Evidence that responses of articular chondrocytes to interleukin-1 and basic fibroblast growth factor are not mediated by protein kinase C

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After exposure to human recombinant interleukin-1 (hrIL-1), chondrocytes increase their synthesis of prostaglandin E_2 (PGE_2) and neutral metalloproteinases (NMPs). This response, known as chondrocyte activation, is also elicited by partially purified preparations of rabbit synovial IL-1, known as 'chondrocyte activating factors' (CAF). CAF activates chondrocytes more strongly than does hrIL-1, probably because it contains additional growth factors. Basic fibroblast growth factor (bFGF) is one such factor, although CAF also contains others which modulate the activation of chondrocytes. Chondrocyte activation by hrIL-1 is strongly potentiated by bFGF and phorbol myristate acetate (PMA). A series of experiments was conducted to examine the possible role of protein kinase C (PKC) in mediating these effects. Inhibitors of PKC partially blocked the induction of NMPs by CAF and completely suppressed the potentiating effect of PMA. However, induction by 10 units of hrIL-1/ml and potentiation by bFGF were not affected by these inhibitors. Furthermore, cells whose PKC had been down-regulated by prolonged exposure to PMA remained responsive to IL-1. Surprisingly, inhibitors of PKC greatly increased the amounts of NMPs produced in response to a low dose (1 unit/ml) of hrIL-1. These inhibitors also enhanced the synthesis of PGE_2 by cells responding to 1 and 10 units of hrIL-1/ml. Phosphorylation of the 80 kDa substrate for PKC was augmented by PMA and CAF, but not by hrIL-1 or bFGF. Moreover, Western-blotting techniques, which confirmed translocation of PKC in response to PMA and CAF, did not detect translocation in cells treated with hrIL-1 or bFGF. Western blotting also demonstrated the presence of PKC isoenzyme type III (α), but not types I (γ) or II (β). These data argue that PKC does not mediate the effects of hrIL-1 or bFGF in chondrocytes. However, CAF contains additional substances which activate this enzyme and whose effects may in part be mediated by PKC.

INTRODUCTION

Cultures of articular chondrocytes increase their synthesis of prostaglandin E_2 (PGE_2) and several neutral proteinases in response to synovial cytokines (Meats *et al.*, 1980; Watanabe *et al.*, 1986). This process, known as chondrocyte activation, may be linked to the catabolin effect, whereby fragments of living cartilage degrade their extracellular matrix following exposure to synovial products (Fell & Jubb, 1977; Dingle *et al.*, 1979). Interleukin-1 (IL-1) is a synovial cytokine which both possesses catabolin activity and activates cultures of articular chondrocytes (Saklatvala *et al.*, 1984; Gowen *et al.*, 1984). However, partially purified preparations of synovial cytokines, which we have previously called 'chondrocyte activating factors' (CAF), activate chondrocytes more powerfully than does purified human recombinant IL-1 (hrIL-1) (Bandara *et al.*, 1989). This discrepancy is explained by the presence in CAF of additional cytokines which potentiate the action of IL-1. Basic fibroblast growth factor (bFGF) is one such cytokine (Phadke, 1987; Chandrasekhar & Harvey, 1989; Bandara *et al.*, 1989; G. Bandara, H. I. Georgescu & C. H. Evans, unpublished work). Blocking the catabolic responses of chondrocytes to synovial cytokines may protect articular cartilage from degradation and thus be of therapeutic value in arthritis. With this in mind, we are

studying the signalling pathways of chondrocyte activation employed by IL-1 and its modulation by other cytokines.

The α and β forms of IL-1 share common high-affinity cell-surface receptors, of which there are at least two types. One class of receptor, present on T-cells and fibroblasts, has a molecular mass of approx. 80 kDa (Bird *et al.*, 1988; Urdal *et al.*, 1988). The other class, found on granulocytes, pre-B-cells and macrophages, has a molecular mass of approx. 59–68 kDa (Bomszyk *et al.*, 1989; Chizzonite *et al.*, 1989). Pig articular chondrocytes possess 7000 IL-1 receptors per cell, with a K_d of 2.5×10^{-11} M (Bird & Saklatvala, 1986), whereas rabbit articular chondrocytes possess around 3000 receptors per cell, with a K_d of $(4-7) \times 10^{-11}$ M (Chin & Horuk, 1990). Although the chondrocyte receptor has not been biochemically characterized, inhibitor studies suggest it to be of the T-cell/fibroblast variety. Thus the recently identified IL-1 receptor antagonist, which prevents binding of IL-1 to T-cells, but not to the pre-B-cell line 70 Z/3 (Eisenberg *et al.*, 1990; Hannum *et al.*, 1990), completely inhibits the activation of rabbit articular chondrocytes by hrIL-1 (Bandara *et al.*, 1991).

Occupancy of the IL-1 receptor appears to activate a G-protein (Chedid *et al.*, 1989; O'Neill *et al.*, 1990a) and to produce changes in protein phosphorylation (Matsushima *et al.*, 1987; Kaur & Saklatvala, 1988; Martin *et al.*, 1989). However, little else is known of the cellular signalling mechanisms which mediate

Abbreviations used: CAF, chondrocyte-activating factors; bFGF, basic fibroblast growth factor; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; hrIL-1, human recombinant interleukin-1; NMPs, neutral metalloproteinases; PBS, phosphate-buffered saline; PGE_2 , prostaglandin E_2 ; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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the response to IL-1. Indeed, this topic is highly controversial (Mizel, 1990; O'Neill *et al.*, 1990b). The literature contains conflicting reports on the possible involvement, in various types of cells, of cyclic AMP (e.g. Carroll, 1985; Didier *et al.*, 1988; Shirakawa *et al.*, 1988, 1989; Zhang *et al.*, 1988; Kasahara *et al.*, 1990; Baratz *et al.*, 1991), InsP_3 and Ca^{2+} (Wijelath *et al.*, 1988; Rosoff *et al.*, 1988; Kester *et al.*, 1989) and protein kinase C (PKC) (see, e.g., Avissar *et al.*, 1985; Bunning *et al.*, 1986; Abraham *et al.*, 1987; Magnuson *et al.*, 1989; Bird & Saklatvala, 1989).

So far as articular chondrocytes are concerned, IL-1 induces very marked changes in protein phosphorylation (Hulkower *et al.*, 1989), but the kinase(s) responsible for this have not been identified. In the present paper, we have directly examined the possible role of PKC in the activation of chondrocytes by IL-1 and its potentiation by bFGF.

EXPERIMENTAL

Materials

The following materials were obtained from the indicated suppliers: sphingosine, phosphatidylserine, palmitoylecarnitine, H-7, histone type III, phorbol 12-myristate 13-acetate (PMA), digitonin, soybean trypsin inhibitor, leupeptin, aprotinin, phenylmethanesulphonyl fluoride, sodium pyruvate, lactate dehydrogenase, NAD^+ , trichloroacetic acid, FGF, ATP, SDS, BSA, phosphate-buffered saline (PBS; 137 mM-NaCl/2.7 mM-KCl/10 mM-phosphate buffer, pH 7.2), β -glycerophosphate, from Sigma Chemical Co. (St. Louis, MO, U.S.A.); 4 α -phorbol 12-myristate 13-acetate (4 α -PMA), from LC Services Corp. (Woburn, MA, U.S.A.); clostridial collagenase and trypsin, from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.); hrIL-1 α , from Genzyme (Boston, MA, U.S.A.); staurosporine, from Boehringer-Mannheim (Indianapolis, IN, U.S.A.); monoclonal anti-PKC isoenzyme antibodies MC-1a, MC-2a and MC-3a, from Seikagaku America (Rockville, MD, U.S.A.); monoclonal anti-PKC antibody MC-5, ^{125}I -labelled sheep anti-mouse IgG, from Amersham (Arlington Heights, IL, U.S.A.); tissue-culture plasticware, HClO_4 , from Fisher Scientific (Pittsburgh, PA, U.S.A.); culture media, antibiotics, sera, trypsin, Gey's solution, from GIBCO (Grand Island, NY, U.S.A.); Electrophoresis equipment and supplies, from Bio-Rad (Richmond, CA, U.S.A.); nitrocellulose membranes, from Schleicher and Schuell (Keene, NH, U.S.A.). hrIL-1 β was generously given by DuPont Co., Wilmington, DE, U.S.A.

Cell culture

Primary monolayer cultures of articular chondrocytes were established from cells derived from the pooled articular cartilages of the knee and shoulder joints of New Zealand White rabbits weighing 2.3–2.8 kg. As described in detail by Green (1971), cartilage was cut into small pieces before digestion sequentially with trypsin (0.2%, w/v) and collagenase (0.2%, w/v). Cells were seeded into 24-well plates or 100 mm-diam. dishes at a density of 10^5 cells/cm ^{2} , in Ham's F12 medium supplemented with 10% (v/v) foetal-bovine serum, 100 units of penicillin/ml and 100 μg of streptomycin/ml. Cultures of chondrocytes were used when confluent, without subculture.

The HIG-82 synovial cell line (Georgescu *et al.*, 1988) was used as a source of CAF. HIG-82 cells were sub-cultured at a 1:2 or 1:4 split ratio. CAF was prepared from medium conditioned by HIG-82 cells by methods that we have described previously (Sung *et al.*, 1988).

Chondrocyte activation

The degree of chondrocyte activation was assessed by measuring PGE_2 content and gelatinolytic activity of the culture

medium conditioned by the chondrocytes. Growth medium was removed from wells of confluent chondrocytes and replaced with the serum-less Neuman-Tytell medium. The various cytokines, PKC agonists or PKC inhibitors were added at this time; 3 days later, conditioned media were recovered and assayed for PGE_2 content and gelatinase activity. PGE_2 was measured by a commercial radioimmunoassay system (New England Nuclear) used according to the manufacturer's instructions. Gelatinase was measured with [^3H]gelatin as substrate as described by Watanabe *et al.* (1986). Aminophenylmercuric acetate (1 mM) was used to activate latent enzyme.

PKC activation

Two methods were employed to assess the state of activation of PKC in chondrocytes: (a) endogenous phosphorylation of the 80 kDa substrate and (b) translocation of enzyme from the soluble to the particulate fraction, as assessed by immunoblotting techniques.

Endogenous phosphorylation of the 80 kDa substrate of PKC (Blackshear *et al.*, 1985; Rozengurt *et al.*, 1983) was employed to assess the degree of activation of PKC. After overnight incubation in serum-less medium, cells in 24-well plates were labelled for a total of 4 h with [^{32}P]P $_i$ (200 $\mu\text{Ci}/\text{ml}$) in minimal essential medium without phosphate or serum. Cytokines or other agents, as indicated in the Results section, were added for the appropriate times, after which the cells were washed twice with Gey's balanced salt solution and scraped directly into 0.5 ml of HClO_4 (2.5%, w/v). After centrifuging at 12000 g for 15 min in a Microfuge, the supernatant was recovered, carrier BSA (200 μg in 200 μl) was added and proteins were precipitated with trichloroacetic acid (20%, w/v). The suspension was re-centrifuged, the supernatant removed, and the pellet dissolved directly into a buffer containing glycerol (10%, w/v), 2-mercaptoethanol (5%, w/v), SDS (0.05%, w/v), Tris/HCl (62.5 mM) and Bromophenol Blue (0.05%, w/v), pH 6.8. The resulting solution was boiled for 3 min before loading on to 5–15% gradient polyacrylamide gels. Electrophoresis was carried out overnight at a constant current of 10 mA. Gels were then dried and subjected to autoradiography.

To assess translocation of PKC from the soluble to the particulate fraction, monolayers in 100 mm culture dishes were exposed to test agents in the presence of 1 mM-leupeptin as described above for endogenous phosphorylation of the 80 kDa substrate. Cells were then washed twice with ice-cold Gey's solution and scraped into 1 ml of a buffer containing β -glycerophosphate (80 mM), EGTA (20 mM), MgCl_2 (15 mM), sodium orthovanadate (2 mM), phenylmethanesulphonyl fluoride (1 mM), aprotinin (25 $\mu\text{g}/\text{ml}$) and soybean trypsin inhibitor (10 $\mu\text{g}/\text{ml}$), pH 7.5. Cells were broken by freeze-thawing three times in liquid nitrogen and centrifuged at 12000 g for 15 min at 4 °C. Supernatants were retained and pellets dissolved in 0.5 ml of the above buffer containing Triton X-100 (0.1%, w/v). Samples of the supernatant and particulate fractions were subjected to SDS/PAGE on 10%-acrylamide gels. Alternatively, whole-cell homogenates were prepared by the hot-SDS method as described by Girard *et al.* (1986).

Western blotting

After electrophoresis, proteins were electrophoretically transferred to nitrocellulose sheets overnight at 4 °C. Non-specific binding sites were blocked by incubating the blots for 1 h in a solution of BSA (1%, w/v, in PBS) containing 0.05% (w/v) Tween-20. The blots were then probed with 1:500 dilutions of primary anti-PKC antibodies. After 4 $\times$ 5 min washes in PBS/Tween, the sheets were incubated with second antibody, a 1:1000

dilution of ¹²⁵I-labelled sheep anti-mouse IgG, for 2 h. After a final set of washes, the blots were subjected to autoradiography.

Other assays

Protein concentrations were measured by the method of Lowry *et al.* (1951), with BSA as a standard.

Lactate dehydrogenase was measured by monitoring the reduction of NAD⁺ to NADH at 340 nm (McMillan *et al.*, 1981).

RESULTS

In agreement with earlier results (Watanabe *et al.*, 1986; Sung *et al.*, 1988), PMA (100 ng/ml) increased the synthesis of PGE₂ by chondrocytes, but failed to induce metalloproteinases. However, PMA strongly potentiated the effects on the production of NMPs and PGE₂ by both α and β forms of hrIL-1 (1–10 units/ml). The inactive phorbol ester 4 α -PMA had no effect (results not shown).

Table 2. Effect of PKC inhibitors on PGE₂ production induced by IL-1 in chondrocytes

Cells were treated with 1 unit of IL-1 α /ml and the PKC inhibitors and assayed for PGE₂ production as described in the Experimental section. Values given are means $\pm$ S.E.M. (*n* = 8).

Treatment	PGE ₂ (ng/10 ⁶ cells)
Control	2.5 $\pm$ 0.3
IL-1	15.5 $\pm$ 2.0
15 μ M-Palmitoylcarnitine	11.6 $\pm$ 3.5
IL-1 + 15 μ M-palmitoylcarnitine	140.2 $\pm$ 27.3
100 μ M-H-7	5.6 $\pm$ 1.2
IL-1 + 100 μ M-H-7	29.4 $\pm$ 4.4
25 μ M-Sphingosine	4.1 $\pm$ 2.1
IL-1 + 25 μ M-sphingosine	75.3 $\pm$ 4.4
10 nM-Staurosporine	32.7 $\pm$ 7.0
IL-1 + 10 nM-staurosporine	74.4 $\pm$ 5.2

Fig. 1. Effect of PKC inhibitors on gelatinase induction by IL-1 and their cytotoxicity in chondrocytes

Cells were preincubated with the inhibitors for 1 h, inoculated with 1 unit of IL-1 α /ml and assayed for gelatinase production 3 days later as detailed in the Experimental section. ●, Gelatinase production by cells treated with staurosporine only; ○, gelatinase production by cells treated with sphingosine only; ▲, gelatinase production by cells treated with IL-1 and staurosporine; △, gelatinase production by cells treated with IL-1 and sphingosine; ■, cytotoxicity of staurosporine as measured by lactate dehydrogenase (LDH) release; □, cytotoxicity of sphingosine as measured by LDH release. Values given are means $\pm$ S.E.M. (*n* = 8).

Several compounds reported to inhibit PKC were evaluated for their effects on chondrocytes. Each of these compounds, sphingosine, staurosporine, palmitoylcarnitine and H-7, was able to suppress phosphorylation of the 80 kDa substrate for PKC (Rozengurt *et al.*, 1983; Blackshear *et al.*, 1985) in response to PMA treatment (results not shown), thereby indicating their efficacy as PKC inhibitors in this system. The effects of inhibitors of PKC on gelatinase induction depended on the concentration of IL-1 employed. When used at a concentration of 1 unit/ml, IL-1 increased the gelatinolytic activity of the conditioned medium only slightly. However, both sphingosine and staurosporine (Fig. 1) strongly augmented the production of gelatinase in response to this dose of IL-1. The shapes of the dose-response curves for these inhibitors probably reflect their toxicity at higher doses, as indicated by the release of lactate dehydrogenase. Similar effects were noted for the inhibitors H-7 and palmitoylcarnitine (results not shown). In contrast, none of the four inhibitors had any effect on the induction of gelatinase by 10 units of IL-1/ml, although they did inhibit the potentiating effect of PMA (results not shown). Sphingosine and staurosporine were also without effect on the potentiating effect of bFGF, whereas gelatinase induction by CAF was partially suppressed by PKC inhibitors (Table 1).

All inhibitors of PKC increased the synthesis of PGE₂ by chondrocytes exposed to either 1 unit of IL-1/ml (Table 2) or 10 units of IL-1/ml (results not shown), although the magnitude of this increase varied from inhibitor to inhibitor. Palmitoylcarnitine showed the greatest effect, and H-7 the least (Table 2). Staurosporine also induced the synthesis of PGE₂ by resting cells (Table 2). The fact that none of the inhibitors suppressed the synthesis of NMPs or PGE₂ in any of these experiments strongly

Table 1. Effect of PKC inhibitors on gelatinase production by FGF, IL-1 and CAF in chondrocytes

Cells were treated with the PKC inhibitors as described in the Experimental section before inoculation with 1 unit of IL-1 α /ml, 100 ng of FGF/ml or 50 μ l of CAF/ml. Values given are means $\pm$ S.E.M. (*n* = 8).

PKC inhibitor	Gelatinase (units/10 ⁶ cells)				
	Control	FGF	IL-1	FGF + IL-1	CAF
None	0.7 $\pm$ 0.1	1.6 $\pm$ 0.9	1.8 $\pm$ 1.1	10.3 $\pm$ 1.8	42.8 $\pm$ 1.9
25 μ M-Sphingosine	0.7 $\pm$ 0.3	1.2 $\pm$ 0.8	7.4 $\pm$ 3.0	9.3 $\pm$ 0.8	27.2 $\pm$ 1.2
10 nM-Staurosporine	1.9 $\pm$ 1.3	1.4 $\pm$ 0.8	5.6 $\pm$ 2.0	10.4 $\pm$ 2.1	26.4 $\pm$ 1.6

Table 3. Effect of PKC down-regulation by 24 h PMA treatment on gelatinase production by IL-1 and CAF in chondrocytes

Cells were pretreated with PMA (100 ng/ml) for 24 h before incubation with 10 units of IL-1 α /ml or 50 μ l of CAF/ml or with 100 ng of PMA/ml and the cytokines. Gelatinase production was assayed as described in the Experimental section. Values given are means $\pm$ S.E.M. ($n = 8$).

Treatment	Gelatinase (units/ 10^6 cells)
Control	0 $\pm$ 0
24 h PMA-pretreated	0.2 $\pm$ 0.1
IL-1	26.5 $\pm$ 2.8
PMA-pretreated, then IL-1	74.2 $\pm$ 3.4
CAF	38.1 $\pm$ 2.6
PMA-pretreated, then CAF	87.4 $\pm$ 5.5
PMA	0.2 $\pm$ 0.1
IL-1 + PMA	79.4 $\pm$ 2.5
PMA-pretreated, then IL-1 + PMA	103.3 $\pm$ 2.7
CAF + PMA	84.1 $\pm$ 6.1
PMA-pretreated, then CAF + PMA	83.0 $\pm$ 3.0

Fig. 2. Effect of PKC down-regulation on 80 kDa protein phosphorylation in chondrocytes

Cells were pretreated with either PMA (100 μ g/ml) or CAF (50 μ l/ml) for 24 h in serum-less media and then were labelled, treated and processed for 80 kDa-protein analysis as described in the Experimental section. Lanes: 1 and 8, basal level of 80 kDa-protein phosphorylation in untreated cultures; 2, PMA (100 ng/ml) treatment for 30 min; 3, PMA treatment for 30 min after 24 h incubation with PMA; 4, CAF (50 μ l) treatment for 30 min; 5, CAF treatment for 30 min after 24 h incubation with PMA; 6, PMA treatment for 30 min after 24 h incubation with CAF; 7, CAF treatment for 30 min after 24 h incubation with CAF. Numbers to the left indicate the migration positions of molecular-mass standards (kDa).

suggests that toxicity is not a complicating factor in the interpretation of these data.

In further experiments, the PKC of chondrocytes was down-regulated by 24 h treatment with PMA (100 ng/ml) before addition of test agents. Induction of PGE₂ and gelatinase by IL-1

Fig. 3. Effect of treatment with IL-1 on 80 kDa-protein phosphorylation in chondrocytes

Cells were treated with 100 ng of PMA/ml for 30 min, or with 10 units of IL-1 α /ml for 1–30 min as indicated. Control cells (C) are untreated chondrocytes and represent basal levels of 80 kDa-protein phosphorylation. Numbers to the left indicate the migration positions of molecular-mass standards (kDa).

was unimpaired by this procedure. Indeed, such chondrocytes responded to IL-1 and CAF in the exaggerated manner of cells treated simultaneously with IL-1 and PMA or CAF and PMA (Table 3). Down-regulation of PKC by 24 h exposure to PMA was confirmed in experiments which monitored the phosphorylation of the endogenous 80 kDa substrate for PKC (Rozengurt *et al.*, 1983; Blackshear *et al.*, 1985). Treatment of chondrocytes with PMA for 30 min enhanced the phosphorylation of a protein of this molecular mass (Fig. 2, lane 2), but it failed to do so in cells that had been down-regulated by prior 24 h exposure to this agent (Fig. 2, lane 3). As described for the 80 kDa substrate of PKC, this protein was soluble in 2.5% HClO₄, and its phosphorylation was inhibited by staurosporine, and sphingosine, palmitoylcarnitine and H-7 at non-cytotoxic dosages (results not shown). CAF also enhanced the phosphorylation of the 80 kDa protein (Fig. 2, lane 4), and its ability to do so was strongly suppressed in cells exposed to PMA for 24 h (Fig. 2, lane 5) or by PKC inhibitors (results not shown). Long-term (24 h) pretreatment with CAF, however, was unable to cause down-regulation of PKC in these cells, as demonstrated by its inability to block phosphorylation of the 80 kDa protein induced by subsequent PMA or CAF treatment (Fig. 2, lanes 6 and 7). 4 α -PMA did not provoke phosphorylation of the 80 kDa protein (results not shown). Doses of IL-1 from 1 to 100 units/ml (results not shown) were all without effect on phosphorylation of the 80 kDa protein at times from 1 to 30 min (Fig. 3). bFGF also did not stimulate phosphorylation of the 80 kDa protein (results not shown).

Immunoreactive PKC was detected by Western blotting with the anti-PKC monoclonal antibody MC-5. This method detected the translocation of PKC provided that leupeptin (1 mM) was added to the cells to prevent proteolysis. PMA and CAF both produced translocation of PKC from the soluble to the particulate fraction, but IL-1 was without effect (Fig. 4). As shown by visual inspection of the autoradiograms, the soluble PKC content of IL-1-treated cells reproducibly exceeded that of resting cells (Fig. 4).

Fig. 4. Effect of cell-activating agents on PKC translocation in chondrocytes

Western blot of cytosolic and membrane fractions of chondrocytes utilizing a 1:500 dilution of Amersham anti-PKC antibody MC-5 which recognizes all isoenzymes of PKC. All treatments were for 15 min in the presence of 1 mM-leupeptin. Equivalent amounts of total protein (75 µg) were loaded in each lane. Lanes: 1, control cytosolic extract; 2, cytosol of cells treated with 100 ng of PMA/ml; 3, cytosol of cells treated with 50 units of IL-1β/ml; 4, cytosol of cells treated with 50 µl of CAF/ml; 5, membrane fraction of control cells; 6, membrane fraction of PMA-treated cells; 7, membrane fraction of IL-1-treated cells; 8, membrane fraction of CAF-treated cells.

Fig. 5. Characterization of PKC isoenzymes present in chondrocytes

Western blot of whole-cell homogenates from chondrocytes utilizing 1:250 dilutions of isoenzyme-specific monoclonal antibodies against PKC. Equal amounts of total protein (75 µg) were added to each lane.

Western blotting with monoclonal antibodies specific for PKC types I (γ), II (β) and III (α) revealed that chondrocytes contain only the type III (α) form (Fig. 5).

DISCUSSION

The biochemical events which follow binding of IL-1 to its cell-surface receptor are as obscure for articular chondrocytes as they are for other types of cells. At face value, our data seem comprehensively to exclude PKC as a mediator of chondrocyte responses to IL-1. Thus, activators of PKC do not mimic the effects of IL-1, inhibitors of PKC do not block responses to IL-1, PKC does not appear to translocate in response to IL-1, and IL-1 does not induce the phosphorylation of the endogenous 80 kDa substrate for PKC. However, the complexities of PKC activation (Bosca *et al.*, 1989; Farago & Nishizuka, 1990) and the problems associated with proving a negative demand a cautious interpretation of the data.

Although we have been unable directly to link PKC activation to the chondrocytes' responses to IL-1, PKC does appear to regulate the magnitude of these responses. However, the details of this regulation are obscure. PMA, for instance, activates PKC and vastly increases the extent of cellular activation produced by IL-1. But inhibitors of PKC also have the same effect. This apparent contradiction might pertain to the ability of PMA, confirmed in the present work, to down-regulate PKC on long-term incubation. As the increase in neutral proteinase and PGE₂ synthesis occurs several hours after addition of the activating agent, there is the possibility that substances such as PMA, which potentiate this increase, do so by decreasing the PKC activity of the cell. The likelihood of this possibility is strengthened by the exaggerated responses to IL-1 of chondro-

cytes whose PKC has been eliminated by prior exposure to PMA. Such speculation is intriguing, as the soluble PKC content of chondrocytes appears slightly to increase in the presence of IL-1.

Although IL-1 augments the synthesis of both PGE₂ and NMPs by articular chondrocytes, there is evidence that these two responses are independently regulated (Sung *et al.*, 1988; Bandara *et al.*, 1989). The present data support this conclusion, as inhibitors of PKC enhanced the synthesis of PGE₂ by cells responding to 10 units of IL-1/ml, but had no effect on the induction of NMPs at this dose of IL-1.

Attempts to measure the translocation of PKC in other types of cells responding to IL-1 have yielded mixed results. Negative findings were reported for a murine T lymphoma cell line (Abraham *et al.*, 1987; Mills *et al.*, 1990), bovine lymph-node lymphocytes (Grove & Mastro, 1989) and rabbit synovial fibroblasts (K. I. Hulkower, R. Sagi-Eisenberg, L. M. Traub, H. I. Georgescu & C. H. Evans, unpublished work). However, positive findings were obtained with human synovial fibroblasts (Bunning *et al.*, 1986) and a murine line of T helper cells (Munoz *et al.*, 1990). Data from the use of inhibitors of PKC have been equally confusing (e.g. Gottlieb *et al.*, 1989; Magnuson *et al.*, 1989; Bird & Saklatvala, 1989). Such conflicting results suggest that IL-1 may trigger different signal-transduction processes in different types of cells and, perhaps, in different species.

Although hrIL-1 failed to activate PKC in chondrocytes, CAF did so. This ability presumably resides with additional cytokines present in the partially purified material. FGF, although present in CAF (G. Bandara, H. I. Georgescu & C. H. Evans, unpublished work) and a potentiator of the action of IL-1 (Phadke, 1987; Chandrasekhar & Harvey, 1989; Bandara *et al.*, 1989), did not activate PKC. This is in agreement with studies which did not detect the activation of PKC by FGF in either rat adrenal PC12 cells (Sharma & Dahiya, 1989) or chemically transformed Balb/c 3T3 fibroblasts (Buchou & Mester, 1990). However, other investigators have reported activation of PKC by FGF in 3T3-L1 cells (Blackshear *et al.*, 1985) and in foetal-bovine aortic endothelial cells (Presta *et al.*, 1989), whereas others have shown that FGF stimulates tyrosine kinase activity in Swiss 3T3 cells (Coughlin *et al.*, 1988). Thus it would appear that FGF, possibly like IL-1, is capable of stimulating different signal-transduction pathways in different types of cells.

In addition to the present data, which provisionally exclude PKC as a mediator of IL-1's effects on chondrocytes, there are grounds for also eliminating cyclic AMP and Ca²⁺. IL-1 does not raise cyclic AMP concentrations in pig articular chondrocytes (Carroll, 1986). Moreover, cyclic AMP analogues do not share the ability of IL-1 to induce the synthesis of NMPs (Sung *et al.*, 1988) or to suppress matrix synthesis (Taylor *et al.*, 1988) by chondrocytes. Furthermore H-7, which does not block chondrocyte activation by IL-1, inhibits protein kinase A more effectively than it inhibits PKC.

Increases in intracellular Ca²⁺ are also unlikely candidates. PKC is classically activated by diacylglycerol generated by the action of phospholipase C on phosphatidylinositol biphosphate. This reaction simultaneously produces InsP₃, which liberates Ca²⁺ from intracellular stores. As PKC is not activated in articular chondrocytes treated with IL-1, it is unlikely, although not impossible, that a Ca²⁺ transient is generated in our cells. Furthermore, IL-1 does not raise cytosolic concentrations of Ca²⁺ in pig chondrocytes (Benton *et al.*, 1989), whereas Ca²⁺ ionophores fail to induce NMPs (Sung *et al.*, 1988).

If none of the well-characterized signal-transduction mechanisms operate for IL-1 in chondrocytes, the changes in protein phosphorylation that accompany exposure of these cells to IL-1 (Hulkower *et al.*, 1989) must be mediated by phosphatases

or novel kinases. Sequence analysis of the 80 kDa IL-1 receptor suggests that its cytoplasmic domain lacks tyrosine kinase activity (Sims *et al.*, 1988). Bird & Saklatvala (1989) have already suggested that IL-1 activates a novel kinase in gingival fibroblasts. Our data support a parallel conclusion for articular chondrocytes. Further analysis of the signalling pathways employed by IL-1 requires the identification and characterization of these kinases.

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