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IL-1β-induced production of metalloproteinases by synovial cells depends on gap junction conductance

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Kolomytkin, Oleg V., Andrew A. Marino, David D. Waddell, J. Michael Mathis, Robert E. Wolf, Kalia K. Sadasivan, and James A. Albright. IL-1β-induced production of metalloproteinases by synovial cells depends on gap junction conductance. *Am J Physiol Cell Physiol* 282: C1254–C1260, 2002. First published January 30, 2002; 10.1152/ajpcell.01166.2000.—Synovial cells can form networks connected by gap junctions. The purpose of this study was to obtain evidence for a necessary role of gap junction intercellular communication in protein secretion by synovial cells. We developed a novel assay to measure the enzymatic activity of metalloproteinases (MMPs) produced by synovial cells in response to interleukin-1β (IL-1β) and employed the assay to explore the biological function of gap junctions. IL-1β produced a dose-dependent increase in MMP activity that was blocked by exposure to the gap junction inhibitors 18α-glycyrrhetinic acid and octanol for as few as 50 min. The inhibitors produced an immediate and marked reduction in intercellular communication, as assessed by transient current analysis using the nystatin perforated-patch method. These observations suggest that communication through gap junctions early in IL-1β signal transduction is critical to the process of cytokine-regulated secretion of MMPs by synovial cells.

perforated patch; HIG-82 cells; gap junction inhibitors; collagen assay

GAP JUNCTIONS ARE PLAQUES of aqueous channels that facilitate direct electrical and metabolic connection between adjacent cells. The plaques consist of a few to many hundreds of individual channels, each capable of passing molecules with mass up to ~1 kDa (2). A channel is formed by noncovalent linkages between annular hemichannels composed of proteins of the connexin family; more than a dozen isoforms have been identified (3). Gap junctions are continuously formed, modified, and removed (12), resulting in restriction or augmentation of gap junction intercellular communication (GJIC).

We used patch clamping, dye transfer, and electron microscopy to demonstrate the existence of functional and structural gap junction channels in rabbit and human synovial cells in culture and in synovial lining cells in tissue (9–11). The specific relevance of intercellular communication to synovial metabolism is unknown, but several lines of evidence suggested to us that GJIC might be involved in signal transduction. First, interleukin-1β (IL-1β) can stimulate protein expression by synovial cells (7) and can depolarize the cell membranes via a GJIC-dependent process (10). These observations raised the possibility that depolarization (hence, GJIC) was necessary for stimulated expression. Second, we showed that synovial lining cells could form highly coupled networks (9), and similar networks have been shown to facilitate regulated secretion in other tissues (4, 14, 15).

The purpose of this study was to obtain evidence for a necessary role of gap junctions in the signaling cascade leading to protein production by synovial cells. This was accomplished by comparing the amount of proteinase activity produced by an established synovial cell line in response to cytokine stimulation in the presence and absence of channel inhibitors using a novel assay system.

MATERIALS AND METHODS

Cells and reagents. Rabbit synovial fibroblasts (HIG-82, American Type Culture Collection, Manassas, VA) were grown at 37°C in 5% CO_2 without antibiotics in 25-ml polystyrene flasks containing F-12 medium (GIBCO BRL, Grand Island, NY) with 10% FCS (growth medium). For passage of cells, confluent cultures were treated with 1 ml of 0.08% trypsin for 3–5 min; then 4 ml of medium were added, and the suspended cells were centrifuged, resuspended, and seeded (10^6 cells) into 4 ml of medium. The experiments were performed using 35-mm petri dishes seeded initially with 5×10^5 cells.

Metalloproteinases (MMPs) were assayed (see below) using 70–80% confluent monolayers in which the growth medium was replaced with serumless Neuman-Tytell medium (GIBCO) containing human recombinant IL-1β or other active substances. After the cells were incubated at 37°C for

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various periods up to 48 h, the proteinase activity of the supernatant was measured.

For electrophysiological measurements, the cells were treated according to our standard procedure (10). The growth medium in 24-h cultures was replaced with bath solution, and all measurements were made in bath solution at 25°C employing aggregates of 15–30 cells that were adherent to the bottom of the dish.

All reagents were obtained from Sigma (St. Louis, MO) unless noted otherwise. The stock solution of IL-1β contained 0.1% bovine albumin as carrier protein. The final albumin concentration in medium and bath solutions did not exceed 10^{-5}%; control experiments showed that 10^{-3}% bovine albumin did not influence the results of the enzyme assay or the current measurements. Phorbol 12-myristate 13-acetate (PMA), 18α-glycyrrhetinic acid (GR), octanol, and *p*-aminophenylmercuric acetate were dissolved in dimethyl sulfoxide at a final concentration not exceeding 0.3%; control experiments showed that this concentration did not influence the measurements.

Enzyme assay. Using collagen film as the substrate, we developed a quantitative non-radioisotope-based assay to measure the enzymatic activity of the MMPs (principally collagenase) produced by HIG-82 cells in response to various stimuli (1, 5, 7). Collagen solution (4 μl, 3.0 mg/ml in 0.012 N HCl; Collagen Biomaterials, Palo Alto, CA) was added to each well (6.4 mm diameter) of 96-well polystyrene plates and precipitated by slowly increasing the pH with 4 μl of H_2O, 4 μl of bath solution, and 8 μl of 0.012 N NaOH. The plate was dried at 23°C for 24 h, washed with H_2O, and dried again at 23°C, resulting in a thin (calculated thickness ~370 nm) film containing ~12 μg of collagen that was tightly bound to the bottom of the wells.

The cell supernatants to be analyzed were filtered, and 1 mM *p*-aminophenylmercuric acetate was added to activate MMPs (1). Enzymatic reactions of the supernatants (200 μl/well) against the collagen films were carried out in quadruplicate at 24°C for various times up to 15 h. The digested portion of the film was washed away, the undigested portion was fixed for 3 h with 0.1% Coomassie brilliant blue G in 10% acetic acid and 45% methanol, and the wells were washed and dried. The undigested film was then dissolved in 50 μl of ethanol for 20 min to obtain a uniform color distribution, and the optical density (OD) of each well was read with a microplate reader (model MR 5000, Dynatec Laboratories) at 570 nm (wavelength of maximum Coomassie brilliant blue G absorbance). Four wells were averaged to characterize the MMP activity of each petri dish, and 4 petri dishes were averaged for each condition.

Calculation of activity. The density of the initial collagen film was $12\ \mu g/3.2 \cdot \pi^2 \cdot mm^2 = 373\ mg/m^2$, and its OD was 0.85 ± 0.02. The amount of collagen degraded per unit area of film was determined from a measurement of OD as follows: $(0.85 - OD)(373/0.85)\ mg/m^2$, and MMP activity was assessed at a standard reaction time of 13 h [$34(0.85 - OD)\ mg \cdot h^{-1} \cdot m^{-2}$]. The supernatants degraded ≤35% of collagen film thickness under the conditions of our experiments.

The assay sensitivity was $0.8 \pm 0.1\ mg \cdot h^{-1} \cdot m^{-2}$. Its specificity was evaluated using *controls a–f*: wells without collagen treated with Neuman-Tytell medium (*a*), supernatant from unstimulated cultures (*b*), wells with untreated collagen films (*c*), collagen films treated with commercial collagenase and gelatinase (*d*), collagen films treated with denatured commercial collagenase and gelatinase (*e*), and collagen films treated with trypsin (*f*). *Controls a, b*, and *d* had no significant OD after Coomassie brilliant blue G treatment, and *controls c, e*, and *f* had OD = 0.85 ± 0.02.

Electrodes. The nystatin perforated-patch method (10) was used to measure the transmembrane current under voltage clamp. The method permitted use of the whole cell configuration for measuring electrical properties of the cell while preserving intracellular regulation by preventing diffusion of small signaling molecules from the cell into the electrode. Glass capillaries 1.0 mm in diameter were pulled in two steps (PB-7, Narishige, East Meadow, NY) and fire polished in a microforge (model MF-9, Narishige). The resistance of the electrodes was 7–9 MΩ in bath solution. The pipette solution contained (in mM) 125 potassium aspartate (monopotassium salt), 30 KCl, 4 NaCl, and 10 HEPES-KOH, pH 7.2 (calculated osmolarity = 318 mosmol/l). The bath solution consisted of (in mM) 145 NaCl, 5.4 KCl, 1.5 $CaCl_2$, 1.0 $MgCl_2$, 5.0 HEPES-NaOH, and 5.0 glucose, pH 7.3 (calculated osmolarity = 328 mosmol/l). Because nystatin interfered with giga-seal formation, the tip of the pipette was filled with a nystatin-free solution before the addition of pipette solution containing nystatin (0.3 μg/ml). The giga-seal was formed during the time needed for the nystatin to diffuse to the tip of the micropipette.

Electrical measurements. Giga-seals (~10 GΩ) were formed under negative pressure (5–10 cmH_2O), typically within 0.5–5 min; the success rate was >50%. After giga-seal formation, nystatin channels formed within 5–15 min; the resistance of the perforated-patch membrane was 40 ± 20 MΩ. Giga-seals and nystatin pores usually remained stable for hours.

Integral gap junction channel conductance between synovial cells was estimated by measuring the time constant of transient current decay induced by applying a 2-mV voltage step (10). A patch-clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA) connected to a computer (Digi-Data 1200, Axon) running commercial software (pCLAMP 6.0, Axon) was used to collect and analyze the data. The transient current trace from a given cell was obtained by averaging the results from 10 voltage steps. The time constants were determined from the average current trace, as described previously (10). Cell aggregates containing 15–30 cells were used, because it was not convenient to restrict the study to aggregates of the same size.

Membrane potential was defined as the potential at zero current. The membrane potentials of the cells studied were −63 ± 5 mV and were stable for hours.

Values are means ± SE ($n = 4$) and were evaluated using the *t*-test at $P < 0.05$.

Northern blot. Total cellular RNA was obtained by TriPure isolation (Roche, Indianapolis, IN). Aliquots (10 μg) of RNA were heat denatured and then size separated in a 1% agarose gel containing 2.2 M formaldehyde. Ethidium bromide was used to stain for the 28S and 18S ribosomal RNA subunit bands to check RNA integrity and loading. The RNA was transferred to a nylon membrane (Schleicher and Schuell, Keene, NH); after uniform transfer was assessed by visual inspection of the ribosomal RNA bands, the RNA was cross-linked to the membrane (Stratalinker, Stratagene, La Jolla, CA).

The membranes were treated for 3 h at 42°C with hybridization buffer [50% formamide, 5× SSC (3 M NaCl, 0.3 M sodium citrate), 10× Denhardt's solution, 1% SDS, 100 μg/ml salmon sperm DNA] before hybridization at 42°C for 16 h with ^{32}P-labeled complementary DNA fragments of pro-MMP-1. The membranes were then washed in 2× SSC-0.1% SDS for 5 min at 25°C and in 0.1× SSC-0.1% SDS for 15 min at 68°C and autoradiographed (Eastman Kodak, Rochester, NY) at −70°C for 5 days. The blots were quantitated (Image-

Quant, Molecular Dynamics, Sunnyvale, CA) and normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA.

RESULTS

MMP assay. Synovial cells incubated for 48 h in the presence of IL-1β released MMPs into the supernatant. The amount of collagen degraded was linearly proportional to the reaction time, as evaluated using our photometric method (Fig. 1); on the basis of this result, we chose 13 h as the standard reaction time. This choice ensured that only a part of the collagen film would be degraded under the conditions studied and that the enzyme reactions would occur with constant speed. The accuracy and sensitivity of the photometric method were compared with the corresponding published values found using a radioisotope-based method (5), with PMA as the MMP stimulus. PMA (0.01–100 ng/ml) elicited dose-dependent production of MMPs, with saturation at ~10 ng/ml (data not shown); half-maximum activity occurred at 1.7 ± 0.5 ng/ml, and the standard error of measurement was ~7% of maximum MMP activity. Effectively the same results were obtained using radioisotopes (5).

IL-1β-induced secretion. Proteinase activity in the supernatants from cells treated with IL-1β was detected after an incubation period of ~24 h. Subsequently, the extent of substrate degradation was dependent on IL-1β concentration (Fig. 2). To determine whether MMP production required the continuous presence of IL-1β in the medium, enzyme activity was measured during 0–48 h of incubation with IL-1β (100 pg/ml); then the medium was replaced with fresh medium containing no IL-1β, and the measurements were continued. MMP production dropped to baseline after removal of the IL-1β and did not rise during the subsequent 96 h, suggesting that the continuous presence of IL-1β was required for MMP production.

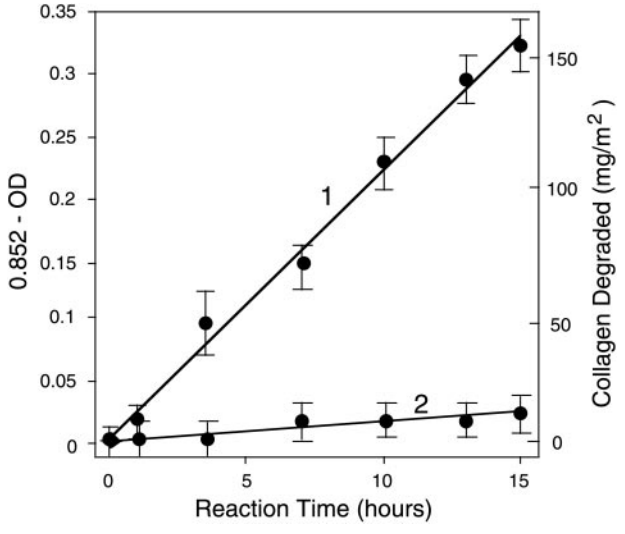

Fig. 1. Effect of reaction time on degradation of collagen film by the supernatant in synovial cells treated with interleukin-1β (IL-1β). *Curve 1*, IL-1β (100 pg/ml) for 48 h; *curve 2*, control (no IL-1β added). OD, optical density.

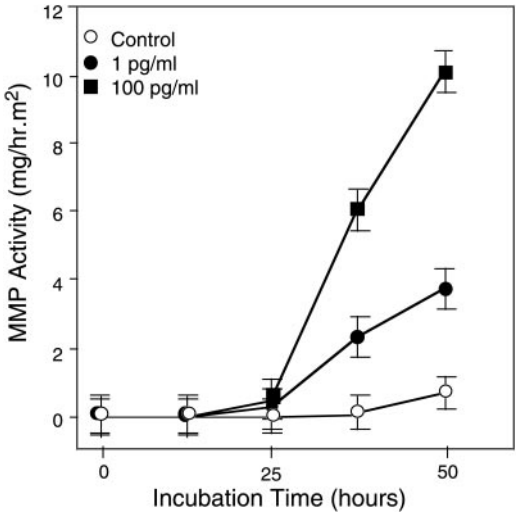

Fig. 2. Metalloproteinase (MMP) activity in the supernatant of synovial cells exposed to IL-1β for 48 h.

Effect of gap junction inhibitors on secretion. The role of GJIC in the stimulated production of MMPs was studied using the gap junction inhibitors GR and octanol. GR was added to the medium in the petri dish at *time 0*, IL-1β (100 pg/ml final concentration) was added 10 min later, and MMP activity was measured as a function of incubation time. Addition of GR resulted in a dose-dependent inhibition of IL-1β-stimulated MMP production (Fig. 3). Half-maximum inhibition occurred at 7.0 ± 0.6 μM GR. GR (5 and 10 μM) alone (without IL-1β) did not induce MMP production (<0.7 mg·h^{-1}·m^{-2}, not shown). The effect of 1 mM octanol on IL-1β-stimulated production of MMPs was similar to that of GR (Fig. 4). Octanol alone did not induce MMP production (not shown).

Effect on secretion of impeding conductance early in transduction. In separate experiments, GR and octanol were added at *time 0* to the medium in dishes contain-

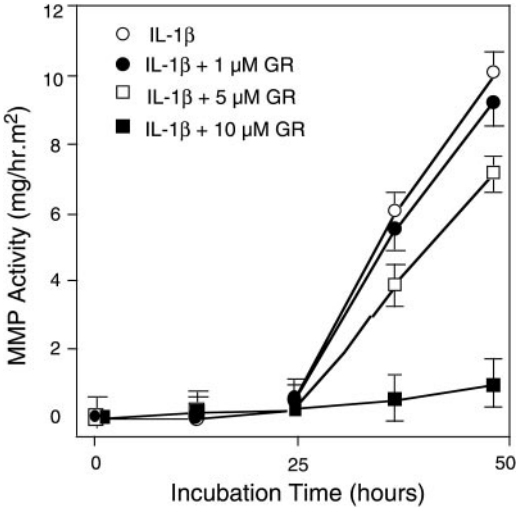

Fig. 3. Effect of 18α-glycyrrhetinic acid (GR) on MMP production by synovial cells in the presence of IL-1β. GR was added at *time 0,* and IL-1β (100 pg/ml) was added 10 min later.

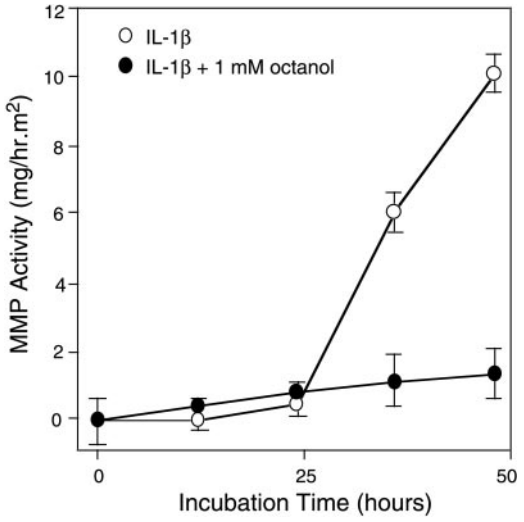

Fig. 4. Effect of octanol on MMP production by synovial cells in the presence of IL-1β. Octanol was added at *time 0,* and IL-1β (100 pg/ml) was added 10 min later.

ing cells, and IL-1β (100 pg/ml) was added 10 min later. After another 40 min, the medium was replaced with medium containing IL-1β but no inhibitor, and the cells were incubated for up to 48 h. We found that the MMP activity of the supernatant was significantly reduced by the brief treatment with the inhibitors (Fig. 5); 50 min of treatment with GR (10 μM) or octanol (1 mM) alone did not induce MMP production (not shown).

Generalized inhibition of MMP release. To evaluate whether the decrease in MMP activity caused by the disruption of GJIC was specific for IL-1β signaling, we investigated whether disruption of GJIC also interfered with PMA-stimulated MMP release. The effect of the gap junction inhibitors on MMPs was essentially the same when PMA was used as the stimulus (Figs. 3, 4, and 6), indicating that the relationship between GJIC and MMP activity did not depend on the specific details of IL-1β signaling.

Effect of inhibitors and IL-1β on gap junction channel conductance. The effect of GR and octanol on intercellular communication was assessed using transient current analysis (10). For aggregated cells in the medium, the time constant after application of a voltage step was 20 ± 5 ms (Fig. 7, *curve 1*), indicating that the cells were initially connected by gap junctions (10). In the presence of GR or octanol, however, the time constant quickly decreased to 3.8 ± 1 ms. At 50 min after addition of the inhibitor, the bath solution was replaced with inhibitor-free solution, and the measurements were repeated, with results identical to those in *curve 1*, Fig. 7, *A* and *B* (not shown).

To further explore the relation between GJIC and regulation of synovial cell responsiveness, we studied the influence of IL-1β on integral gap junction channel conductance during the first 30 h of treatment. The transient current response to 2-mV steps was not changed 0.5 h after addition of IL-1β (1 ng/ml), but the cells were essentially uncoupled after 30 h (Fig. 8).

Northern blot. The cells synthesized inducible but not constitutive MMP-1 mRNA in response to treatment with IL-1β (Fig. 9, *lanes 2* and *1*, respectively). Addition of the gap junction inhibitors had no significant effect on the total amount of cytokine-induced cellular mRNA for MMP-1 (Fig. 9, *lanes 3* and *4*).

DISCUSSION

The effect of PMA on MMP production by HIG-82 cells was the same as that described previously (5). The result can therefore be viewed as a positive control for our assay method. Using our assay method, we found that addition of IL-1β to the cell medium resulted in a

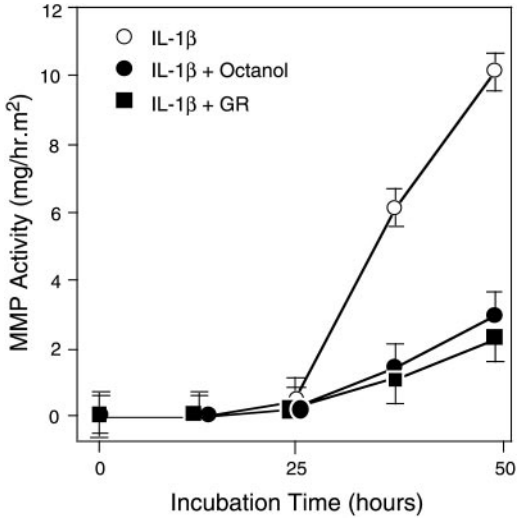

Fig. 5. Effect of short-term application (50 min, beginning at *time 0*) of 10 μM GR and 1 mM octanol on MMP production by synovial cells in the presence of IL-1β (100 pg/ml). GR and octanol were added at *time 0*, and IL-1β (100 pg/ml) was added 10 min later. After another 40 min, medium was replaced with medium containing IL-1β (100 pg/ml) but no inhibitors.

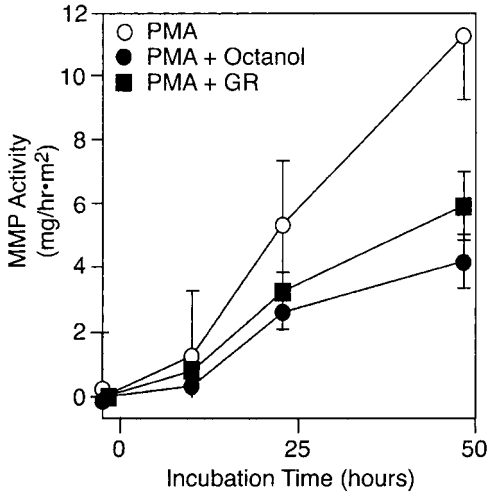

Fig. 6. MMP activity in the supernatant of synovial cells exposed to phorbol 12-myristate 13-acetate (PMA) for 48 h in the presence and absence of gap junction inhibitors. Inhibitors were added at *time 0*, and PMA was added 10 min later. PMA, 10 ng/ml; GR, 10 μM; octanol, 1 mM.

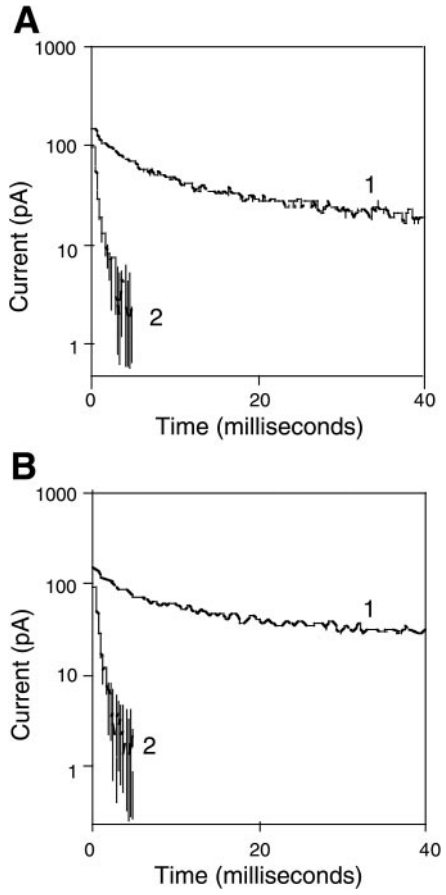

Fig. 7. Influence of 10 μM GR (*A*) and 1 mM octanol (*B*) on transient current induced in synovial cells by a 2-mV voltage step. Currents were measured on cells aggregated with >30 other cells, using perforated-patch recording; voltage step was applied to the electrode at *time 0*. Each curve was produced by averaging transient current for 10 voltage steps for 3 different aggregates. Individual records were filtered with a 10-kHz low-pass 4-pole Bessel filter. SD was 20% (not shown on curves for clarity). *Curve 1*, before addition of inhibitor; *curve 2*, 10 min after addition. At 50 min after addition of inhibitor, bath solution was replaced with inhibitor-free bath solution, and measurement was repeated, with results identical to those shown in *curve 1* (not shown).

dose-dependent increase of MMP activity (Fig. 2). The IL-1β concentrations (1 and 100 pg/ml) were comparable to those found in marrow plasma (200–300 pg/ml) (8) and synovial fluid (150 pg/ml) (13), suggesting that the processes underlying the observed secretion were physiologically relevant. Previous electrophysiological studies indicated that the first steps of IL-1β signal transduction in HIG-82 cells occurred within 10 min (10). Thus the delay in MMP production (Fig. 2) did not imply that the initial steps of IL-1β signal transduction were slow processes.

Removing IL-1β from the medium caused termination of production of the enzymes that degraded the collagen film, indicating that IL-1β was not simply a trigger that initiated a signal cascade leading to secretion. Rather, the continuous presence of IL-1β in the medium was required for MMP production. This conclusion is in general agreement with our earlier data showing that IL-1β-induced depolarization was reversed when the cytokine was removed from the medium (10).

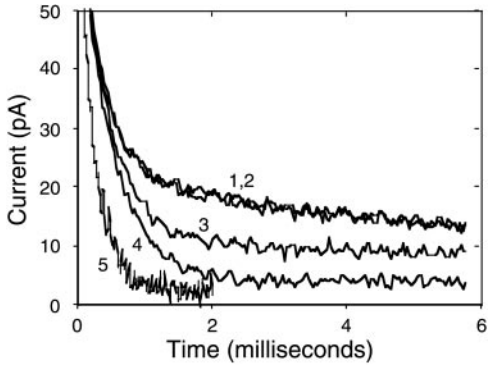

Fig. 8. Transient current responses of synovial cells to a 2-mV step after exposure to IL-1β. Currents were measured on cells aggregated with 3 other cells using perforated-patch recording; voltage step was applied to the electrode at *time 0*. *Curve 1*, before addition of IL-1β; *curve 2*, 30 min after addition (1 ng/ml); *curve 3*, 1 h after addition; *curve 4*, 30 h after addition; *curve 5*, an isolated cell. Individual records were filtered with a 10-kHz low-pass 4-pole Bessel filter. Each curve was produced by averaging transient current for 10 voltage steps for 3 different aggregates. SDs were 20% (not shown on curves for clarity).

Synovial cells are connected to each other by an extensive network of gap junctions (9), and it is possible that such networks mediate some cellular processes. We examined whether IL-1β-induced MMP production might be an example and found that the stimulated secretion was eliminated by each of two chemically disparate gap junction inhibitors (Figs. 3 and 4). They rapidly blocked GJIC, and the effect was reversible after treatment for 50 min (Fig. 7). This result confirmed previous data from others (2, 4, 6) that for treatment times <1 h the inhibitors worked via the same mechanism, namely, reversible inhibition of GJIC, and did not change other cell properties. Expo-

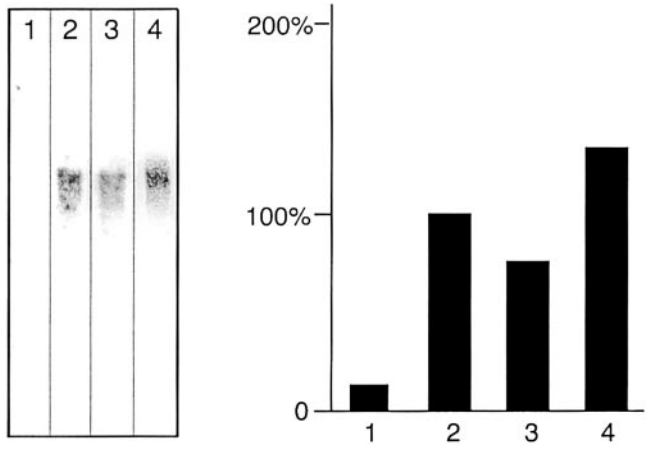

Fig. 9. *Left*: Northern blot for MMP-1 mRNA extracted from synovial cells. Cells were incubated for 2 days as follows: control, no active substance (*lane 1*); 300 pg/ml IL-1β (*lane 2*); 10 μM GR and 300 pg/ml IL-1β (*lane 3*); and 1 mM octanol and 300 pg/ml IL-1β (*lane 4*). Experiment was repeated twice, and the difference between band intensities was ~20%. Blot represents 1 experiment. *Right*: amount of MMP-1 mRNA for each lane averaged for 2 experiments and presented relative to amount in *lane 2*.

sure to GR at concentrations higher than that used in our study (30 μM) for a longer period (4 h) reduced connexin43 expression and promoted disassembly of gap junction plaques (6). Because the cells in our study were incubated in the presence of GR for 48 h (Figs. 3 and 4), we considered the possibility of an effect on MMP expression that did not involve gap junction communication. This was accomplished by exposing the cells to the inhibitors only for the period of time during which their effects on GJIC were reversible. Application of GR or octanol for only 50 min caused significant inhibition of IL-1β-induced MMP production (Fig. 5), thereby showing the importance of gap junction communication in signal transduction and the temporal localization of GJIC in the early stages of transduction.

We showed previously that gap junction channel conductance between HIG-82 cells could be calculated from an induced transient cellular current by fitting the current to the sum of two exponentials and using the time constant of the slower exponential as a measure of integral cell conductance (10). The time constant was ~0.2 ms for isolated cells, but cells connected by gap junctions exhibited much longer time constants. We found that the time constant for aggregated cells initially was 20 ± 5 ms (Fig. 8). After application of IL-1β, the time constant was unchanged after 30 min and decreased only slightly during the 1st h, indicating that the cytokine did not significantly influence channel conductance. With GR and octanol, in contrast, the time constant decreased almost immediately to 3.8 ± 1 ms, indicating a dramatic decrease in channel conductance. When the inhibitors were removed, the time constants returned to their initial values. This result provided a possible explanation for the effect of the inhibitors in the MMP assay (Fig. 5). The gap junction channels were closed at the time IL-1β was added and remained closed for another 40 min, after which the channels were opened as a consequence of removal of the blocker from the medium. Nevertheless, the period of inhibition was enough to significantly inhibit MMP production in the presence of IL-1β, suggesting that GJIC in the early stages of IL-1β signal transduction facilitated signal amplification.

GJIC was necessary for IL-1β-induced protease production, as determined from the results of treatment with GJIC inhibitors (Figs. 3–5). We measured the effect of treatment with GJIC inhibitors on the amount of MMP-1 mRNA to address the question of the level of regulation of induced expression that was affected by inhibitors. The data in Fig. 9 suggest that the differences in MMP-1 mRNA in cells treated with the two inhibitors were too small to explain the effect of the inhibitors on protease production. Consequently, we believe that it is likely that the effect was produced by changes at some other level of regulation, for example, secretion or translation. Future studies involving other approaches are necessary to resolve the issue.

On the basis of our previous electrophysiological studies, we can suggest several mechanisms by which intercellular gap junction communication could lead to signal amplification. We found that IL-1β switched the cell membrane potential from −63 to −30 mV and that preexposure of the cells to low membrane potential (−30 mV) using voltage clamp increased the sensitivity of the cells to IL-1β, probably by opening Ca^{2+} channels and increasing intracellular Ca^{2+} concentration (10, 11). It is possible that the change of membrane potential could spread between cells connected by gap junction channels, because they have high conductance for monovalent ions, thereby increasing the sensitivity of adjacent cells to IL-1β. The diffusion of second messengers through gap junction channels is an alternative mechanism for signal amplification. This argument requires a nonuniform distribution of at least one element in the signaling cascade, and we previously presented evidence that intracellular Ca^{2+} concentration could be such an element (10).

Perspectives

Overproduction of MMPs in response to inflammatory cytokines is probably an important cause of arthritis. A possible strategy for treating arthritis involves the inhibition of IL-1β receptors, but the clinical effectiveness of this approach remains low. The results of this study suggest the possibility that additional inhibition of intercellular communication would increase treatment effectiveness.

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