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Differential effects of protein kinase C inhibitors on chemokine production in human synovial fibroblasts

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1 Rheumatoid arthritis is associated with the accumulation and activation of selected populations of inflammatory cells within the arthritic joint. One putative signal for this process is the production, by resident cells, of a group of inflammatory mediators known as the chemokines.

2 The chemokines interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1) and RANTES (regulated on activation normal T-cell expressed and presumably secreted) are target-cell specific chemoattractants produced by synovial fibroblasts in response to stimulation with interleukin-1α (IL-1α) or tumour necrosis factor α (TNFα). The signalling pathways involved in their production are not well defined. We therefore used four different protein kinase C inhibitors to investigate the role of this kinase in the regulation of chemokine mRNA and protein expression in human cultured synovial fibroblasts.

3 The non-selective PKC inhibitor, staurosporine (1-300 nM) significantly increased the production of IL-1α-induced IL-8 mRNA and protein. A specific PKC inhibitor, chelerythrine chloride (0.1-3 μM), also caused a small concentration-dependent increase in IL-8 mRNA and protein production. In contrast, 3-[1-[3-(amidinothio)propyl]-3-indolyl]-4-(1-methyl-3-indolyl)-1H-pyrrole-2,5-dione methanesulphonate (Ro 31-8220) and 2[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide (GF 109203X), two selective PKC inhibitors of the substituted bisindolylmaleimide family had a concentration-dependent biphasic effect on IL-1α or TNFα-induced chemokine expression. At low concentrations they caused a stimulation in chemokine production, which was especially evident at the mRNA level. At higher concentrations both inhibited IL-1α or TNFα-induced chemokine mRNA and protein production. Ro 31-8220 was 10 fold more potent than GF 109203X, with an IC_{50} of 1.6±0.08 μM (mean±s.e.mean, $n=4$) for IL-1α induced IL-8 production. Ro 31-8220 also inhibited the expression of IL-1α or TNFα-induced MCP-1 and RANTES mRNA with a similar potency.

4 The stimulatory effect of staurosporine is discussed in relation to the known poor selectivity of this inhibitor for PKC. It is proposed that activation of an isoform of PKC, possibly PKC epsilon or zeta, which is inhibited by higher concentrations of the bisinodolylmaleimides, plays a role in the regulation of chemokine expression induced by IL-1α or TNFα in synovial cells.

5 The inhibition of chemokine production by bisindolylmaleimide compounds heralds a novel approach for future anti-inflammatory therapies.

Keywords: Protein kinase C inhibitors; chemokine; synovial fibroblasts; interleukin 1α; tumour necrosis factor α; bisindolylmaleimide; Ro 31-8220; GF 109203X; staurosporine; chelerythrine chloride

Introduction

The accumulation and activation of selected populations of inflammatory cells in the synovial cavity appears to play a pivotal role in the pathogenesis of rheumatoid arthritis (Palmer *et al.*, 1986). This process requires a series of coordinated signals leading to the local generation of chemotactic factors. An important group of recently identified chemotactic proteins is the chemokine family (Lindley *et al.*, 1993a,b). Members of this family include interleukin-8 (IL-8), which is selectively chemotactic for neutrophils and a subset of lymphocytes (Yoshimura *et al.*, 1987; Bacon *et al.*, 1990), monocyte chemotactic protein-1 (MCP-1) which attracts monocyte derived macrophages (Yoshimura *et al.*, 1989) and RANTES which attracts eosinophils and CD4/CD45RO T-cells (Schall *et al.*, 1990; Kameyoshi *et al.*, 1992). The production of chemokines may be relevant to the pathogenesis of rheumatoid arthritis, since in patients with this disease, interleukin-8 (IL-8) and MCP-1 have been detected in synovial fluid and synovial cells from these patients have been found to express mRNA for IL-8, MCP-1 and RANTES (regulated on activation normal T-cell expressed and presumably secreted) (Brennan *et al.*, 1990; Schall *et al.*, 1991; Koch *et al.*, 1992; Villiger *et al.*, 1992).

In cultured synovial cells the most important stimuli for chemokine induction are the pro-inflammatory cytokines IL-1α and tumour necrosis factor α (TNFα) (Watson *et al.*, 1988; DeMarco *et al.*, 1991; Bédard & Golds, 1993; Rathanaswami *et al.*, 1993). Their signalling mechanisms are not well defined, thus identification of intracellular events involved in chemokine regulation may identify potential targets for the action of novel anti-inflammatory agents (Saklatvala & Guesdon, 1992). Both IL-1α and TNFα cause intracellular proteins to be phosphorylated on serine/threonine residues which implies activation/inactivation of protein kinases and phosphatases respectively (Guy *et al.*, 1991). The relevance of protein kinase C (PKC) activation in these IL-1α and TNFα-induced phosphorylation events remains uncertain.

PKC is a generic term for a family of serine/threonine kinases, which can be further subdivided into related groups (Dekker & Parker, 1994). The conventional PKC's (α, βI, βII, and γ) are activated by Ca^{2+} and diacylglycerol (DAG) or phorbol esters. The novel PKC's (delta, epsilon, eta and theta) are Ca^{2+}-independent but can be activated by DAG and phorbol esters. The atypical PKC's (zeta, iota) are both Ca^{2+}-independent and unresponsive to phorbol esters. A recently identified isoform, PKCμ, may be classed in a separate group

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due to atypical structural features (Johannes *et al.*, 1994). Attempts to establish whether PKC plays a role in IL-1α and TNFα signal transduction have been contradictory. This is largely the result of the use of poorly selective 'PKC inhibitors', such as staurosporine and H7 and the exogenously applied activator, phorbol 12 myristate 13 acetate (PMA) (Wilkinson & Hallam, 1994).

Recently, a number of synthetic derivatives of staurosporine have been developed, these compounds show improved selectivity for PKC (Toullec *et al.*, 1991; Davies *et al.*, 1992; Bit *et al.*, 1993). The substituted bisindolylmaleimides 3-[1-[3-(amidinothio)propyl]-3-indolyl]-4-(1-methyl-3-indolyl)-1H-pyrrole-2,5-dione methanesulphonate (Ro 31-8220) and 2[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide (GF 109203X), are similar to staurosporine in that they inhibit ATP binding competitively but they are also highly selective for PKC over Ca^{2+}/calmodulin-dependent protein kinase and phosphorylase kinase and have inhibitory activity against all isoforms of PKC (Toullec *et al.*, 1991; Martiny-Baron *et al.*, 1993; Wilkinson & Hallam, 1994). Another specific PKC inhibitor used in this study is chelerythrine chloride, a benzophenanthridine alkaloid which acts at the protein substrate binding site, hence does not block phorbol ester binding to PKC and is a non-competitive inhibitor of ATP binding (Herbert *et al.*, 1990). Chelerythrine chloride is also selective for PKC over tyrosine protein kinases, adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinases and Ca^{2+}/calmodulin-dependent protein kinases.

We have investigated the ability of PKC inhibitors to affect TNFα and IL-1α-induced chemokine gene expression and protein production in synovial fibroblasts. The results indicate that a bisindolylmaleimide-sensitive protein kinase is involved in IL-1α/TNFα signalling and that these compounds have potential for use as anti-inflammatory agents.

Methods

Synovial cell culture

Human synovial tissue was obtained from patients undergoing joint replacement as treatment for rheumatoid or osteoarthritis (Bath and Wessex Orthopaedic Research Unit, Royal United Hospital, Bath). Tissue was minced and subjected to sequential digestion at 37°C with periodic shaking in Dulbecco's modified Eagle's medium (DMEM) containing 1 mg ml^{-1} collagenase (type 1) for 3 h followed by digestion in 0.25 mg ml^{-1} trypsin/0.1 mg ml^{-1} EDTA for a further hour (Dayer *et al.*, 1976). Dispersed cells were recovered by centrifugation and resuspended in DMEM containing 10% HI-FBS, 10 u ml^{-1} penicillin and streptomycin and 0.5 μg ml^{-1} fungizone, which was the medium used for routine culture. Confluent cells were routinely passaged by detaching in trypsin/EDTA. Cells were used between passages 4–17 when the cultures contained predominantly fibroblast-like cells. The cultures of synovial cells used were from 12 different patients.

Measurement of secreted IL-8, MCP-1 and RANTES protein by enzyme-linked immunosorbent assay (ELISA)

Synovial fibroblasts were grown to confluence in 24 well plates, when there were approximately 1.6×10^4cells/well. The culture medium was removed and replaced with fresh DMEM containing 5% HI-FBS. The cells were pretreated for 2–4 h with PKC inhibitors or vehicle at the concentrations indicated in the figure legends, then TNFα or IL-1α were added to the wells and the incubation was continued for 20 h. Supernatants were collected and stored at −70°C until assayed by ELISA. The cell monolayers were examined microscopically to check for cell damage.

IL-8 protein was measured by ELISA as described previously (Ceska *et al.*, 1989; Brown *et al.*, 1991). A mouse monoclonal anti-IL-8 antibody was used to coat the plates (Nunc MaxiSorp, Gibco BRL) and a polyclonal goat anti-IL-8 antibody conjugated to alkaline phosphatase was used for detection. MCP-1 protein was measured in a sandwich ELISA as previously described (Yoshimura *et al.*, 1991). Briefly, plates were coated with monoclonal MCP-1 antibody (4.5 μg ml^{-1} in sodium bicarbonate buffer pH9.6). The plates were blocked with 0.2% bovine serum albumin in Tris buffered saline (TBS, 42 mM Tris, 150 mM NaCl pH7.3). Samples and standards in TBS + 0.05% Tween were added for 90 min at 37°C, the standard curve range was from 0.25–8.0 ng ml^{-1}. Plates were washed and incubated with polyclonal rabbit anti-MCP-1 (90 min 37°C). Binding was detected with alkaline phosphatase labelled anti-rabbit IgG followed by *p*-nitrophenylphosphate as the substrate.

For the RANTES ELISA, plates were coated overnight at 4°C with monoclonal anti-RANTES antibody (1 μg ml^{-1} in sodium bicarbonate buffer pH9.6). Plates were washed and incubated for 2 h at 37°C with samples and standards. The detecting antibody was a polyclonal goat IgG anti-RANTES added at 1 μg ml^{-1} for 1 h at 37°C. Binding was detected with alkaline phosphatase conjugated rabbit anti-goat with *p*-nitrophenylphosphate as the substrate. The standard curve of the assay ranged from 0.2–2.0 ng ml^{-1}. All ELISA estimates were made from duplicates.

Northern blot analysis

Synovial fibroblasts were grown to confluence in 10 cm diameter Petri dishes ($0.5-1 \times 10^6$ cells ml^{-1}). The culture medium was removed and replaced with 5 ml of fresh medium containing 5% HI-FBS. The cells were pretreated with PKC inhibitors for 1–4 h, then IL-1α or TNFα was added for the time shown in the figure legends. Initial time course studies revealed that the optimum time for detection of IL-8 and MCP-1 mRNA was 6 h whereas RANTES mRNA was detected following 24 h stimulation. Cells were lysed with 4 M guanidinium thiocyanate extraction buffer (Chomczynski & Sacchi, 1987). Total cellular RNA was extracted with phenol/chloroform and chloroform/isoamylalcohol as described by Strieter *et al.* (1989). RNA was ethanol precipitated, dissolved in water and the concentration measured by absorbance at 260 nm. Approximately 10 μg total RNA was loaded per lane of a 1% agarose/formaldehyde gel. Ethidium bromide was included in each sample, enabling equal loading to be assessed by densitometric analysis of 18S and 28S ribosomal RNA. Results were corrected for differences in loading when necessary. RNA was transferred overnight to nylon membrane (Boehringer Mannheim) by capillary blotting and fixed by baking at 120°C for 20 min.

The hybridization protocol was essentially as described in the DIG luminescent detection kit (Boehringer Mannheim). Briefly, membranes were prehybridized for 1 h at 42°C then hybridized overnight at the same temperature with DIG-labelled oligonucleotide probes (10 ng ml^{-1}). Bound probes were detected with anti-DIG Fab conjugated to alkaline phosphatase followed by a chemiluminescent substrate (CSPD) (Boehringer Mannheim). Bands were visualized by exposure to X-ray film for 1–2 h and quantified by scanning densitometry.

Materials

All PCK inhibitors were prepared as concentrated stock solutions stored at −20°C and diluted in culture medium just before use. Staurosporine was stored as a 1 mM solution in dimethylsulphoxide (DMSO) (Sigma, Poole, UK). Chelerythrine chloride was stored as a 10 mM stock solution in water (Calbiochem, Nottingham, UK). GF 109203X was a 5 mM stock solution in DMSO (Calbiochem). Ro 31-8220 was a gift from Dr J.S. Nixon (Roche, Welwyn Garden City, UK) and was prepared as a 50 mM stock solution in DMSO. Phorbol 12 myristate 13 acetate (PMA) was from Sigma. Human re-

combinant IL-1α (specific activity 5×10^7 u mg^{-1}) and TNFα (specific activity 6×10^7 u mg^{-1}) were generous gifts from Glaxo (Greenford, UK) and Bayer (Slough, UK) respectively. All cell culture reagents and heat inactivated foetal bovine serum (HI-FBS) were from Gibco BRL (Paisley, Scotland). Linbro 24 well tissue culture plates were from ICN (Thame, UK) and Falcon petri dishes were from Becton Dickinson (Oxford, UK). The Digoxigenin (DIG) chemiluminescent detection kit for Northern blotting was from Boehringer Mannheim (Lewes, UK). 5' Digoxigenin labelled probes for IL-8, MCP-1 and RANTES mRNA were cocktails containing 3 antisense 30-mer oligonucleotides (R&D Systems, Abingdon, UK). Antibodies for the IL-8 enzyme linked immunosorbent assay (ELISA) were a generous gift from Dr I.J.D. Lindley (Sandoz Forschungsinstitut, Vienna, Austria). Specific anti-RANTES antibodies for the RANTES ELISA were from R&D Systems. All other chemicals were from Sigma or Fisons (Loughborough, UK).

Statistics

Where appropriate, the effects of drug treatment compared to control were assessed by two way analysis of variance. Dunnett's test was used for comparison of control to treatment groups of square root transformed data, where each datum unit was the mean protein level from 2 ELISA estimates.

Results

Chemokine production by cultured human synovial fibroblasts

Unstimulated human synovial fibroblasts obtained from patients with either rheumatoid or osteo arthritis, which had been kept in culture for 4 or more passages, secreted < 0.5 ng ml^{-1} IL-8 into the culture supernatant over a 20 h period. There were differences in the amount of IL-8 secreted by the different cultures and although cells from rheumatoid patients produced more IL-8 initially, after 4 passages any differences did not correlate with the disease status of the patient from whom the synovial tissue was obtained. IL-8 secretion increased in a time and concentration-dependent manner when cells were stimulated with either TNFα or IL-1α (Figure 1). Little IL-8 could be detected in the culture supernatants after 3 h of stimulation, but secretion increased over the next 24 h. Chemokine secretion was routinely measured at 20 h. Stimulation with IL-1α elicited twice as much IL-8 protein as compared with TNFα treatment and was effective at a 100 fold lower concentration. This increased efficacy of IL-1α has also been shown in our laboratory in mesangial cells (Brown *et al.*, 1991). The synovial cells shown in Figure 1b responded to 20 h treatment with IL-1α (3 ng ml^{-1}) and TNFα (300 ng ml^{-1}) by secreting 62 ± 8.5 and 28 ± 5 ng ml^{-1} IL-8 respectively. Stimulation with IL-1α or TNFα also induced synovial fibroblasts to secrete the C-C chemokines MCP-1 and RANTES. Stimulation for 20 h with IL-1α (3 ng ml^{-1}) or TNFα (30 ng ml^{-1}) induced 18 ± 4 and 19 ± 4 ng ml^{-1} of MCP-1 respectively (means ± s.e.mean for 3 experiments). Cytokine stimulation over the same time period induced little RANTES protein. Twenty hours stimulation with TNFα (30 ng ml^{-1}) induced the secretion of 3.0 ± 0.8 ng ml^{-1} RANTES (mean ± s.e.mean for 3 experiments) and IL-1α (3 ng ml^{-1}) induced < 1 ng ml^{-1} RANTES protein.

This increase in IL-1α and TNFα-induced protein was preceded by an increase in mRNA expression. MCP-1 and IL-8 mRNA was induced maximally by IL-1α at 4 to 6 h and expression decreased by 24 h, whereas mRNA induced by TNFα remained maximally expressed between 2 and 24 h. RANTES gene expression differed from that of the other chemokines, in that peak levels of expression were not obtained before 24 h of stimulation with TNFα (data not shown).

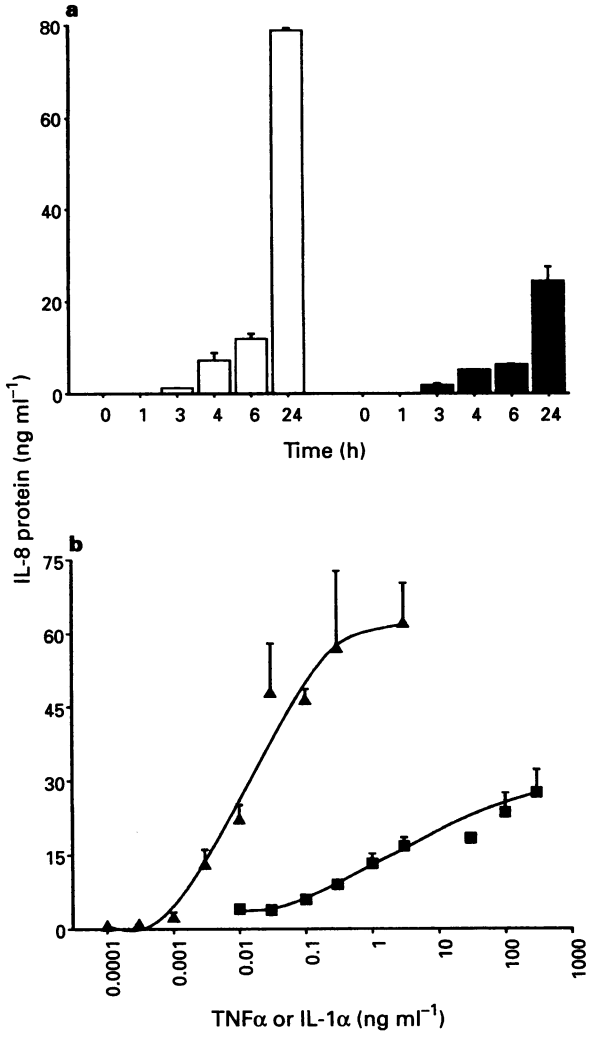


Figure 1 IL-8 production by human cultured synovial fibroblasts stimulated with IL-1α or TNFα. (a) Synovial fibroblasts were grown to confluence in 24 well dishes then treated with 3 ng ml^{-1} TNFα (solid columns) or 0.3 ng ml^{-1} IL-1α (open columns). Supernatants were removed after the times shown and IL-8 was measured by ELISA. The results shown are means of duplicate samples measured in duplicate and are expressed as ng ml^{-1} IL-8 (mean ± s.d.). (b) Synovial fibroblasts were grown to confluence in 24 well dishes then treated with TNFα (■) or IL-1α (▲) at the concentration shown. Supernatants were removed after 20 h and IL-8 was measured by ELISA. The results shown are means of duplicate samples measured in duplicate and are expressed as ng ml^{-1} IL-8 (mean ± s.d.).

IL-1α was a poor inducer of RANTES mRNA, even when stimulation was extended beyond 24 h (Jordan *et al.*, 1995). It has been reported that TNFα-induced RANTES gene expression requires protein synthesis, which may explain the late induction of this chemokine mRNA by TNFα (Rathanaswami *et al.*, 1993). These authors have also reported that more RANTES protein was produced in synovial fibroblasts stimulated with TNFα compared to IL-1α.

Induction of IL-8 protein by the phorbol ester PMA

The phorbol ester PMA, a known activator of PKC, was also able to induce the production of IL-8 protein in synovial fibroblasts, albeit at a much lower level than either IL-1α or TNFα. An optimum concentration of PMA (10 nM) induced 18.7 ± 4.8 ng ml^{-1} IL-8 (mean ± s.d. for 2 experiments). When simultaneously treated with TNFα or IL-1α and PMA, a synergistic increase in IL-8 secretion occurred which was 2.4 fold greater than that produced by either agent alone (Table 1).

Table 1 IL-8 secretion by cultured human synovial fibroblasts treated with PMA

PMA (nM)	*IL-8* (ng ml^{-1})
0	0.35
0.1	0.8±4.2
1.0	2.65±0.5
3.0	7.15±3.5
10	18.7±4.8
30	16.9±7.5
100	15.9±10.6
300	14.4±6.5
TNF (30 ng ml^{-1})	46±9.9
TNF (30 ng ml^{-1})+PMA (10 nM)	159±22
IL-1 (3 ng ml^{-1})	77.5±57
IL-1 (3 ng ml^{-1})+PMA (10 nM)	228±120

Synovial fibroblasts were grown to confluence in 24 well plates, then stimulated with the indicated concentrations of PMA, either alone or in combination with TNFα (30 ng ml^{-1}) or IL-1α (3 ng ml^{-1}) for 20 h. IL-8 protein was measured by ELISA. The results are means±s.d. (ng ml^{-1}). from 2 experiments in which each sample was measured in duplicate.

The PKC inhibitors staurosporine and chelerythrine chloride enhance IL-1α induced IL-8 expression

Staurosporine added to unstimulated synovial fibroblasts did not affect basal secretion of IL-8. This non-selective PKC inhibitor caused an increase in IL-1α-induced IL-8 protein production over a concentration-range (1–100 nM) previously shown to be effective at inhibiting PKC in fibroblasts (Taylor *et al.*, 1990). As shown in Figure 2a, a concentration of 3 nM staurosporine caused a 2 fold increase in IL-1α induced IL-8 production ($P<0.05$). Staurosporine also caused a concentration-dependent bell shaped increase in IL-8 mRNA expression. In the experiment shown in Figure 2b, 30 nM staurosporine increased IL-1α-induced IL-8 mRNA expression 4 fold. At concentrations >300 nM staurosporine was cytotoxic.

Chelerythrine chloride also caused a concentration-dependent increase in IL-1α-induced IL-8 production (Figure 3a). A 3 μM concentration of chelerythrine chloride increased IL-8 expression 1.5 fold ($P<0.01$). The increase in mRNA expression was even more pronounced with 1 μM chelerythrine causing a 2 fold increase in IL-8 mRNA expression (Figure 3b). A 10 μM concentration of chelerythrine chloride was cytotoxic.

Effect of bisindolylmaleimide PKC inhibitors on IL-1α and TNFα-induced chemokine production

We investigated the effects of two closely related specific PKC inhibitors of the bisindolylmaleimide family, GF 109203X and Ro 31-8220 on IL-1α and TNFα induced chemokine production. Neither compound affected basal levels of chemokine production. As shown in Figure 4a, GF 109203X had a concentration-dependent biphasic effect on the secretion of IL-8 protein. At low concentrations, <3 μM, it caused a small but not statistically significant increase in IL-1α-induced IL-8 protein secretion. Increasing the concentration of GF 109203X to 10 μM caused a significant inhibition of IL-8 protein and almost complete inhibition resulted at 30 μM. Hence GF 109203X had an IC_{50} for IL-1α-induced IL-8 production of 17±1.7 μM (mean±s.e.mean for 3 experiments). GF 109203X also inhibited the accumulation of IL-1α-induced IL-8 mRNA at concentrations above 10 μM (Figure 4b).

The inhibition of both IL-1α and TNFα-induced IL-8 and MCP-1 and TNFα-induced RANTES protein by Ro 31-8220 was concentration-dependent. Unlike GF 109203X, lower concentrations of Ro 31-8220 did not increase chemokine protein secretion. The IC_{50} for the inhibition of IL-1α-induced IL-8 was 1.6±0.08 μM (mean±s.e.mean for 4 experiments) and 1.33±0.33 μM (mean±s.e.mean for 3 experiments) for TNFα-induced IL-8, with almost total inhibition of all of the chemokines being caused by 10 μM Ro 31-8220 (Figure 5). Thus Ro 31-8220 was approximately 10 fold more potent than GF 109203X. This inhibition was not due to cytotoxicity, as assessed by observation of the cell morphology and trypan blue viability staining.

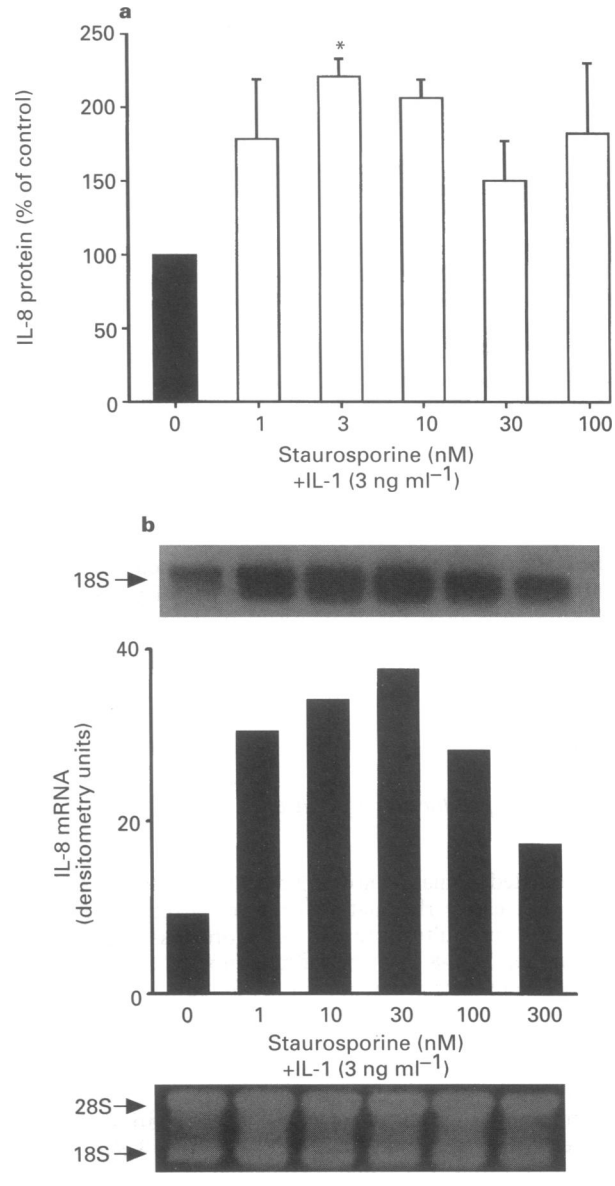


Figure 2 Effect of staurosporine on IL-1α-induced IL-8 mRNA and protein production. (a) Cells were pretreated for 2 h with staurosporine at the indicated concentrations. IL-1α (3 ng ml^{-1}) was added and the incubation continued for 20 h. Supernatants were removed and IL-8 protein was measured by ELISA. The results are means±s.e.mean for 3 experiments, which have been expressed as a percentage of the IL-1α control, which was 86±50 ng ml^{-1} (mean± s.e.mean). *$P<0.05$ compared to IL-1α control. (b) Cells were pretreated for 1 h with medium containing staurosporine at the concentrations shown, then IL-1α (3 ng ml^{-1}) was added for a further 6 h. Total RNA was extracted and IL-8 mRNA was analysed by Northern blotting. Relative amounts of IL-8 mRNA were quantified by scanning densitometry and equal loading was confirmed by ethidium bromide staining of the 18S and 28S rRNA shown in the lower panel. The data shown are from a single experiment and are representative of 2 similar experiments.

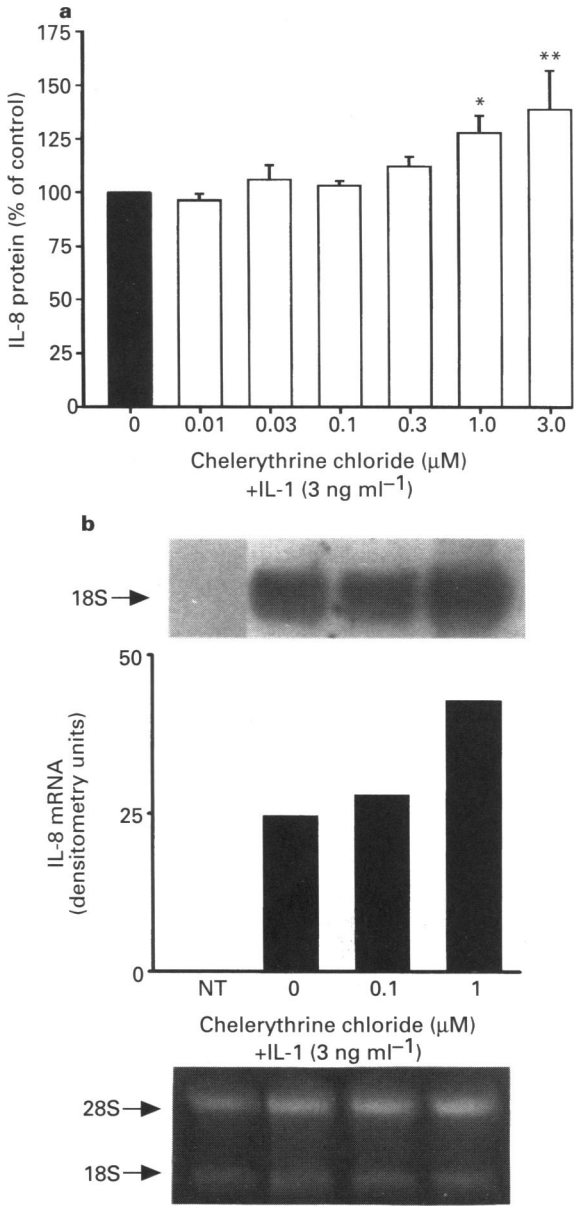


Figure 3 Effect of chelerythrine chloride on IL-1α-induced IL-8 mRNA and protein production. (a) Cells were pretreated for 2–4 h with chelerythrine chloride at the indicated concentrations, before the addition of IL-1α (3 ng ml^{-1}) for a further 20 h. Supernatants were removed and IL-8 protein was measured by ELISA. The results shown are means ± s.e.mean of 3 separate experiments, expressed as a percentage of the IL-1α control, which was 40 ± 4 ng ml^{-1} (mean ± s.e.mean). *P < 0.05, **P < 0.01 compared to the IL-1α control. (b) Cells were pretreated for 1 h with chelerythrine chloride at the indicated concentrations, then IL-1α (3 ng ml^{-1}) was added for a further 6 h. NT = no treatment. Total RNA was extracted and IL-8 mRNA analysed by Northern blotting. Relative amounts of IL-8 mRNA were quantified by scanning laser densitometry and equal loading was confirmed by ethidium bromide staining of the 18S and 28S rRNA shown in the lower panel. The data shown are from a single experiment and are representative of 2 similar experiments.

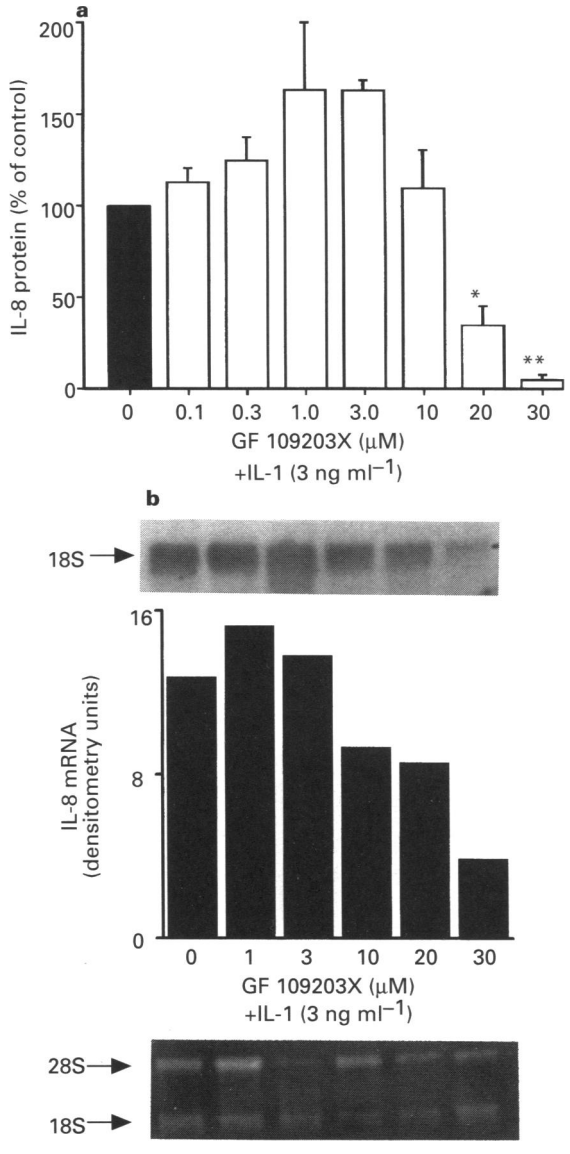


Figure 4 Effect of GF 109203X on IL-1α-induced IL-8 mRNA and protein production. (a) Cells were pretreated for 4 h with the indicated concentrations of GF 109203X. IL-1α (3 ng ml^{-1}) was added and the incubation continued for 20 h. IL-8 in the supernatants was measured by ELISA. The results shown are means ± s.e.mean for three separate experiments, expressed as a percentage of the IL-1α control, which was 41 ± 11 ng ml^{-1} (mean ± s.e.mean). *P < 0.05; **P < 0.01 compared to IL-1 control. (b) Cells were pretreated for 1 h with GF 109203X at the indicated concentrations before the addition of IL-1α (3 ng ml^{-1}) for 6 h. Total cellular RNA was extracted and IL-8 mRNA analysed by Northern blotting. Relative amounts of IL-8 mRNA were quantified by scanning densitometry and corrected for any small differences in loading as assessed by densitometric analysis of the 18S and 28S rRNA shown in the lower panel. The results shown are from a representative experiment.

Ro 31-8220 had a concentration-dependent biphasic effect on the accumulation of IL-1α or TNFα-induced IL-8, MCP-1 and RANTES mRNA (Figure 6). Concentrations of Ro 31-8220 up to 0.3 μM enhanced the expression of chemokine mRNA, but above 1 μM inhibition was observed.

Reduced expression of the chemokine mRNA did not affect the integrity of the 18S and 28S rRNA or the expression of β-actin mRNA (not shown).

Discussion

The controversial role of PKC in IL-1α and TNFα signalling pathways may have arisen from the former assumption that PKC was a single enzyme which required Ca^{2+} and DAG for its activation; an effect which could be mimicked experimentally by the phorbol esters. However, it is now evident that phorbol esters activate only some isoforms of PKC and can have additional non-PKC-related effects (Wilkinson & Hallam, 1994). Although PMA can mimic many IL-1α and TNFα-

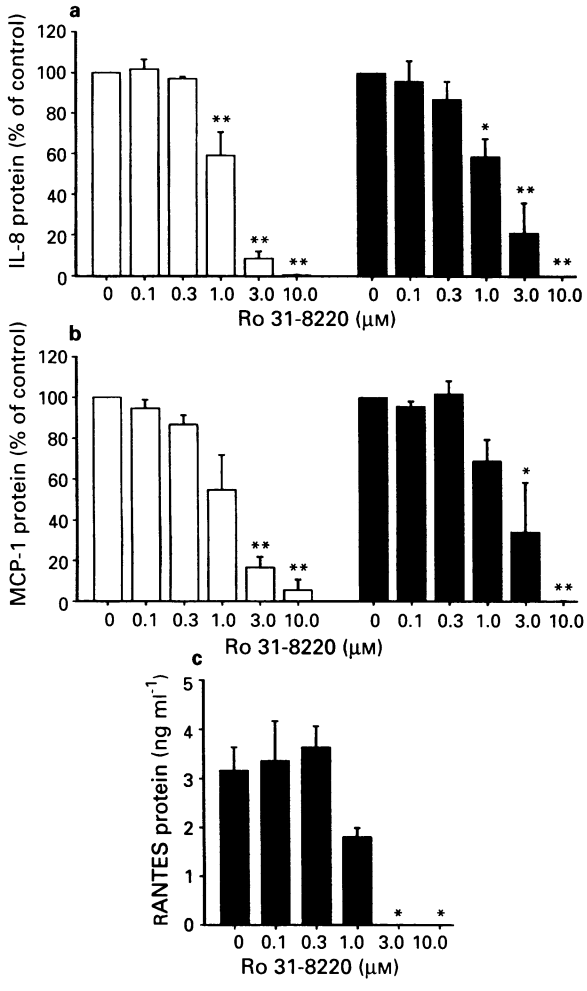


Figure 5 Effect of the PKC inhibitor Ro 31-8220 on TNFα and IL-1α-induced chemokine protein production. Cells were pretreated for 4 h with the indicated concentrations of Ro 31-8220. The incubation was continued for a further 20 h following the addition of 3 ng ml^{-1} IL-1α (open columns) or 30 ng ml^{-1} TNFα (solid columns). The culture supernatants were analysed by ELISA for IL-8 (a), MCP-1 (b) and RANTES protein (c). The results shown are means ± s.e.mean of at least 3 experiments. IL-8 and MCP-1 protein has been expressed as a percentage of the IL-1α control. IL-1α induced 51 ± 6 ng ml^{-1} IL-8 and 18 ± 4 ng ml^{-1} MCP-1. TNFα induced 28 ± 3 ng ml^{-1} IL-8 and 19 ± 4 ng ml^{-1} MCP-1. IL-1α induced RANTES was < 1 ng ml^{-1} (not shown). *P<0.05; **P<0.01 compared to IL-1α or TNFα controls.

induced responses including the accumulation of MCP-1 mRNA in synovial fibroblasts (Villiger *et al.*, 1992) and IL-8 in skin fibroblasts (Zhang *et al.*, 1992), it does so with a different time course and magnitude to their induction by cytokines, suggesting that the activation of a phorbol ester-sensitive isoform of PKC is not the only signalling pathway activated by IL-1α or TNFα. This is also supported by our finding that PMA-stimulated synovial fibroblasts produced much less IL-8 than fibroblasts stimulated with IL-1α and that co-stimulation with these 2 stimuli synergistically enhanced IL-8 secretion.

IL-1α binding to its receptor does not activate the classical inositol phosphate and calcium pathway normally associated with DAG production and PKC activation. This too has been taken as evidence that cytokine signalling does not involve PKC. However DAG can be generated in the absence of Ca^{2+} by treatment of fibroblasts with IL-1α. This DAG appears to be produced from an alternative pathway involving the activation of a phosphatidyl choline-specific phospholipase C (Rosoff *et al.*, 1988). It has been shown that DAG from this source, although unable to activate PKCα in fibroblasts (Ha & Exton, 1993), can activate a recently identified isoform of PKC, designated PKC epsilon (Dekker & Parker, 1994). Fibroblasts also express the isoform PKC zeta (Ha & Exton, 1993), an isoform which is not activated by phorbol esters (Ways *et al.*, 1992). Studies with dominant negative mutants have shown that the induction of NFkB by TNFα in fibroblasts requires PKC zeta (Diaz-Meco *et al.*, 1993). Since NFkB is a transcription factor important in IL-8 (Muikada *et al.*, 1990) and MCP-1 (Ueda *et al.*, 1994) gene expression, the PKC zeta isoform may also be involved in IL-1α/TNFα-induced chemokine expression.

We have used PKC inhibitors to investigate the role of PKC activation in IL-1α/TNFα-induced chemokine expression.

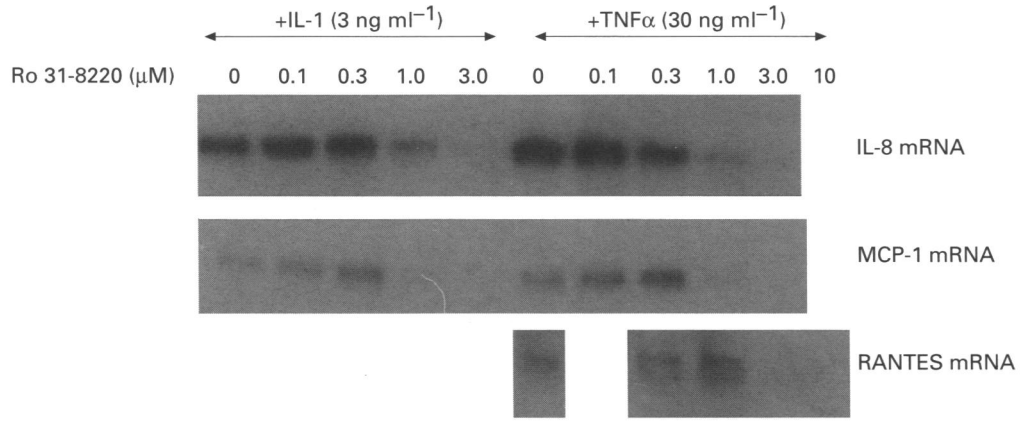
Our results show that while staurosporine and chelerythrine chloride enhance IL-1α-induced chemokine mRNA production, the bisindolylmaleimides Ro 31-8220 and GF 109203X have a biphasic effect. Lower concentrations cause an increase in chemokine mRNA expression and higher concentrations cause a significant inhibition in protein and mRNA expression.

These differences may relate to variations in the selectivity of these inhibitors. Staurosporine is known to be poorly selective for PKC. In addition to brain PKC, which is inhibited with an IC_{50} of 22 nM (Wilkinson *et al.*, 1993), staurosporine also inhibits protein kinase A (PKA), phosphorylase kinase and myosin light chain kinase, with IC_{50} values of 120 nM, 0.5 nM and 55 nM respectively (Bradshaw *et al.*, 1993). Both the bisindolylmaleimides GF 109203X and Ro 31-8220 are selective for PKC over PKA and phosphorylase kinase (Toullec *et al.*, 1991; Davies *et al.*, 1992). However, although the action of chelerythrine chloride and the bisindolylmaleimides is currently thought to be restricted to PKC isoforms, the relative newness of these compounds means that they have not been tested against a wide enough variety of protein kinases to ensure that they are absolutely specific for PKC. The possibility must be considered that presently unknown kinases may be the targets responsible for the results obtained in this study. For example, chelerythrine is currently claimed to be selective for PKC (Herbert *et al.*, 1990), but the nature of its inhibition, which is via the catalytic domain which is homologous with other protein kinases (Von Stebut *et al.*, 1994) may render it potentially less selective. Indeed, chelerythrine has been observed to have a paradoxical stimulatory effect on the phosphorylation of a 20 kDa protein (Lombardini, 1995).

Differential effects of the inhibitors against the different isoforms of PKC may also be the cause of some of the results that we have reported. Ro 31-8220 and GF 109203X are active against most of the PKC isoforms. Both are very potent inhibitors of purified cell free PKCα with IC_{50} values of 5 and 8 nM for Ro 31-8220 and GF 109203X respectively. The two compounds have similar activity against PKCβ with an IC_{50} of 24 and 18 nM. They differ in their ability to inhibit PKC epsilon which Ro 31-8220 inhibits with an IC_{50} of 24 nM and GF 109203X inhibits with an IC_{50} of 132 nM (Martiny-Baron *et al.*, 1993; Wilkinson *et al.*, 1993). Ro 31-8220 also appears to be much more active against fractions of pituitary that contain PKC zeta (IC_{50} 103–169 nM) (Ison *et al.*, 1993) when compared to GF 109203X which inhibits isolated PKC zeta with an IC_{50} of 5800 nM (Martiny-Baron *et al.*, 1993).

The enhanced chemokine production induced by staurosporine is probably a result of the poor selectivity of this inhibitor. Staurosporine has previously been shown to double the secretion of IL-8 protein in the U937 monocytic cell line (Pleass & Westwick, 1990) and enhance prostaglandin E_2 (PGE_2) production in synovial fibroblasts (Taylor *et al.*, 1990). This increased PGE_2 may then upregulate cyclic AMP with concomitant protein kinase A (PKA) activation. Since the IL-8 gene contains binding sites for the transcription factor AP-1, which can be activated by PKA, (Muikada *et al.*, 1990) this could be an indirect mechanism by which staurosporine increases IL-8 expression in synovial fibroblasts.

The significant inhibition of both IL-1α and TNFα-induced chemokine expression by the bisindolylmaleimide PKC inhibitors, GF 109203X and Ro 31-8220, strongly suggests that



	IL-1 (3 ng ml^{-1})					*TNF* (30 ng ml^{-1})					
Ro 31-8220 μM	0	0.1	0.3	1.0	3.0	0	0.1	0.3	1.0	3.0	10
IL-8 mRNA (densitometry units)	9.3	11.9	13.3	4.7	1.6	14.9	17.5	12.3	3.9	1.7	ND
MCP-1 mRNA (densitometry units)	1.5	2.2	4.0	1.0	0.5	3.3	5.4	6.9	2.0	1.5	ND
RANTES mRNA (densitometry units)	ND	ND	ND	ND	ND	4.3	ND	4.7	7.0	1.2	0.1

Figure 6 Effect of Ro 31-8220 on IL-1α and TNFα-induced chemokine mRNA expression. Synovial fibroblasts were pretreated for 1 h with Ro 31-8220 at concentrations between 0.1 – 10 μM as shown, then IL-1α (3 ng ml^{-1}) or TNFα (30 ng ml^{-1}) was added. Total cellular RNA was extracted after 6 h for IL-8 and MCP-1 and after 24 h for RANTES and mRNA was analysed by Northern blotting. The table shows the results obtained by densitometric analysis of the Northern blots. Corrections were made for any small differences in loading as determined from densitometric analysis of the ethidium bromide stained 18S and 28S rRNA (not shown). The results shown are from representative experiments. ND = not determined.

activation of an isoform of PKC does play an important role in chemokine regulation. It is possible that different agonists may preferentially activate only one specific subtype of PKC, which in turn regulates the expression of specific genes. Since the bisindolylmaleimides only inhibited chemokine expression when used at relatively high concentrations, the PKC isoforms inhibited less potently by these compounds may have been those that played an important role in inducing chemokine gene expression in response to IL-1α and TNFα. It is equally possible that gene expression could be negatively regulated by a different isoform of PKC. If this were so it would provide an explanation for the observed stimulation of IL-8 mRNA accumulation which could be induced by low concentrations of Ro 31-8220, GF 109203X and staurosporine. Since these inhibitors are all most potent against PKCα and PKCβ, these isoforms may actually function as inhibitors in IL-1α and TNFα-induced chemokine expression.

Our results have shown that Ro 31-8220 is at least 10 fold more potent than GF 109203X in inhibiting IL-8 expression in synovial fibroblasts. Since it has been shown that the PKC isoforms epsilon and zeta, both of which are present in fibroblasts, are inhibited more potently by Ro 31-8220 than GF 109203X, we propose that one or both of these PKC isoforms may be important in IL-1α-induced chemokine expression.

Recently the potential of specific PKC inhibitors to be used therapeutically as selective immunomodulators has become apparent (Bit *et al.*, 1993; Bradshaw *et al.*, 1993). Conditions which may benefit from treatment with PKC inhibitors include the autoimmune diseases rheumatoid arthritis, multiple sclerosis and diabetes mellitus (Bradshaw *et al.*, 1993). A bisindolylmaleimide compound closely related to Ro 31-8220 has recently been shown to inhibit selectively the secondary T-cell-mediated response in developing adjuvant arthritis in rats (Bit *et al.*, 1993).

Our results support a signalling pathway in which an isoform of PKC, possibly PKC epsilon or zeta, which is inhibited by the bisindolylmaleimide compounds, plays an important role in activating chemokine gene expression in response to IL-1α and TNFα. We propose that one of the ways that bisindolylmaleimide PKC inhibitors may reduce inflammation is by reducing the expression of the chemokines MCP-1, IL-8 and RANTES which attract subsets of leukocytes responsible for initiating the augmenting inflammatory processes. We have found no significant difference in the potency of Ro 31-8220 against any of the chemokines investigated, and therefore suggest that this compound could be of use in any disease in which chemokine activity is implicated.

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