The protein phosphatase inhibitor calyculin A stimulates chemokine production by human synovial cells

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Cultured human synovial fibroblasts express mRNA for the chemotactic cytokines (chemokines) interleukin-8 (IL-8), monocyte chemotactic protein 1 (MCP-1) and regulated upon activation normal T-cell expressed and presumably secreted (RANTES), when stimulated with IL-1 or tumour necrosis factor α (TNFα). Calyculin A, a potent type 1/2A protein serine/threonine phosphatase inhibitor, was used to examine the role of protein phosphatases in the regulation of chemokine gene expression. Calyculin A (1 nM) mimicked IL-1 by inducing IL-8 and MCP-1 mRNA expression in synovial cells. IL-8 mRNA was induced over a similar time period (1–6 h) in response to IL-1 or calyculin A, whereas MCP-1 mRNA was induced more rapidly (1–2 h) by calyculin A than by IL-1 (4–6 h). Expression of RANTES mRNA occurred in response to TNFα, but could not be induced by stimulation with calyculin A alone. These results suggest that inhibition of protein phosphatase type 1/2A may have a differential role in the regulation of the expression of each of the chemokine genes. Synovial fibroblasts also secreted IL-8 and IL-6 peptide when stimulated with either IL-1/TNFα or calyculin A. The amount of IL-8 and IL-6 peptide produced in response to calyculin A was significantly increased above that produced by untreated synovial cells, though it was much less than the amount induced by IL-1 or TNFα. Calyculin A also acted synergistically with IL-1 or TNFα to cause a 2-fold potentiation of IL-1- or TNFα-induced IL-8 mRNA and peptide and RANTES mRNA expression. These results suggest that although inhibition of a protein phosphatase may be able to regulate the magnitude of IL-1-induced chemokine gene expression, the IL-1 signal transduction pathway involves components in addition to phosphatase inhibition, possibly including the activation of a protein kinase, the action of which may be opposed by a protein phosphatase inhibited by calyculin A.

INTRODUCTION

Rheumatoid arthritis is characterized by the accumulation and activation of selected populations of inflammatory cells in the synovial cavity and hyperplastic synovial tissue [1]. This requires a series of co-ordinated signals, including the generation of a chemotactic gradient by the cells of the extravascular compartment.

A family of target-cell-specific chemotactic peptides, now known as chemokines, has been identified [2]. These small peptides are structurally characterized by the location of four cysteine residues [3]. Members of the chemokine family include interleukin 8 (IL-8), which is an attractant for neutrophils and a population of lymphocytes [4–6], monocyte chemotactic protein-1 (MCP-1), which attracts monocytes/macrophages [7], and regulated upon activation normal T-cell expressed and presumably secreted (RANTES), which is a chemo-attractant for $CD45^+$ RO^+ T-cells and eosinophils [8,9].

Elevated levels of the chemokines IL-8, MCP-1 and RANTES have been detected in the synovial tissue of rheumatoid arthritis patients, but not in normal synovial tissue [10–12]. Since the discovery that IL-1- or tumour necrosis factor α (TNFα)-treated human synovial fibroblasts produced neutrophil- and monocyte-stimulating activity [13,14], these cells have provided a convenient system in which to examine the mechanisms regulating chemokine production [15,16]. In most cells examined, including synovial fibroblasts, IL-1 and TNFα are the major pro-inflammatory cytokines which induce the production of chemokines. One of the fundamental changes that both TNFα and IL-1 induce on binding to fibroblasts is an increase in the serine/threonine phosphorylation of a number of proteins [17], including a small heat shock protein (hsp27) and the epidermal growth factor receptor [18].

The steady-state level of phosphorylation of any protein depends on the relative activities of both protein kinases and phosphatases. Although IL-1 and TNFα are known to activate many kinases, comparatively little is known about the role of protein phosphatases in cell regulation. Four classes of protein serine/threonine phosphatases have been identified, PP1, PP2A, PP2B and PP2C [19]. Research on the protein serine/threonine phosphatases PP1 and PP2A (PP1/2A) has recently been facilitated by the use of the specific inhibitors, okadaic acid and calyculin A. Okadaic acid and calyculin A can mimic the effects of IL-1 and TNFα by inducing the phosphorylation of the same proteins, for example hsp27, and the activation of the nuclear transcription factors NFkB, erg, jun and fos [18,20–22]. Protein phosphatase inhibitors have also been shown to up-regulate expression of the IL-6, haeme oxygenase, IL-1 and TNFα genes [18,20,21,23].

In the present study we have demonstrated that the pro-inflammatory cytokines IL-1 or TNFα induce the expression of the chemokines IL-8, MCP-1 and RANTES in synovial fibroblasts. We have investigated the role of PP1/2A in IL-1 and TNFα signalling pathways by demonstrating that the phosphatase inhibitor calyculin A can mimic IL-1/TNFα by inducing chemokine mRNA and peptide expression in synovial fibroblasts.

Abbreviations used: PKC, protein kinase C; IL, interleukin; TNFα, tumour necrosis factor α; MCP-1, monocyte chemotactic protein-1; RANTES, regulated upon activation normal T-cell expressed and presumably secreted; PP1/2A, protein phosphatases type 1 or 2A; hsp, heat shock protein; MAP kinase, microtubule-associated protein kinase/mitogen activated protein kinase; HI-FBS, heat inactivated fetal bovine serum; DIG, digoxigenin; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbeccco's modified Eagle's medium.

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EXPERIMENTAL

Materials

Human recombinant TNFα (specific activity 6×10^7 U/mg) and IL-1α (specific activity 5×10^7 U/mg) were gifts from Bayer (Slough, U.K.) and Glaxo (Greenford, U.K.) respectively. All cell culture reagents and heat-inactivated fetal bovine serum (HI-FBS) were from Gibco BRL (Paisley, Scotland, U.K.). Linbro 24-well tissue-culture plates were from ICN-Flow (Thame, U.K.) and Falcon Petri dishes from Becton Dickinson (Oxford, U.K.). Calyculin A and okadaic acid were purchased from Calbiochem (Nottingham, U.K.). These drugs were stored at −20 °C as concentrated stock solutions in dimethyl sulphoxide and diluted in culture medium as required. Digoxigenin (DIG)-labelled probes for IL-8, MCP-1 and RANTES were probe cocktails containing three anti-sense 30mer oligonucleotides purchased from R&D Systems (Abingdon, U.K.). Northern-blot analysis was performed using reagents for DIG chemiluminescent detection from Boehringer Mannheim (Lewes, U.K.). Antibodies for the IL-8 enzyme-linked immunosorbent assay (ELISA) were provided by Dr. I. J. D. Lindley (Sandoz Forschungsinstitut, Vienna, Austria). IL-6 antibody was obtained from Dr. S. Poole (National Institute for Biological Standards and Controls, U.K.). Antibodies for the RANTES ELISA were from R&D Systems. All other reagents were from Sigma (Poole, Dorset, U.K.) or Fisons (Loughborough, U.K).

Synovial cell culture

Human synovial tissue was obtained from patients undergoing joint replacement as treatment for osteo-arthritis or rheumatoid arthritis (Bath and Wessex Orthopaedic Research Unit, Royal United Hospital, Bath, U.K.). The tissue was minced, and subjected to sequential digestion at 37 °C with 1 mg/ml type I collagenase (Sigma) for 3 h and 0.25 mg/ml trypsin/0.1 mg/ml EDTA for 1 h [24]. The dispersed cells were washed, then cultured until a monolayer was obtained. Cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) HI-FBS, 0.5 μg/ml fungizone and 10 U/ml penicillin and streptomycin. Cells were used in these studies between passages 3 and 15 when the cultures contained predominantly fibroblast-like synovial cells and the basal level of IL-8 secretion was less than 0.5 ng/ml.

Measurement of secreted IL-8, IL-6 and RANTES by ELISA

Synovial fibroblasts were grown in 24-well plates until confluent, when there were approximately 1.5×10^4 cells/well. Culture medium was removed and experiments were carried out in fresh DMEM containing 5 % HI-FBS. Cells were treated with calyculin A (0.1–1 nM) or okadaic acid (1–100 nM) for 20 h, or the cells were pretreated with calyculin A for 1 h before the addition of TNFα or IL-1, as indicated in the Figure legends. Supernatants were collected after 20 h, centrifuged to remove any detached cells, then stored at −70 °C until assayed.

The IL-8 ELISA used mouse monoclonal anti-IL-8 as the coating antibody and polyclonal anti-IL-8 antibody conjugated to alkaline phosphatase as the detecting antibody. *p*-Nitrophenyl phosphate (Sigma) was used as the substrate. The standard curve ranged from 0.2 to 2 ng/ml [25].

The IL-6 ELISA used affinity-purified polyclonal goat anti-IL-6 as a coating antibody and the same antibody biotinylated as the detecting antibody. Antibody-binding was detected using streptavidin horseradish peroxidase (Sigma) followed by detection with phenylenediamine dihydrochloride (Sigma), as described previously [26]. The standard curve range was from 0.06 to 2 ng/ml.

The RANTES ELISA used mouse monoclonal anti-RANTES (R&D Systems) as the coating antibody. The detecting antibody was a polyclonal goat IgG anti-RANTES (R&D Systems). Binding was detected using alkaline phosphatase conjugated rabbit anti-(goat IgG) (Sigma) with *p*-nitrophenyl phosphate as the substrate. The standard curve range was from 0.2 to 2 ng/ml.

Northern-blot analysis

Synovial fibroblasts were grown to confluence in 10-cm diam. Petri dishes [$(0.5–1) \times 10^6$ cells/dish]. Culture medium was removed and experiments were carried out in fresh medium containing 5 % HI-FBS. In some experiments cells were treated with calyculin A (0.1–1 nM) for 30 min–24 h. In other experiments the cells were pretreated with calyculin A for approx. 1 h then TNFα or IL-1 were added for the length of time shown in the Figure legends. At the end of the incubation period cells were lysed into a guanidinium thiocyanate buffer [27] and frozen at −70 °C overnight. Total RNA was then extracted with phenol/chloroform and chloroform/isoamyl alcohol [28]. RNA was ethanol-precipitated and dissolved in water and the concentration determined by measuring the absorbance at 260 nm. A 10 μg amount of total RNA was loaded per lane and separated by electrophoresis through a 1 % agarose/formaldehyde gel. Ethidium bromide was included in each sample, enabling equal loading to be assessed by densitometric analysis of the 18S and 28S ribosomal RNA. Results were corrected for differences in loading where necessary. RNA was transblotted overnight to a nylon membrane (Boehringer Mannheim) and fixed by baking at 120 °C for 20 min.

The hybridization protocol used was essentially as described by Boehringer Mannheim in the DIG luminescent detection kit. The membranes were pre-hybridized at 42 °C then hybridized overnight with DIG-labelled oligonucleotide probes (at 10 ng/ml). The bound probes were detected using anti-DIG Fab fragments conjugated to alkaline phosphatase; lumigen PPD (Boehringer Mannheim) was used as the chemiluminescent substrate. Blots were exposed to X-ray film for 1–2 h and the autoradiographs were quantified by scanning laser densitometry.

Statistics

Where appropriate, the effects of drug treatment were assessed by Friedman's two-way analysis of variance by ranks followed by multiple comparison of groups with control [29].

RESULTS

Calyculin A induces the expression of chemokine mRNA in synovial fibroblasts

Untreated human synovial fibroblasts in culture did not constitutively express IL-8 mRNA, as measured by Northern-blot analysis. When the cells were stimulated with IL-1 (0.3 ng/ml), IL-8 mRNA was expressed in a time-dependent manner (Figure 1). This increase in IL-8 mRNA expression could be mimicked by the addition of the protein phosphatase inhibitor calyculin A. The expression of IL-8 mRNA induced in response to calyculin A followed a similar time-course to that induced by IL-1: both stimuli induced detectable IL-8 mRNA by 1 h and maximum expression at 6 h (Figure 1). Calyculin A-induced IL-8 mRNA expression was also concentration dependent, with 1 nM being the optimum concentration. As shown in Figure 2,

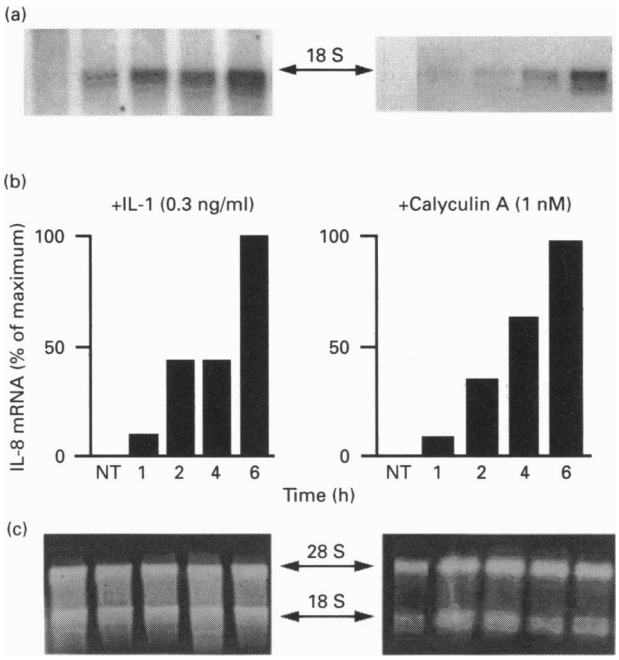

Figure 1 Comparison of the time-course of IL-8 mRNA expression induced by calyculin A or IL-1

Synovial fibroblasts were grown to confluence in Petri dishes and treated with either IL-1 (0.3 ng/ml) or calyculin A (1 nM). After the indicated times, total RNA was extracted and 10 μg were loaded per lane of a 1% agarose/formaldehyde gel and IL-8 mRNA was detected by Northern analysis (**a**). Relative amounts of IL-8 mRNA were measured by scanning densitometry of autoradiographs. The values shown have been corrected for any small differences in loading and expressed as a percentage of the maximum amount of IL-8 which was taken as 100% (**b**). The ethidium bromide-stained 18 S and 28 S rRNA was quantified by densitometry and used to correct for differences in loading (**c**). The data shown are from single experiments each representative of three similar experiments. NT, no treatment.

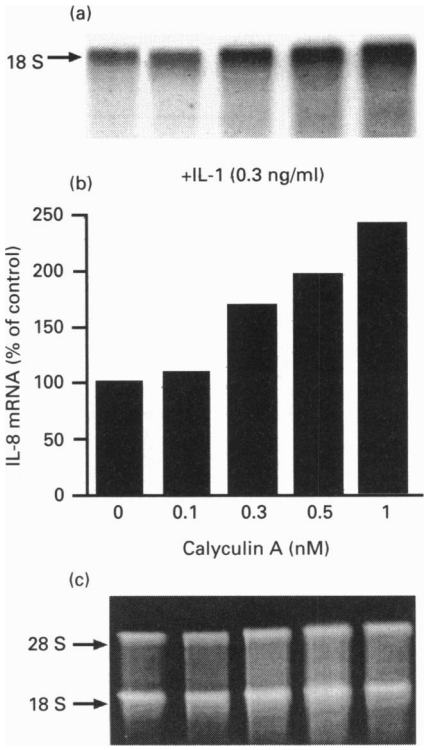

Figure 2 Calyculin A enhances IL-1-induced IL-8 mRNA expression in synovial fibroblasts

Synovial fibroblasts were pretreated for 1 h with 0.1, 0.3, 0.5 and 1.0 nM calyculin A before exposure to IL-1 (0.3 ng/ml) for 6 h. Total RNA was extracted and IL-8 mRNA detected by Northern analysis (**a**). Relative amounts of IL-8 mRNA were measured by scanning densitometry. The values shown have been corrected for any differences in loading and expressed as a percentage of the IL-1-treated control which has been taken as 100% (**b**). The ethidium bromide-stained 18 S and 28 S rRNA was quantified by densitometry and used to correct for differences in loading. The data shown are from a single experiment and are representative of two similar experiments.

calyculin A was also able to enhance IL-1-induced IL-8 mRNA expression in a concentration-dependent manner. Cells treated with IL-1 (0.3 ng/ml) and an optimum concentration of calyculin A (1 nM) expressed 2.3-fold as much IL-8 mRNA as cells treated with IL-1 alone. Calyculin A was similarly found to enhance TNFα-induced IL-8 mRNA expression in synovial fibroblasts (results not shown).

Synovial fibroblasts in culture also expressed mRNA for the chemokine MCP-1. Expression by untreated cells was variable: in some cultures low levels of MCP-1 mRNA were detectable, as in the representative experiment shown in Figure 3(I); in other cultures none was present. In all the synovial fibroblast cultures tested, MCP-1 mRNA could be induced by both calyculin A (Figure 3, I) and IL-1 (Figure 3, II). The time-courses for MCP-1 induction by these two stimuli were however quite different. Induction of MCP-1 by calyculin A was rapid and the response was short-lived, reaching a maximum between 1 and 2 h. In contrast, IL-1-induced MCP-1 mRNA expression did not peak until 4 h and high levels of MCP-1 mRNA were still being expressed by 6 h.

The effect of treating cells simultaneously with IL-1 (3 ng/ml) and calyculin A (1 nM) is shown in Figure 3(III). Between 1 and 4 h, MCP-1 mRNA expression appeared to be the result of the summation of the two stimuli added separately, but mRNA expression was decreased by 6 h in the presence of calyculin A compared with IL-1 alone. Quantification of the MCP-1 mRNA levels by densitometry indicated that the presence of calyculin A reduced the total amount of IL-1-induced MCP-1 mRNA by approx. 35%, largely as a result of the reduction in MCP-1 expression at 6 h. This may have been caused by the depletion of factors essential for continued IL-1 signalling, or by the initiation of feedback controls.

In synovial fibroblasts, mRNA for the chemokine RANTES was induced weakly in response to IL-1α and more strongly following 24 h stimulation with TNFα (N. J. Jordan, M. L. Watson and J. Westwick, unpublished work). Untreated cells did not express RANTES, and in four separate experiments we were unable to detect any RANTES mRNA expression in cells treated with calyculin A at concentrations from 0.2 to 1.0 nM and over a time-course of 4–24 h (not shown). Calyculin A was however able to increase, in a concentration-dependent manner, the amount of RANTES mRNA expressed after 24 h stimulation with TNFα. More than twice as much RANTES mRNA was expressed when TNFα (30 ng/ml) and calyculin A (0.8 nM) were added simultaneously compared with the RANTES expression in cells stimulated with TNFα alone (Figure 4).

Production of IL-8, IL-6 and RANTES peptide by synovial fibroblasts treated with calyculin A

In order to quantify the effect of calyculin A on synovial fibroblast chemokine expression, we have used specific ELISAs

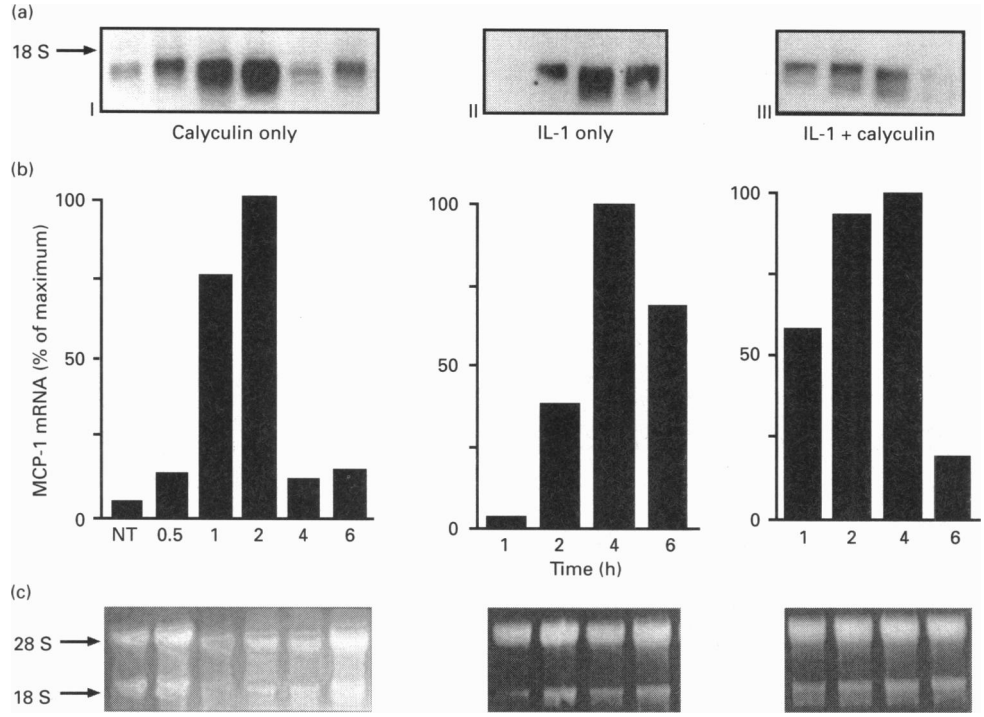

Figure 3 Calyculin A induces MCP-1 mRNA expression in synovial fibroblasts

Synovial cells were treated with 1 nM calyculin A (I), 3 ng/ml IL-1 (II) or 1 nM calyculin A plus 3 ng/ml IL-1 (III). Total RNA was extracted after the indicated times and MCP-1 mRNA analysed by Northern blotting (**a**). MCP-1 mRNA was quantified by scanning densitometry of autoradiographs (**b**). The data shown have been corrected to adjust for the differences in the amounts of total RNA loaded, which were determined by densitometric analysis of the ethidium bromide-stained 18S and 28S rRNA bands (**c**). These results are representative of 2–4 independent experiments. Results are expressed as a percentage of the maximum amount of MCP-1 produced by each treatment, which has been taken as 100%.

to measure IL-8, IL-6 and RANTES peptide production. Supernatants from synovial cells cultured for 20 h in DMEM containing 5% HI-FBS contained < 0.5 ng/ml IL-8 and IL-6. Treatment with IL-1 induced a concentration-dependent release of IL-8 and IL-6 (Table 1). These data are from a typical experiment showing that IL-1 was a more effective stimulus for the production of IL-8 compared with IL-6. The actual amount of IL-8 and IL-6 produced varied between cultures. When averaged over five different cultures, in 20 h, IL-1 (0.3 ng/ml) induced synovial fibroblasts to secrete 74 ± 7 ng/ml IL-8 compared with 36 ± 7.5 ng/ml IL-6.

As shown in Table 2, the treatment of synovial fibroblasts with calyculin A also caused a significant increase ($P < 0.05$) in IL-6 and IL-8 peptide production above basal levels at calyculin A concentrations greater than 0.6 nM. An optimum concentration of 0.8 nM calyculin A induced 9.0 ± 4.4 ng/ml IL-8 ($n = 6$) which was a 20-fold increase above the amount produced by untreated cells ($P < 0.01$). The data shown in Table 2 are means obtained from between 5 and 10 different synovial cell cultures. A 0.8 nM concentration of calyculin A induced a range of IL-8 from 0.3 to 27 ng/ml ($n = 6$), depending on the culture tested. This variation did not correlate with the cell passage number, nor was it dependent on whether the cells were derived from rheumatoid or osteo-arthritic patients. The effective concentration range of calyculin A was very narrow: below 0.4 nM it had little effect and above 1 nM (or at 1 nM in some cultures) it caused the cells to round up and detach from the culture surface. These rounded cells were still viable as assessed by Trypan Blue exclusion, but their IL-8 production was reduced.

Like IL-1, calyculin A also induced the production of lower amounts of IL-6 peptide than IL-8 peptide. The maximum amount of IL-6 (2 ng/ml) secreted in response to 1 nM calyculin A was still significantly increased ($P < 0.01$) above the amount of IL-6 produced by untreated cells. A comparison between Table 1 and Table 2 clearly demonstrates that the maximum amount of IL-8 and IL-6 induced by calyculin A was significantly less than that induced by IL-1. This quantitative difference was not apparent by measurement of the mRNA levels.

In five independent experiments, RANTES peptide could not be detected in supernatants collected after 24 or 48 h, either from unstimulated synovial fibroblasts or from cells treated with calyculin A over a concentration range of 0.2–1 nM (not shown). This correlated with the lack of RANTES mRNA expression in calyculin A-treated synovial cells.

Production of IL-8 peptide by synovial fibroblasts treated with okadaic acid

To investigate whether the stimulatory effect of calyculin A on IL-8 generation was likely to be attributable to the inhibition by this compound of protein phosphatases, synovial fibroblasts were also treated with okadaic acid. This compound is another PP-1/2A inhibitor with a different chemical structure to calyculin A. Okadaic acid also caused a concentration-dependent increase in IL-8 production, although it was approx. 100-fold less potent than calyculin A. An optimum concentration of 100 nM okadaic acid induced the secretion of 9.4 ± 4.7 ng/ml IL-8 (mean $\pm$ S.D. for two experiments).

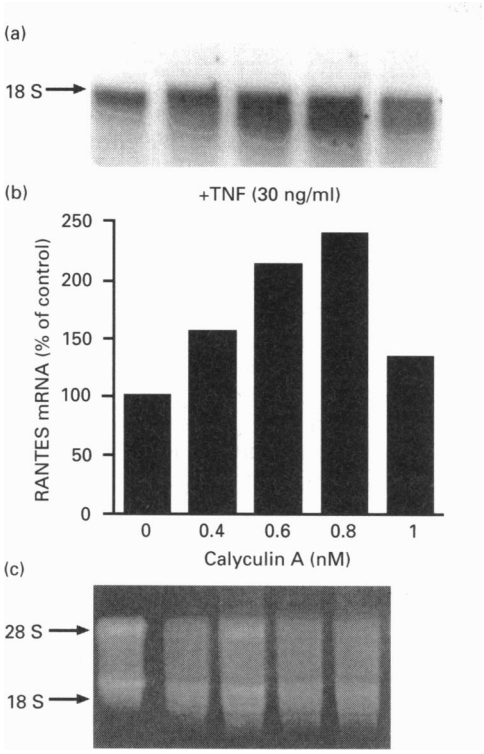

Figure 4 Calyculin A enhances the expression of TNFα-induced RANTES mRNA in synovial fibroblasts

Synovial fibroblasts were pretreated with 0.4, 0.6, 0.8 and 1.0 nM calyculin A for 1 h before the addition of 30 ng/ml TNFα for a further 24 h. Total RNA was extracted and RANTES mRNA detected by Northern blotting (**a**). RANTES mRNA was quantified by laser densitometry (**b**). The values shown have been corrected to adjust for differences in the amounts of total RNA loaded, which were determined by densitometric analysis of the ethidium bromide-stained 18S and 28S rRNA bands (**c**). The data shown are representative of two similar experiments. Results are expressed as a percentage of the TNFα-treated control.

Table 1 Induction of IL-8 and IL-6 peptide in synovial fibroblasts treated with IL-1

Synovial fibroblasts were grown to confluence in 24-well plates, then treated for 20 h with the indicated concentrations of IL-1 in DMEM containing 5% HI-FBS. IL-8 and IL-6 peptide were measured in the supernatants by ELISA. The data shown are means ± S.D. (ng/ml) of duplicate samples. Similar results were found in two additional cultures.

IL-1 (ng/ml)	IL-8 peptide (ng/ml)	IL-6 peptide (ng/ml)
0	6.77 ± 2.8	5.51 ± 0.74
0.0001	7.83 ± 0.09	7.05 ± 0.01
0.001	16.35 ± 1.01	12.15 ± 0.83
0.01	25.35 ± 1.20	27.50 ± 0.42
0.1	99.52 ± 1.45	50.55 ± 0.35
1.0	108.62 ± 16.94	55.20 ± 2.50
10	129.00 ± 50.45	62.40 ± 4.40

Effect of calyculin A on IL-1- and TNFα-induced IL-8, IL-6 and RANTES peptide production

Calyculin A added simultaneously with IL-1 or TNFα caused an increase in the secretion of IL-8 peptide (Table 3). This corresponded to the increased IL-8 mRNA expression. Since calyculin A alone induced only relatively low amounts of IL-8 and IL-6 peptide (Table 2), the increase in IL-1- or TNFα-induced IL-8 and IL-6 peptides was not simply an additive effect of calyculin

Table 2 Induction of IL-8 and IL-6 peptide in synovial fibroblasts treated with calyculin A

Synovial fibroblasts were grown to confluence in 24-well plates, then treated for 20 h with the indicated concentrations of calyculin A in DMEM containing 5% HI-FBS. IL-8 and IL-6 peptides were measured in the supernatants by ELISA. The data are means ± S.E.M. (ng/ml) for 5–10 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with untreated control.

Calyculin A (nM)	IL-8 peptide (ng/ml)	IL-6 peptide (ng/ml)
0	0.45 ± 0.17	0.45 ± 0.13
0.2	0.46 ± 0.15	0.69 ± 0.12
0.4	2.58 ± 1.42	0.96 ± 0.17
0.6	7.44 ± 3.95*	1.22 ± 0.21*
0.8	9.07 ± 4.41**	1.75 ± 0.32**
1.0	6.88 ± 1.95*	1.99 ± 0.44*

Table 3 Calyculin A synergistically enhances TNFα- and IL-1-induced IL-8 and IL-6 secretion in synovial fibroblasts

Synovial fibroblasts were grown to confluence in 24-well plates, then the indicated concentrations of calyculin A were added 1 h before the addition of IL-1 (0.3 ng/ml) or TNFα (30 ng/ml). After a 20 h incubation period supernatants were collected and IL-8 and IL-6 were measured by ELISA. The data shown are means ± S.E.M. obtained from 3–5 experiments. IL-8 and IL-6 production is expressed as a percentage of the amount produced by cells treated with TNFα or IL-1 alone, which is taken as 100%. TNFα (30 ng/ml) alone induced 50 ± 15 and 14 ± 3 ng/ml of IL-8 and IL-6 respectively and IL-1 (0.3 ng/ml) induced 77 ± 7 and 36 ± 8 ng/ml of IL-8 and IL-6 respectively.

Calyculin A (nM)	IL-1 (0.3 ng/ml)		TNFα (30 ng/ml)	
	IL-8	IL-6	IL-8	IL-6
0	100	100	100	100
0.2	110 ± 13	112 ± 12	118 ± 16	124 ± 14
0.4	138 ± 21	126 ± 18	162 ± 68	131 ± 8
0.6	154 ± 10	145 ± 21	210 ± 65	127 ± 12
0.8	256 ± 38	169 ± 28	243 ± 73	152 ± 17
1.0	264 ± 23	165 ± 23	292 ± 153	161 ± 30

A and TNFα/IL-1, but was the result of a synergistic response to IL-1 or TNFα and calyculin A. An optimum concentration of calyculin A caused a 2.5-fold increase in IL-8 production and a 1.6-fold increase in IL-6 generation above that produced by IL-1 alone. To enable data from different cultures to be compared, the results shown in Table 3 have been expressed as a percentage of a control (TNFα or IL-1 treatment only), which has been taken as 100%. The actual amount of IL-8 secreted in response to IL-1 (0.3 ng/ml) was 77 ± 7 ng/ml ($n = 5$); this increased to 205 ± 24 ng/ml ($n = 4$) when calyculin A (1 nM) was present. Cells treated with TNFα (30 ng/ml) alone produced 59 ± 15 ng/ml IL-8 ($n = 4$) compared with 147 ± 37 ng/ml ($n = 3$) in the presence of calyculin A (1 nM) and TNFα. Calyculin A caused a similar enhancement of IL-1- and TNFα-induced IL-6 production (Table 3).

The effect of calyculin A on IL-8 production was also examined over a range of concentrations of IL-1 (Table 4). Treatment of cells with 0.8 nM calyculin A alone produced 9 ± 4.4 ng/ml IL-8 and 0.4 nM calyculin A produced 2.6 ± 1.4 ng/ml IL-8 ($n = 6$). When calyculin A was added simultaneously with any concentration of IL-1 it caused a synergistic increase in IL-8 production that was greater than the sum of the IL-1 and

Table 4 Calyculin A synergistically potentiates IL-1-induced IL-8 expression over a range of IL-1 concentrations

Cells were stimulated with a range of IL-1 concentrations from 0.003 to 0.3 ng/ml in the presence of 0.4 or 0.8 nM calyculin A or vehicle control, as indicated. After 20 h, supernatants were collected and IL-8 was measured by ELISA. The results shown are means ± S.D. of two samples measured in duplicate, except the data for 0.4 and 0.8 nM calyculin A treatment alone which are means ± S.D. for six samples.

	IL-8 (mean ng/ml)		
IL-1 (ng/ml)	IL-1 only	IL-1 + calyculin A (0.4 nM)	IL-1 + calyculin A (0.8 nM)
0	1.03 ± 0.09	2.60 ± 1.40	9.0 ± 4.40
0.003	3.24 ± 0.03	12.7 ± 0.94	17.1 ± 0.85
0.01	6.93 ± 0.06	26.8 ± 2.68	32.2 ± 1.20
0.03	15.2 ± 2.14	35.7 ± 5.60	52.3 ± 4.52
0.1	23.4 ± 1.97	56.3 ± 9.19	64.9 ± 3.60
0.3	37.9 ± 7.00	71.6 ± 10.8	96.5 ± 1.13

calyculin A treatments separately. This calyculin-A-induced synergistic potentiation of IL-1-induced IL-8 production occurred over the range of IL-1 concentrations examined.

The amount of RANTES peptide produced by synovial fibroblasts following 24 h stimulation with TNFα (30 ng/ml) was 11 ± 4 ng/ml ($n = 2$), but in these experiments no increase in this level could be detected when calyculin A, over the range 0.2–1.0 nM, was added simultaneously with TNFα (not shown). This did not reflect the calyculin A-induced potentiation of TNFα-induced RANTES mRNA expression.

DISCUSSION

The serine/threonine protein phosphatase inhibitors calyculin A and okadaic acid have previously been shown to mimic the early events of IL-1 and TNFα signal transduction by inducing the expression of early response genes and phosphorylation of intracellular proteins [18,23]. We show here that calyculin A is also able to induce the accumulation of chemokine mRNA and increase production of chemokine peptides. Okadaic acid has similar potency to calyculin A as an inhibitor of PP2A but, in cell-free systems, is approx. 20–300-fold less active than calyculin A as an inhibitor of PP1 [30]. The results in this paper indicate a 100-fold difference in potency as stimuli for IL-8 production, suggesting that a type 1 protein phosphatase regulates chemokine expression in human synovial fibroblasts.

Guy et al. [18] found that inhibition of protein phosphatases induced changes in intracellular protein phosphorylation that were both quantitatively and qualitatively similar to those caused by TNFα. We also found that calyculin A and IL-1 or TNFα caused qualitatively similar responses in fibroblasts in terms of enhancing expression of the same chemokines. There were however some important differences between the calyculin A- and IL-1/TNFα-induced responses. MCP-1 mRNA was induced more rapidly by calyculin A than by IL-1, suggesting that calyculin A may be able to bypass the early part of the IL-1 signalling pathway and enable MCP-1 mRNA to accumulate more rapidly. MCP-1 mRNA was expressed in untreated synovial fibroblasts, probably because of the presence of FBS in the culture medium [31]. Hence, calyculin A may enhance MCP-1 mRNA accumulation rapidly by the stabilization of existing mRNA. Although IL-1 and calyculin A induced IL-8 mRNA with similar time-courses and efficacy, IL-1 appeared to be a much more effective stimulus for IL-8 peptide release. This may have been due to greater accuracy in the measurement of the latter or to additional post-transcriptional effects of IL-1. The calyculin A effect may also have been shorter lived than that stimulated by IL-1, which is known to sustain IL-8 mRNA expression over 24 h. It was not possible to assess the effect of increasing the concentration of calyculin A, since this agent was cytotoxic at concentrations above 1 nM, probably due to hyperphosphorylation of cytoskeletal elements [32] which may have been the cause of the cell rounding which was observed.

The activities of many enzymes involved in signalling pathways are regulated by phosphorylation. It would therefore appear likely that the effects of phosphatase inhibitors would be similar to those of activators of protein kinases, since both agents would tend to increase the levels of protein phosphorylation. The action of both IL-1 and calyculin A could therefore be to increase the phosphorylation of proteins involved at any step in the initiation of gene transcription or in post-transcriptional events by affecting mRNA stabilization or translation. While TNFα can decrease protein phosphatase activity in cytosolic extracts of human fibroblasts [18], this may not be the principal mechanism of IL-1- or TNFα-induced chemokine production. Calyculin A synergistically augments TNFα-induced apoptosis in tumour cell lines [33] and okadaic acid synergistically increases TNFα mRNA expression in combination with lipopolysaccharide and phorbol ester [20,21]. In the present study, calyculin A was found to act synergistically with IL-1 and TNFα to induce almost twice as much IL-8 as was induced by IL-1/TNFα and calyculin A added separately. These results suggest that both calyculin A- and IL-1- or TNFα-stimulated responses are regulated by the same phosphorylation-dependent pathway, but at different levels.

Although early events in IL-1/TNFα signal transduction, such as hsp27 phosphorylation, seem to require only the inactivation of a phosphatase and are unaffected by inhibitors of protein kinase [18], we propose that downstream events, such as the production of chemokine peptide, also require the activation of a kinase. We have obtained evidence which shows that a protein kinase (possibly an isoform of protein kinase C (PKC) is necessary for IL-1/TNFα-induced IL-8, IL-6, MCP-1 and RANTES expression, since a bisindolylmaleimide protein kinase inhibitor Ro 31-8220 [34], which is a potent inhibitor of PKC, decreased the expression of these cytokines in synovial fibroblasts (N. J. Jordan, M. L. Watson and J. Westwick, unpublished work). We therefore propose a signalling pathway in which the primary role of IL-1/TNFα is the activation of a kinase, the action of which may be enhanced by inhibiting a phosphatase with calyculin A. If the putative IL-1/TNFα-sensitive kinase has a low basal activity in untreated cells, phosphatase inhibition by calyculin A may result in the accumulation of a phosphorylated target protein to a level sufficient to trigger the expression of chemokine mRNA in the absence of IL-1 or TNFα.

There are many reports showing that kinases are activated by IL-1 or TNFα. In addition to PKC, IL-1 and TNFα activate a number of kinases, including the microtubule-associated protein kinase/mitogen-activated protein kinase (MAP) kinase cascade [35,36]. This system is also activated by okadaic acid [37] and may be the site at which phosphatase inhibitors are able to impinge on the IL-1/TNFα signalling pathway. Activation of MAP kinase may occur by inhibition of the phosphatase product of the CL-100 gene [38]. Both IL-1 and TNFα increase chemokine mRNA accumulation by stimulating increased gene transcription in synovial fibroblasts [16]. The genes for IL-8, IL-6, MCP-1 and RANTES all contain potential binding sites for the nuclear factors NFkB and AP-1 [39–42]. Both these factors are influenced by protein phosphorylation. AP-1 comprises products of the c-jun and c-fos genes [43] which are activated by the MAP kinase

cascade and by protein phosphatase inhibitors [23]. NFkB activity is regulated by the phosphorylation of a cytoplasmic retention protein IkBα [44], and can be activated in transformed cells by okadaic acid or calyculin A [22]. Since all the genes we investigated can be controlled by these same transcription factors, regulation via AP-1 or NFkB does not explain the observed differential regulation of the cytokine genes, in particular the inability of calyculin A to induce RANTES mRNA accumulation.

Post-transcriptional events are also implicated in cytokine gene regulation. Message stabilization is commonly used to regulate cytokine gene expression and may be one of the ways in which phosphatase inhibitors can up-regulate cytokine expression [21]. The accumulation of TNFα mRNA, which contains tandem repeats of the destabilizing sequence AUUUA, is increased by treatment of B-cells with okadaic acid [20], probably as a result of the specific binding of phosphorylation regulated AU-binding proteins [45–47]. Since IL-8 and IL-6 genes contain several AUUUA sequences [48,49] they may be regulated in this way by calyculin A. Consistent with this hypothesis, calyculin A was unable to stimulate accumulation of RANTES mRNA, which lacks multiple AUUUA sequences [50]. However MCP-1 mRNA, which was up-regulated, does not contain multiple AUUUA repeats either [51], so other mechanisms must also exist which account for the accumulation of MCP-1 mRNA. RANTES mRNA expression may be dependent on the synthesis of an unidentified intermediary protein [16] which is not induced by calyculin A in the absence of IL-1/TNFα.

In summary, these results indicate a role for a calyculin A-sensitive protein phosphatase in the expression of chemokine and other cytokine genes, although there are clearly differences in the way that IL-8, IL-6, MCP-1 and RANTES gene expression is regulated. These differences may enable differential control of the leucocyte infiltration to occur during the course of an inflammatory response. Since the inhibition of protein phosphatases was insufficient to completely mimic IL-1- or TNFα-induced cytokine accumulation, we suggest that additional signals, probably involving activation of a kinase, are normally required for IL-1- and TNFα-induced chemokine production in synovial fibroblasts.

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