+}]_i$ ranging from 0 to 39.8 μM. Sf_2 is the fluorescence of the Ca^{2+}-free sample and Sb_2 is the fluorescence of the Ca^{2+}-bound sample. The maximal 346/380 nm ratio (R_{max}) was measured after saturation of intracellular fura 2 with Ca^{2+} by adding 3 μM ionomycin to allow equilibration with extracellular Ca^{2+} ions. The minimal 346/380 nm ratio (R_{min}) measured during the complete dissociation of fura 2 from Ca^{2+} was obtained by adding 5 mM EGTA to the bathing buffer for each measured cell. R is the ratio of fluorescence intensities at 346/380 nm. In each experiment, background was estimated by the incubation of cells with 10 mM Mn^{2+}; this value was subtracted from the cellular fluorescence values before the 346/380 nm ratios were calculated.

Immunofluorescence staining and confocal microscopy

Multichamber glass slides were coated with bovine plasma fibronectin (10 μg/ml), poly-(L-lysine) (1 mg/ml) or collagen type II (500 μg/ml) when required and allowed to dry under sterile conditions before the cells were plated. Cells were plated for 24 h, fixed in 2% (w/v) paraformaldehyde for 30 min at room temperature, permeabilized with 0.3% Triton X-100 and thoroughly rinsed with PBS. Immunofluorescence staining for vinculin was performed with a mouse anti-vinculin antibody (1:20 dilution) for 1 h at 37 °C followed by a FITC-conjugated goat anti-mouse antibody (1:50 dilution) for 1 h at 37 °C. The coverslips were washed with PBS and mounted with an anti-fade mounting medium (ICN). Non-specific control staining was performed on the same slide by using secondary antibody only.

Cells attached to poly-(L-lysine) were also incubated with fibronectin- or BSA-coated latex beads (described above) [32] for 3 h before immunostaining. Briefly, one drop of beads was incubated at room temperature for 1 h with 1 ml of fibronectin or BSA solution, and then the beads were sonicated to eliminate clumps. Beads were rinsed and resuspended in PBS before being added to cells. At the end of the incubation time with beads, the cells were washed three times to remove unbound beads.

The spatial distribution of staining for vinculin in focal adhesions was imaged in single cells on a confocal microscope (Leica CLSM). For FITC-labelled antibodies, excitation was set at 488 nm and emission at 530 nm. Cells were imaged with a $\times$63 oil immersion lens (numerical aperture 1.4); transverse optical sections (nominal thickness 0.5 μm) were obtained at the level of cell attachment to the substratum.

Isolation of focal adhesions

Ferric oxide microparticles (hereafter 'magnetic beads'; Fe_3O_4; Aldrich Chemicals, Milwaukee, WI, U.S.A.) were coated with fibronectin (10 μg/ml) or BSA (1 mg/ml) for 1 h at room temperature. The beads were then sonicated to eliminate clumps and resuspended in PBS. Beads were added to attached cells for 1 h and then the cells were washed three times to remove unbound beads. By using the protocol of Plopper and Ingber

[33], cells and the attached magnetic beads were collected by scraping cells into cold cytoskeleton extraction buffer (CSKB) [0.5% Triton X-100/50 mM NaCl/300 mM sucrose/3 mM $MgCl_2$/20 μg/ml aprotinin/1 μg/ml leupeptin/1 μg/ml pepstatin/1 mM PMSF/10 mM Pipes (pH 6.8)]. The isolation procedure was performed at 4 °C with a side-pull magnetic isolation apparatus (Dynal, Lake Placid, NY, U.S.A.). The cell–bead suspension was sonicated for 10 s (output setting 3, power 15%; Sonifier 185; Branson, Danbury, CT, U.S.A.), and homogenized in a 2 ml Dounce homogenizer (20 strokes). The beads were magnetically pelleted and washed five times with cytoskeleton extraction buffer before protein analysis.

Western blot analysis

Proteins in the bead complexes were dissolved in SDS sample buffer [2% (w/v) SDS/2 mM urea/15 mg/ml dithiothreitol/0.003% Bromophenol Blue). All the samples were heated to 95 °C for 3 min and equal amounts of protein were loaded on individual lanes. Proteins were separated by SDS/PAGE [12% (w/v) gel] and then transferred to nitrocellulose membrane (0.45 μm pore). For immunoblotting, the membrane was blocked with 5% (w/v) non-fat milk in washing buffer (0.5%, v/v, Tween-20 in PBS) for 2 h. The first antibody (mouse monoclonal antibody to α_5-integrin, talin, vinculin and β-actin) was diluted 1000-fold in an incubation buffer [3% (w/v) non-fat milk and 0.5% Tween-20 in PBS] and incubated with the membrane for 1 h. The membrane was washed for 30 min, incubated with the peroxidase-conjugated anti-mouse antibody (1:1000 dilution) for 1 h, and detected by enhanced chemiluminescence (Amersham).

IL-1 receptor binding studies

Binding assays were performed as described by Dower et al. [34], with some modifications. Confluent cultures in 60 mm culture dishes were washed three times with binding buffer (F-12 containing 1% BSA, pH 7.4) after 1 h incubation with fibronectin- or BSA-coated beads at 37 °C. The cells were incubated at 4 °C with 0.5 nM ^{125}I-labelled IL-1β in 1 ml for 4 h under gentle rotatory shaking. Non-specific binding was determined in the presence of a 100-fold excess of unlabelled IL-1β. At the end of the incubation period, free ligand was removed, the cells were washed with ice-cold PBS and incubated with 200 μg/ml dithiobis-succinimidyl propionate for 1 h at 4 °C. Cells were washed three times with PBS, and the focal adhesion complex was isolated by using the procedure described above. Sample buffer (20 μl) was added to each sample; the samples were then boiled for 3 min. Samples were counted in a gamma counter and subjected to SDS/PAGE [12% (w/v) gel] under non-reducing conditions. Gels were dried under vacuum and exposed in a phosphorimager for 3 days at room temperature.

Statistical analysis

For $[Ca^{2+}]_i$, the means and S.E.M. were computed and comparisons between two groups were evaluated with an unpaired *t* test.

RESULTS

Ca^{2+} responses to IL-1β

In bovine chondrocytes grown for 24 h on coverslips coated with fibronectin (10 μg/ml), the basal $[Ca^{2+}]_i$ was 100 ± 3 nM (n = 20). Basal $[Ca^{2+}]_i$ was detectably increased by 0.1 and 1 nM IL-1, but at 10 nM IL-1 there was a nearly 3-fold increase in $[Ca^{2+}]_i$ (183 ± 30 nM above baseline; n = 7) (Figure 1). At 50 nM IL-1, the Ca^{2+} increase was less than at 10 nM IL-1 ($P < 0.05$). For all subsequent experiments, IL-1 was added to cells at 10 nM. To investigate whether the IL-1-induced Ca^{2+} increase was due to release from intracellular stores or entry from the extracellular bathing medium, we examined the effect of IL-1β on $[Ca^{2+}]_i$ in the presence (1.8 mM) and in the absence of extracellular Ca^{2+} (Ca^{2+}-free medium with 0.5 mM EGTA for 15 min). The amplitudes of Ca^{2+} transients above baseline after incubation in EGTA without Ca^{2+} were significantly decreased (74 ± 11 nM above baseline; $P < 0.05$; n = 4) (Figure 2). Preincubation for 30 min with 1 μM thapsigargin, an inhibitor of the sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPase, completely blocked the Ca^{2+} response to IL-1β in normal Ca^{2+}-containing medium (n = 5) (Figure 2). Thus extracellular Ca^{2+} was not wholly required for Ca^{2+} flux, and most of the increased $[Ca^{2+}]_i$ was released from internal compartments.

In cells plated on type II collagen, the principal collagen synthesized by chondrocytes, there was no significant change in the amplitude of IL-1-induced $[Ca^{2+}]_i$ compared with cells plated on fibronectin. Cells plated for 24 h on collagen type II exhibited

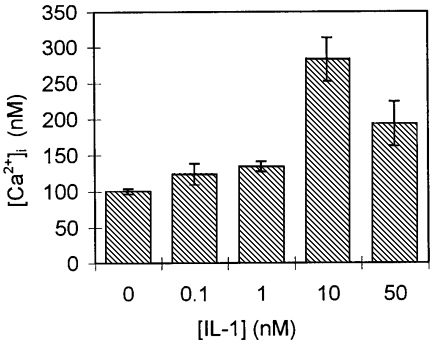

Figure 1 Histogram of peak intracellular Ca^{2+} amplitudes in response to IL-1β at indicated concentrations

$[Ca^{2+}]_i$ values were measured in groups of five chondrocytes plated on fibronectin. Cells were loaded with fura 2/AM and measured by ratio fluorimetry. Results are means ± S.E.M.

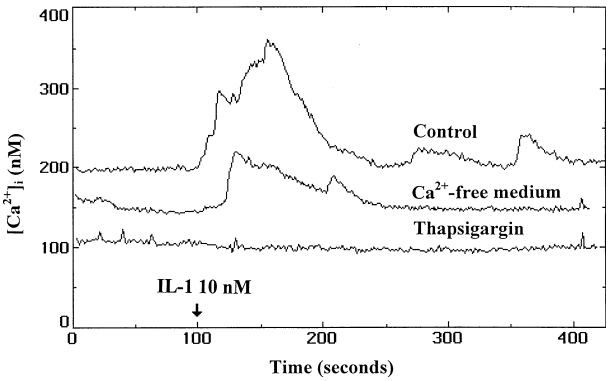

Figure 2 Intracellular Ca^{2+} concentrations of groups of five chondrocytes after incubation with IL-1β when indicated

Incubation in nominally Ca^{2+}-free medium with 0.5 mM EGTA decreased, but did not eliminate, Ca^{2+} transients, whereas thapsigargin (1 μM) completely blocked Ca^{2+} transients. The two upper traces have been offset by 50 nM vertically from each other to facilitate visualization of the different responses, but the baseline Ca^{2+} was similar.

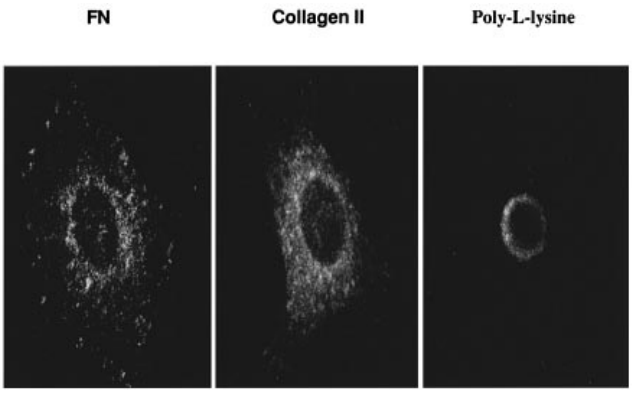

Figure 3 Confocal micrographs of chondrocytes stained for vinculin

Cells were plated on fibronectin (FN), type II collagen or poly-(L-lysine). Note the presence of focal adhesions, seen as discrete, punctate staining in cells on fibronectin or collagen but not on poly-(L-lysine).

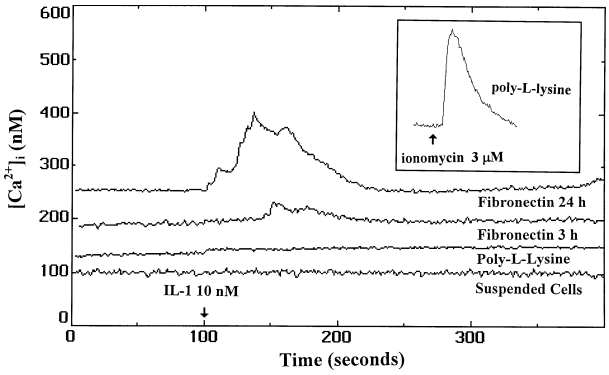

Figure 4 $[Ca^{2+}]$ of cell suspensions (approx. 10^6 cells) or groups of five chondrocytes plated on fibronectin or poly-(L-lysine)

Cells were loaded with fura 2/AM and stimulated with IL-1β when indicated, or with ionomycin in cells on poly-(L-lysine) (inset). The three upper traces have been offset vertically for clarity, as described in the legend to Figure 2.

a 233 ± 90 nM increase of $[Ca^{2+}]_i$ above baseline in response to 10 nM IL-1β ($n = 4$). There were no significant differences in Ca^{2+} response between high- and low-density cells cultured on either fibronectin ($n = 10$), or type II collagen-coated dishes ($P > 0.2$; $n = 4$).

Dependence of Ca^{2+} signalling on focal adhesions

We examined whether IL-1-induced Ca^{2+} responses were dependent on focal adhesions by plating cells for 24 h on dishes coated with poly-(L-lysine) (1 mg/ml), a protocol that mediates non-specific cell adhesion that is independent of integrins. As expected, in low-density cultures, cell morphology was influenced by the type of substrates used for plating: cells remained rounded on poly-(L-lysine), whereas cells on collagen or fibronectin were well spread. Immunolocalization of the focal adhesion protein vinculin by confocal microscopy showed that cells on either fibronectin or collagen exhibited bright, punctate staining in presumptive focal adhesions (Figure 3). In contrast, cells plated on poly-(L-lysine) showed only diffuse staining and no discrete sites that were greatly enriched for vinculin.

Cells plated on poly-(L-lysine) showed no Ca^{2+} increase, in spite of prolonged plating times ($n = 6$) (Figure 4). These cells were viable, as shown by their robust Ca^{2+} responses to ionomycin (Figure 4, inset). The Ca^{2+} responses of cells on poly-(L-lysine) to ionomycin were indistinguishable from responses on fibronectin, indicating that IL-1 responses were modified selectively by substrate. Previous results have also shown that integrin-independent prostaglandin E_2 signalling on polylysine is similar to that on fibronectin [20], indicating that poly-(L-lysine)-mediated adhesion is probably not involved in integrin-dependent IL-1 signalling. In contrast, cells grown on fibronectin-coated coverslips exhibited IL-1-induced Ca^{2+} responses that corresponded temporally to the time-dependent cell spreading after plating on fibronectin. The IL-1-evoked Ca^{2+} increase above baseline in cells plated on fibronectin for 3, 6 and 24 h were 21 ± 9 nM ($n = 4$), 41 ± 18 nM ($n = 4$) and 183 ± 30 nM ($n = 7$) respectively, indicating that the IL-1-induced Ca^{2+} responses were associated with spreading rather than cell attachment alone. Cells in suspension incubated with soluble fibronectin (10 μg/ml) and stimulated with 10 nM IL-1β also showed no Ca^{2+} flux (Figure 4), demonstrating a requirement for substrate attachment and not just occupation of fibronectin receptors for promotion of an IL-1-induced Ca^{2+} flux.

As cell shape profoundly affects cellular response to exogenous signals, we assessed whether cell rounding induced by poly-(L-lysine) was solely responsible for the failure to induce Ca^{2+} signalling after IL-1 stimulation. Fibronectin-coated latex beads were added to cells previously plated on poly-(L-lysine) for 3 h at 37 °C. Confocal optical sections of vinculin staining showed that in these rounded cells there was aggregation of vinculin at bead–cell attachment sites (Figure 5). In contrast, little or no clustering of vinculin was observed with beads coated with BSA. We assessed whether, in identical cell preparations, focal adhesions induced by bead complexes would mediate Ca^{2+} signalling by IL-1. Cells plated on poly-(L-lysine) and incubated with fibronectin-coated beads demonstrated an increase in $[Ca^{2+}]_i$ of 136 ± 39 nM above baseline in response to 10 nM IL-1β ($n = 4$) (Figure 6), although the response rate was somewhat slower than for cells plated on fibronectin, perhaps because of differences in the numbers of focal adhesions or because of variations in IL-1 receptor accessibility. In contrast there was no IL-1-induced Ca^{2+} increase when cells on poly-(L-lysine) were treated with the following bead loading protocols: (1) incubation of fibronectin

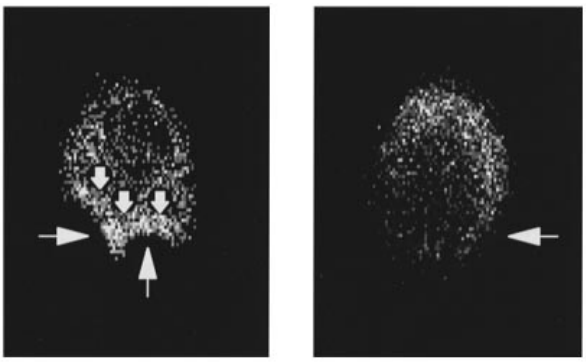

Figure 5 Confocal micrographs of single chondrocytes plated on poly-(L-lysine) and incubated with beads coated with fibronectin (left panel) or BSA (right panel)

Cells were stained for vinculin. Note the staining for vinculin adjacent to the fibronectin-coated beads, but not the BSA-coated beads.

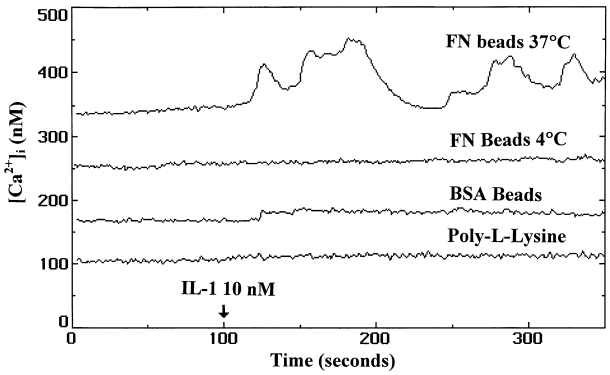

Figure 6 [Ca^{2+}] of groups of five chondrocytes plated on poly-(L-lysine) or plated on poly-(L-lysine) and incubated with fibronectin (FN)- or BSA-coated beads as described in Figure 5

Note that only cells that were preincubated with fibronectin-coated beads at 37 °C are capable of IL-1-induced Ca^{2+} signalling. As described in the legend to Figure 2, the upper three traces have been offset vertically for clarity.

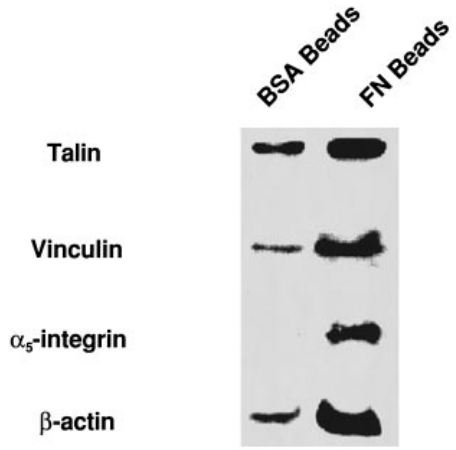

Figure 7 Western blots of focal adhesion proteins prepared by the bead isolation method show the presence of α_5-integrin and 3–8-fold enrichment for talin, vinculin and β-actin when beads were coated with fibronectin (FN)

Note the absence of α_5-integrin staining and much decreased talin, vinculin and β-actin in focal adhesion complexes purified from BSA beads. Focal adhesion complexes were prepared by incubating cells with ferric oxide beads, followed by the use of a magnet to strip beads from cells. Equal amounts of protein were loaded on gels from BSA and fibronectin-coated bead complexes before immunoblotting.

beads with cells for 15 min at 37 °C; (2) incubation of fibronectin beads with cells for 3 h at 4 °C, a procedure that inhibited redistribution of focal adhesion proteins to the dorsal surface; (3) incubation with BSA beads for 3 h at 37 °C; or (4) pretreatment of cells with cytochalasin D (1 μg/ml for 15 min). These results indicate that IL-1-induced Ca^{2+} signalling is dependent on focal adhesion formation and the ability of focal adhesion proteins such as vinculin, integrins and talin to redistribute in the cell membrane, but is independent of cell attachment.

IL-1 receptors in focal adhesions

As IL-1 signalling might also be dependent on the physical association of IL-1 receptors with focal adhesions, we examined

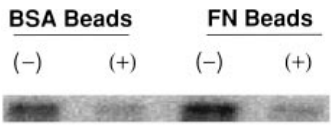

Figure 8 Focal adhesion complexes are enriched 3-fold in amounts of radiolabelled IL-1 receptor when beads coated with fibronectin (FN) instead of BSA were used for isolation

Cells were incubated with radiolabelled IL-1β before bead isolation (−). Note the virtual disappearance of radiolabelled IL-1 receptor when cells were preincubated with 100-fold higher concentrations of unlabelled IL-1 before incubation with the radiolabelled IL-1β (+).

the levels of focal adhesion proteins and IL-1 receptors in purified focal adhesion complexes. We used magnetic beads coated with fibronectin or BSA (1 h at 37 °C) to isolate focal adhesion proteins. Immunoblotting of isolated proteins from the magnetic beads showed that fibronectin beads induced a strong enrichment for talin (200 kDa), vinculin (118 kDa) and β-actin (45 kDa) compared with BSA beads (Figure 7). In contrast with cells incubated with fibronectin-coated beads, there was no detectable α_5-integrin (140 kDa) in the BSA bead preparation. We determined whether incubation with fibronectin-coated beads recruits IL-1 receptors into focal adhesions. ^{125}I-labelled IL-1β was used as a ligand to assess IL-1 receptor association with fibronectin- or BSA-coated beads. In cells incubated with fibronectin-coated beads, ^{125}I-IL-1β labelled a protein in the isolated focal adhesions with a molecular mass of approx. 100 kDa. Compared with cells incubated with BSA-coated beads, there was 3-fold more labelling in the 100 kDa bands of cells incubated with fibronectin-coated beads (Figure 8), indicating that IL-1 receptor binding and/or the number of IL-1 receptors was increased in focal adhesions (Figure 8). The ^{125}I-IL-1β labelling was specific, because preincubation of cells with 100-fold higher concentrations of unlabelled IL-1β abolished the labelled band on the gel.

DISCUSSION

Most of our understanding of the role of IL-1 in joint diseases originates from relatively simplified tissue-culture models; consequently there is a need to obtain a more detailed view of how cells bound to matrix ligands respond to cytokines such as IL-1 and regulate chondrocyte function [14]. Our principal finding is that in chondrocytes, Ca^{2+} signalling is dependent on focal adhesion formation. These results support the notion that cellular interactions with matrix ligands regulate the generation of signals after IL-1 interaction with its cell-surface receptor. This idea is consistent with the data of Qwarnstrom et al. [15], who found that IL-1 receptors in fibroblasts are concentrated near regions of cell attachment. Thus a requirement for Ca^{2+} signalling and focal-adhesion formation might provide a restriction mechanism by which only cells appropriately attached to their matrix can generate downstream signals, including Ca^{2+} fluxes. In the context of signalling through cytoskeletal elements, it is interesting to note that IL-1 induces serine/threonine kinase activity and that some of the phosphorylated target proteins include actin-related or focal adhesion proteins [12,19]. Further, Werb et al. [35] demonstrated that ligation of the fibronectin receptor induced changes in the cytoskeleton and in the expression of genes encoding for matrix metalloproteinases such as collagenase and stromelysin. Thus the binding of various ligands to integrins seems to be involved in the expression of genes regulating cell

behaviour and perhaps other adhesive receptors in the extracellular environment [36].

Focal adhesions are discrete, specialized adhesive domains that are thought to be important not only as structural links between the extracellular matrix and the cytoskeleton, but also as specialized sites of signal transduction [37]. These structures contain protein receptors that bind extracellular ligands such as integrins [38], and those proteins that mediate attachment of the receptors to the cytoskeleton, such as vinculin, paxillin, talin and α-actinin [39]. We showed, both in immunocytochemical preparations and in purified fibronectin-coated beads complexes, that actin, talin, vinculin and α_5-integrin were present, confirming that the structures we examined in chondrocytes were indeed focal adhesions. In addition, several potential regulatory enzymes involved in signal transduction have also been identified in focal adhesions, including tyrosine kinases such as $pp125^{fak}$ [40], Src and Lyn, and type 3 protein kinase C. As IL-1 might affect the interactions of fibroblasts with the extracellular matrix by modulating cell–matrix interactions at focal adhesions [16], it is notable that IL-1 causes a transient increase in phosphorylation and redistribution of the focal adhesion protein talin by rapid post-translational modification [19]. Conversely, the novelty of our findings is that, after very short-term incubations with IL-1 (i.e. less than 30 s), Ca^{2+} signalling is detected and that the physical association of focal adhesion proteins with IL-1 receptors is required. Further, the mobility of these focal adhesions and their ability to form on the ventral surfaces of rounded cells indicate that cell spreading and attachment are not prerequisites for signalling. Instead, it is the close physical association of IL-1 receptor with focal adhesion proteins that is the central determinant.

The results presented here emphasize the importance of cell–extracellular-matrix interactions through integrin proteins and the formation of focal adhesions in the regulation of IL-1-induced responses by chondrocytes. It is well established that chondrocytes localized to the cartilage surface have a flatter morphology and are more sensitive to IL-1 stimulation than are deeper chondrocytes [10,41]. Surface chondrocytes contain higher levels of IL-1 receptors [10], but it is not known whether surface chondrocytes are enriched with focal adhesions. However, our findings raise the possibility that an increased number of focal adhesions might contribute to the increased susceptibility of surface chondrocytes to IL-1 stimulation. In diseased states, the generation of focal adhesions enriched in IL-1 receptors and focal adhesion proteins involved in cell signalling might provide a mechanism to increase the sensitivity of chondrocytes to exogenous levels of cytokines that would not normally induce a signal.

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