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Induction of neutrophil chemotactic factor production by staurosporine in rat peritoneal neutrophils

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1 Incubation of rat peritoneal neutrophils in medium containing various concentrations of staurosporine (6.4–64 nM) increased the neutrophil chemotactic activity in the conditioned medium in a time- and concentration-dependent manner.

2 Separation of the neutrophil chemotactic activity in the conditioned medium by isoelectric focusing revealed that staurosporine (64 nM) stimulated the production of basic (pH > 8) neutrophil chemotactic factors, while TPA (12-*O*-tetradecanoylphorbol 13-acetate, 49 nM) stimulated the production of both basic (pH > 8) and acidic (pH 5) neutrophil chemotactic factors.

3 Determination by immunoassay of cytokine-induced neutrophil chemoattractant (CINC)-1, -2_α, -2_β and -3 in the conditioned medium at 4 h revealed that staurosporine (64 nM) and TPA (49 nM) strongly stimulated the production of CINC-3 (staurosporine, 133.0 ± 3.8; TPA, 26.7 ± 1.0; control, 0.32 ± 0.01 ng ml^{-1}, means ± s.e.mean from four samples) compared to CINC-1 (staurosporine, 55.0 ± 1.2; TPA, 12.2 ± 0.3; control, 0.56 ± 0.01 ng ml^{-1}), and CINC-2_α (staurosporine, 1.09 ± 0.03; TPA, 0.90 ± 0.02; control, <0.10 ng ml^{-1}). CINC-2_β was below the detectable amount (<0.078 ng ml^{-1}).

4 The level of CINC-3 mRNA in the peritoneal neutrophils was determined by reverse transcription-polymerase chain reaction. Staurosporine (64 nM) and TPA (49 nM) enhanced the level of CINC-3 mRNA time-dependently, but had no effect on GAPDH mRNA levels.

5 Production of staurosporine-induced neutrophil chemotactic factor was inhibited by the protein kinase C inhibitors, H-7 (IC_{50}, 12.3 μM), calphostin C (IC_{50}, 0.77 μM) and Ro 31-8425 (24.3% inhibition at 10 μM), and by the tyrosine kinase inhibitor, genistein (IC_{50}, 68.5 μM). Production of TPA-induced neutrophil chemotactic factor was also inhibited by both inhibitors.

6 Both the staurosporine- and the TPA-induced increase in CINC-3 mRNA levels were suppressed by H-7 and genistein.

Keywords: Staurosporine; neutrophil chemotactic factor; CINC-3; MIP-2; protein kinase C; tyrosine kinase

Introduction

It has been found that a family of chemokines, consisting of interleukin (IL)-8 (Koch *et al*., 1991), epithelial neutrophil-activating peptide-78 (ENA-78) (Koch *et al*., 1994) and growth related gene product (gro) (Koch *et al*., 1995), exist in the synovial fluid of joints from patients with rheumatoid arthritis, suggesting that these chemokines serve as chemotactic factors to induce neutrophil infiltration into the synovial fluid. In rats, a chemokine equivalent to human IL-8 (Matsushima *et al*., 1988) has yet to be identified. However, cytokine-induced neutrophil chemoattractant (CINC) (Watanabe *et al*., 1989) and macrophage inflammatory protein-2 (MIP-2) (Tekamp-Olson *et al*., 1990) have been described as rat homologues to human gro and both contribute to the neutrophil infiltration in glomerulonephritis (Wu *et al*., 1994; Feng *et al*., 1995). Recently, we found that leukocytes that had infiltrated into the inflammatory locus in an air pouch-type allergic inflammation model in rats, produced two types of neutrophil chemotactic factors, leukocyte-derived neutrophil chemotactic factor (LDNCF)-1 and LDNCF-2 (Tanabe *et al*., 1994), and the latter has been identified as MIP-2 (Tanabe *et al*., 1995).

Although the biological activities of such chemokines are fully analysed, the regulatory mechanism for their production remains to be elucidated. In general, chemokines are not stored in the cells, but are *de novo* synthesized by various stimuli such as lipopolysaccharide, tumour necrosis factor α, IL-1β and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Matsushima *et al*., 1988; Mukaida *et al*., 1989). The activation of nuclear factor-κB (NF-κB), which is indispensable for the induction of several chemokines (Kunsch & Rosen, 1993; Ohmori *et al*., 1995) including MIP-2 (Widmer *et al*., 1993), requires activation of certain protein kinases (Ishikawa *et al*., 1995). Previously, we found that the production of MIP-2 (CINC-3) by leukocytes that had been infiltrated into the pouch fluid in the air pouch-type allergic inflammation model in rats was inhibited by the protein kinase C (PKC) inhibitor H-7, and by the tyrosine kinase inhibitor, genistein, but not by the adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-dependent protein kinase inhibitor, H-89 (Tanabe *et al*., 1994), suggesting that the activation of PKC and tyrosine kinase is a prerequisite for the production of MIP-2 (CINC-3). During the course of the study to clarify further the mechanism of chemokine production, we found that staurosporine, known to be a powerful inhibitor of PKC (Tamaoki *et al*., 1986), stimulates chemokine production. We describe here the pharmacological analysis of the mechanism of action of staurosporine on chemokine production in rat peritoneal neutrophils.

Methods

Preparation of rat peritoneal neutrophils

Male Sprague-Dawley strain rats, specific pathogen-free, and weighing 350–450 g (Charles River Japan, Kanagawa, Japan)

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were used. The rats were treated in accordance with procedures approved by the Animal Ethics Committee of the Faculty of Pharmaceutical Sciences, Tohoku University, Japan. Rat peritoneal neutrophils were harvested 16 h after injection of 30 ml of Ca^{2+}-free Krebs-Ringer solution containing 1% casein (casein from milk, vitamin-free, Wako Pure Chemical Ind., Osaka, Japan) which had been sterilized by autoclaving at 120°C for 15 min. The peritoneal cells were washed twice with Ca^{2+}-free phosphate-buffered saline (PBS, pH 7.4) and finally suspended in RPMI 1640 medium (Nissui Seiyaku Co., Tokyo, Japan) containing 0.25% (w/v) bovine serum albumin (BSA, essentially fatty acid-free, Sigma Chemical Co., St. Louis, MO, U.S.A.) at 1×10^7 cells ml^{-1}.

Cell culture

Two milliliters of the cell suspension (2×10^7 cells) was incubated at 37°C for various periods of time in the presence or absence of drugs. After appropriate periods of incubation, the cell suspension was centrifuged at 1500 *g* and 4°C for 5 min. The supernatant fraction obtained was diluted ten fold with RPMI-1640 medium containing 0.25% (w/v) bovine serum albumin (BSA), and used for the measurement of neutrophil chemotactic activity.

Measurement of neutrophil chemotactic activity

Neutrophil chemotactic activity in the diluted supernatant fraction of the conditioned medium was assayed by use of modified Boyden chambers as described previously (Tanabe *et al*., 1994).

Isoelectric focusing of neutrophil chemotactic factor in the conditioned medium

Peritoneal neutrophils (5×10^8 cells) were incubated for 4 h at 37°C in RPMI 1640 medium containing 0.25% (w/v) BSA at a concentration of 1×10^7 cells ml^{-1} in the presence or absence of drugs. After incubation, the cell suspension was centrifuged at 1,500 *g* and 4°C for 5 min. The supernatant fraction (49 ml) was dialyzed against 10 mM NaCl, mixed with Bio-Lyte ampholytes (pH range 3–10, Bio-Rad Lab., Richmond, CA, U.S.A.) at a concentration of 2% (v/v), and the mixture was loaded into the focusing chamber of a Rotfor cell (Bio-Rad Lab.). Isoelectric focusing was carried out at 12 W constant power for 3 h at 4°C. Twenty fractions were harvested and their pH values were determined. Each fraction (2 ml) was dialyzed against 1 M NaCl to remove ampholytes, and further dialyzed against PBS at 4°C. The neutrophil chemotactic activity in each fraction was then determined.

Measurement of CINC concentrations in the conditioned medium

The concentrations of CINC-1, -2α, -2β and -3 in the conditioned medium were measured by ELISA kits for each type of CINC (Immuno-Biological Laboratories Co., Tokyo, Japan), following the manufacturer's instructions. In brief, the assay of plates precoated with capture antibody (rabbit anti-CINC, C terminus-specific), and a detection antibody (rabbit anti-CINC, IgG Fab conjugated to horseradish peroxidase, N terminus-specific). The ELISA was developed colorimetrically with H_2O_2 and *o*-phenylenediamine, and read by comparison with CINC standards. There was no cross-reactivity between antibody and protein, other than cognate antigen.

Semiquantitation of CINC-3 mRNA by reverse transcription polymerase chain reaction

Peritoneal neutrophils (8×10^7 cells) were incubated for various periods of time at 37°C in RPMI 1640 medium containing 0.25% (w/v) BSA at a concentration of 1×10^7 cells ml^{-1} in the presence or absence of drugs. After incubation, the cells were collected by centrifugation at 350 *g* and 4°C for 5 min. Total RNA was prepared from each sample by acid guanidinium-phenol-chloroform extraction, and the yield of RNA extracted was determined by spectrophotometry. One microgram of RNA from each sample was reverse transcribed at 37°C for 1 h in 20 μl of the buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl and 3 mM $MgCl_2$) containing 5 μM of random hexamer oligonucleotides (Gibco BRL, Gaithersburg, MD, U.S.A.), 200 u of the reverse transcriptase from moloney murine leukaemia virus (Gibco BRL), 0.5 mM deoxyribonucleoside triphosphates (dNTP, Pharmacia Biotechn Uppsala, Sweden) and 10 mM dithiothreitol. Polymerase chain reaction (PCR) primers for CINC-3 were designed (Tanabe *et al*., 1995) from rat MIP-2 cDNA sequence obtained from EMBL/Genbank/DDBJ databases. The sequences of primers used were: (former) 5′-GCCTAGCGCCATGGCCCCTCCCACT-3′ and (reverse) 5′-GGCACATCAGGTACGATCCAGGCTT-3′, which amplify a 413 base pair (bp) CINC-3 fragment. PCR was performed for 16 cycles in 50 μl of the PCR buffer (2.5 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$) containing 5 μM of the reverse transcribed RNA solution, 0.25 μM of each primer, 170 μM dNTP and 1.25 u Taq polymerase (Takara Shuzo Co., Shiga, Japan) with a thermal cycler (GeneAmp PCR System 2400, Perkin Elmer Cetus, Norwalk, CT, U.S.A.). Each cycle consisted of 30 s denaturation at 94°C, 1 min annealing at 55°C and 1 min extension at 70°C.

The rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (a housekeeping gene) was used as an internal standard gene. As the internal standard gene was also amplified, relative levels of CINC-3 mRNA were quantified. PCR primers for rat GAPDH were described by Robbins and McKinney (1992); primers used were (former) 5′-TGATGACATCAAGAAGGTGGTGAAG-3′ and (reverse) 5′-TCCTTGGAGGCCATGTAGGCC-3′, which amplify a 240 bp GAPDH fragment. PCR was performed for 18 cycles; 30 s denaturation at 94°C, 1 min annealing at 57°C and 1 min extension at 72°C. Other conditions were the same as for CINC-3. After the PCR performance, 10 μl of the PCR reaction mixture was loaded onto a 2% agarose minigel, and the PCR products were visualized by ethidium bromide staining after electrophoresis. The levels of mRNA for CINC-3 and GAPDH were quantified by scanning densitometry, and the ratio of the CINC-3 mRNA density versus the GAPDH mRNA density in each point was calculated.

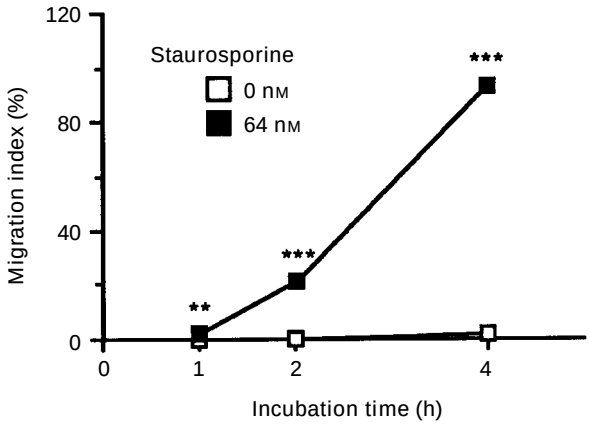


Figure 1 Time course of the effect of staurosporine on chemotactic factor production by rat peritoneal neutrophils. Peritoneal neutrophils (2×10^7 cells) were incubated for the indicated periods at 37°C in 2 ml of medium in the presence and absence of staurosporine (64 nM). Neutrophil chemotactic activity in the conditioned medium was determined after 10 fold dilution. Values are the means from four samples. The s.e.mean were too small to depict, i.e. they lie within the symbols. Statistical significance: $^{**}P<0.01$, $^{***}P<0.001$ vs corresponding control.

Drug treatment

Drugs used were the PCK activator TPA (12-*O*-tetradecanoylphorbol 13-acetate (Nishizuka 1992)) (Sigma Chemical Co.), the protein synthesis inhibitor cycloheximide (Wako Pure Chemical Ind.), the nonselective protein kinase C inhibitor staurosporine (Tamaoki *et al*., 1986) (Kyowa Medex Co., Tokyo, Japan), the more selective protein kinase C inhibitor H-7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochlo- ride) (Hidaka *et al*., 1991) (Seikagaku Kogyo, Tokyo, Japan), the selective protein kinase C inhibitors calphostin C (2 - [12-[2- (benzyloxy)-propyl]-3,10- dihydro-4,9- dihydroxy-2,6, 7,11- tetramethoxy-3,10 - dioxo -1-perylenyl]-1- methylethylcarbonic acid 4-hydroxyphenyl ester) (Kobayashi *et al*., 1989) (BIOMOL Res. Lab. Inc., Plymouth Meeting, PA, U.S.A.), and Ro 31-8425 (3-[8-(aminomethyl)-6,7,8,9-tetrahydropyridol [1,2-a]-indol-10-yl]-4-(1-methyl-3-indolyl)-1H-pyrrole-2,5-dione hydrochloride) (Wilkinson *et al*., 1993) (a gift from Dr Kohji Yamada at Eisai Co., Tsukuba, Japan), and the tyrosine kinase inhibitor genistein (Tremblay *et al*., 1992) (Wako Pure Chemical Ind.). H-7 was dissolved in water, and the rest were dissolved in dimethysulphoxide (DMSO). An aliquot of each solution was added to medium, and the final concentration of the vehicle in the medium was adjusted to 0.1% (v/v). The control medium contained the same amount of the vehicle. After treatment with drugs, the viability of the neutrophils was examined by trypan blue exclusion test; no cytotoxic effect was observed.

Statistical analysis

Results were analysed for statistical significance by Dunnett's test for multiple comparisons and Student's *t* test for unpaired observations.

Results

Induction of neutrophil chemotactic factor production by staurosporine

Incubation of rat peritoneal neutrophils in RPMI 1640 medium containing 64 nM staurosporine increased the neutrophil chemotactic activity in the conditioned medium in a time-

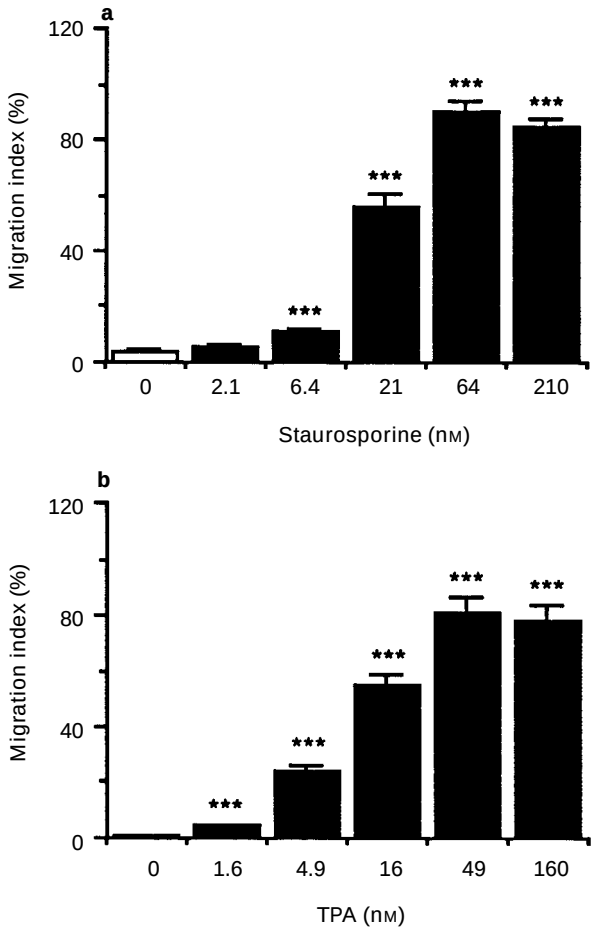


Figure 2 Effects of various concentrations of staurosporine and TPA on chemotactic factor production by rat peritoneal neutrophils. Peritoneal neutrophils (2×10^7 cells) were incubated for 4 h at 37°C in 2 ml of medium containing the indicated concentrations of staurosporine (a) or TPA (b). Neutrophil chemotactic activity in the conditioned medium was determined after 10 fold dilution. Values are the means from four samples with s.e.mean shown by vertical lines. Statistical signficiance: $***P<0.001$ vs corresponding control.

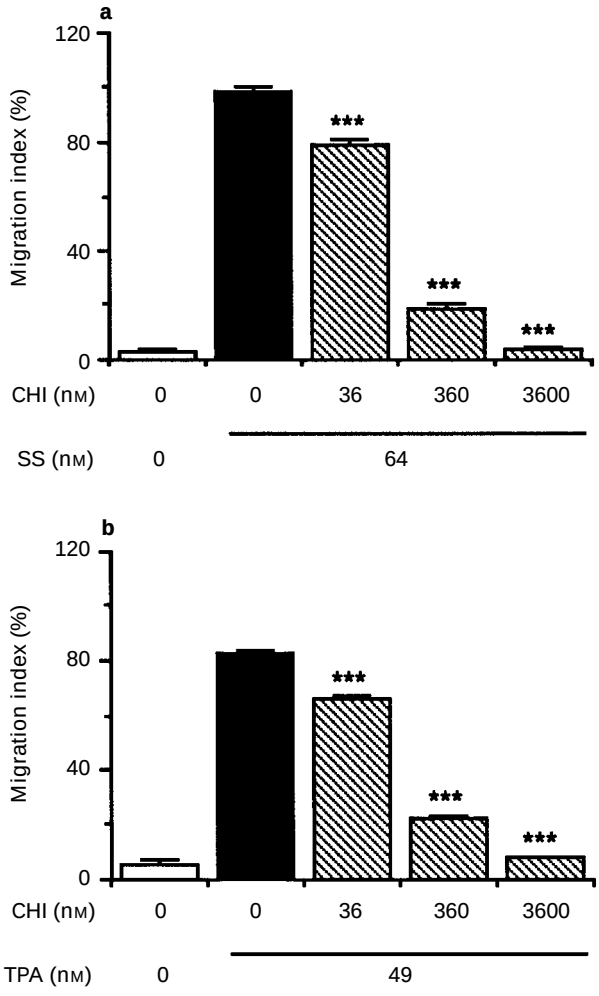


Figure 3 Effects of cycloheximide on staurosporine and TPA-induced chemotactic factor production by neutrophils. Peritoneal neutrophils (2×10^7 cells) were incubated for 4 h at 37°C in 2 ml of medium containing the indicated concentrations of cycloheximide (CHI) in the presence and absence of staurosporine (SS) (a) or TPA (b). Neutrophil chemotactic activity in the conditioned medium was determined after 10 fold dilution. Values are the means from four samples with s.e.mean shown by vertical lines. Statistical significance: $***P<0.001$ vs corresponding control.

dependent manner (Figure 1). When determined at 4 h, the neutrophil chemotactic activity in the conditioned medium was increased by staurosporine in a concentration-dependent manner (Figure 2a). Incubation with TPA also induced the production of neutrophil chemotactic factor in a concentration-dependent manner when determined at 4 h (Figure 2b). Staurosporine and TPA at such concentrations showed no direct effect on neutrophil chemotaxis (data not shown).

The increase in the production of neutrophil chemotactic factor induced by staurosporine (64 nM) or TPA (49 nM) was inhibited by cycloheximide (36–3600 μM) (Figure 3), but not by the arachidonate 5-lipoxygenase inhibitor AA861, at concentrations up to 30 μM (data not shown). These findings suggest that staurosporine and TPA induce the production of proteinaceous neutrophil chemotactic factor(s).

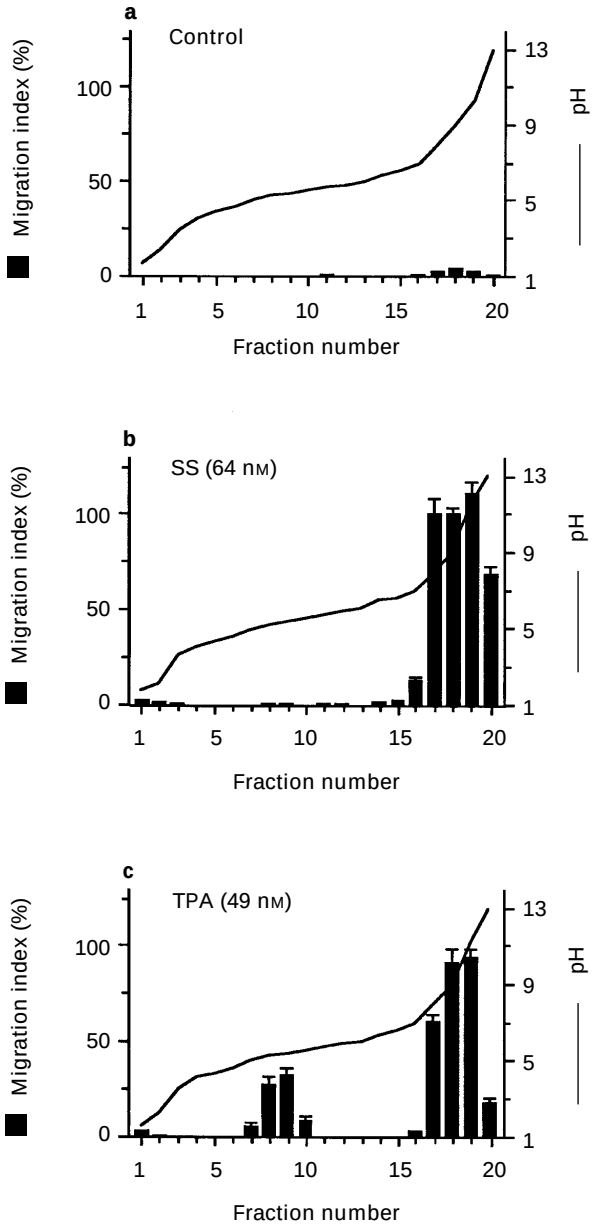


Figure 4 Separation profiles obtained by isoelectric focusing of neutrophil chemotactic activity in the conditioned medium. Peritoneal neutrophils (5×10^8 cells) were incubated for 4 h at 37°C in 50 ml of medium alone (a, control), medium containing staurosporine (SS, 64 nM) (b), or TPA (49 nM) (c). After focusing proteins in the conditioned medium, neutrophil chemotactic activity in each fraction was determined after 10 fold dilution. Values are the means from four assays with s.e.mean shown by vertical lines. Representative profiles of three separate experiments are shown.

By isoelectric focusing, neutrophil chemotactic activity in the conditioned medium of the staurosporine-treated neutrophils was recovered in the basic (pH > 8) fractions, whereas that of the TPA-treated neutrophils was recovered in both the acidic (pH 5) and the basic (pH > 8) fractions (Figure 4).

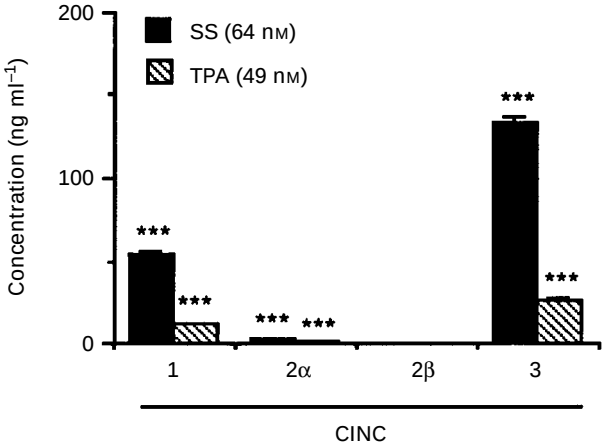


Figure 5 Concentrations of CINC-1, -2α, -2β and -3 in the conditioned medium. Peritoneal neutrophils (2×10^7 cells) were incubated for 4 h at 37°C in 2 ml of medium in the presence and absence of staurosporine (SS, 64 nM) or TPA (49 nM). Concentrations of each CINC were determined by ELISA. Values are the means from four samples with s.e.mean shown by vertical lines. CINC-2β concentrations in the conditioned medium of staurosporine- or TPA-treated neutrophils were below detectable levels (<0.078 ng ml^{-1}). In the absence of staurosporine or TPA, concentrations of each CINC in the conditioned medium were as follows: CINC-1, 0.56 ± 0.01; CINC-2α, <0.10; CINC-2β, <0.078; CINC-3, 0.32 ± 0.01 (ng ml^{-1}, means$\pm$s.e.mean from four samples). Statistical significance: $^{***}P<0.001$ vs corresponding control.

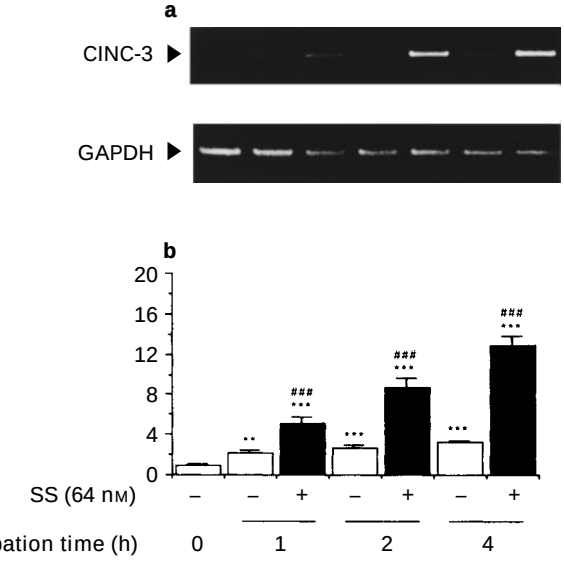


Figure 6 Levels of mRNA for CINC-3 and GAPDH in neutrophils. Peritoneal neutrophils (8×10^7 cells) were incubated for the indicated periods at 37°C in 8 ml of mediumin the presence (+) and absence (−) of staurosporine (SS, 64 nM). Total RNA was extracted and RT-PCR for CINC-3 mRNA and GAPDH mRNA was performed (a) as described in Methods. The ratio of CINC-3 mRNA density to GAPDH mRNA density is shown in (b). The ratio of the mean value of the 0 h control is exprsesed as 1.0. Histograms are the means$\pm$s.e.mean of three separate experiments. Statistical significance: $^{**}P<0.01$, $^{***}P<0.001$ vs the 0 h control. $^{\#\#\#}P<0.001$ vs corresponding SS-free control.

Determination of CINC-1, -2α, -2β and -3 in the conditioned medium

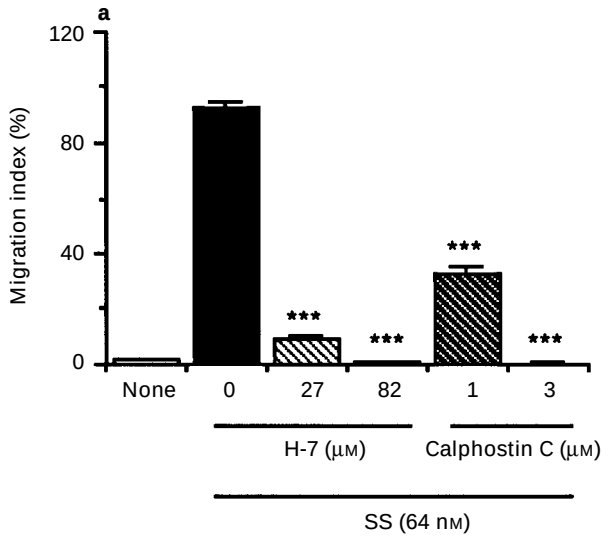
Rat basic chemokines have been shown to constitute a CINC familiy (Nakagawa *et al*., 1994). Thus, we determined the concentration of each CINC in the conditioned medium. At 4 h, the concentration of each CINC in the conditioned medium of the non-treated neutrophils was very low. Treatment with staurosporine (64 nM) or TPA (49 nM) enhanced the production of CINC-1 and CINC-3 (Figure 5). Production of CINC-2α also was stimulated by staurosporine (64 nM) or TPA (49 nM), but the level of CINC-2α was much lower than that of CINC-1 or CINC-3. However, the concentration of CINC-2β was below the detectable levels (<0.078 ng ml^{-1}) and did not increase by treatment with staurosporine or TPA.

Semiquantitation of CINC-3 mRNA levels in neutrophils

Among the four CINCs, on treatment with staurosporine or TPA, CINC-3 was most prominently produced (Figure 5). Therefore, CINC-3 mRNA levels in neutrophils were examined by RT-PCR. Treatment with staurosporine (64 nM) increased the levels of CINC-3 mRNA in a time-dependent manner, but had no effect on the mRNA levels of GAPDH, a housekeeping gene product (Figure 6a). The ratio of CINC-3 mRNA density to GAPDH mRNA density was increased with time in the presence of staurosporine (Figure 6b). Without staurosporine treatment, the ratio was only slightly increased with time.

Effects of protein kinase inhibitors on neutrophil chemotactic factor production induced by staurosporine

Incubation of the peritoneal neutrophils in medium containing staurosporine (64 nM) and various concentrations of the PKC



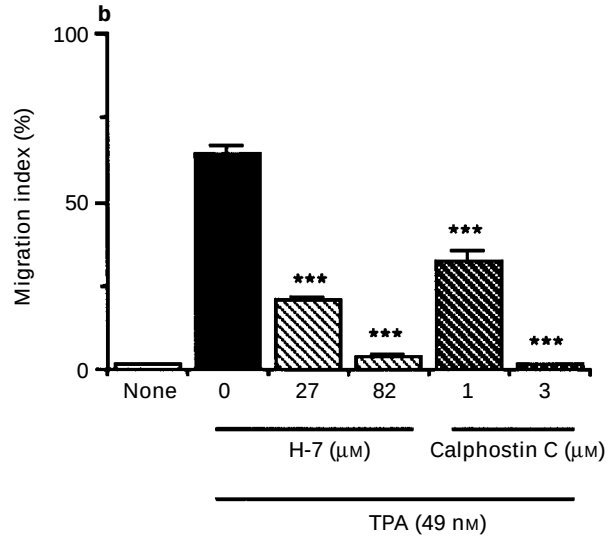
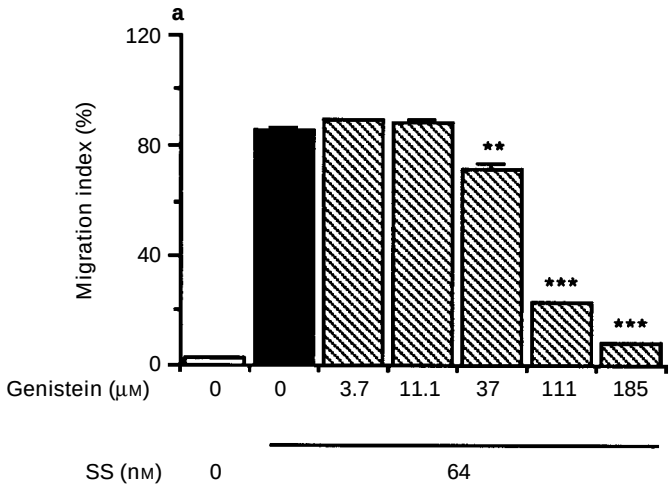


Figure 7 Effects of H-7 and calphostin C on staurosporine- and TPA-induced chemotactic factor production by neutrophils. Peritoneal neutrophils (2×10^7 cells) were incubated for 4 h at 37°C in 2 ml of medium containing the indicated concentrations of H-7 or calphostin C in the presence and absence of staurosporine (SS, 64 nM) (a), or TPA (49 nM) (b). Neutrophil chemotactic activity in the conditioned medium was determined after 10 fold dilution. Values are the means from four samples with s.e.mean shown by vertical lines. Statistical significance: $***P<0.001$ vs corresponding control. The results were confirmed in three independent experiments.



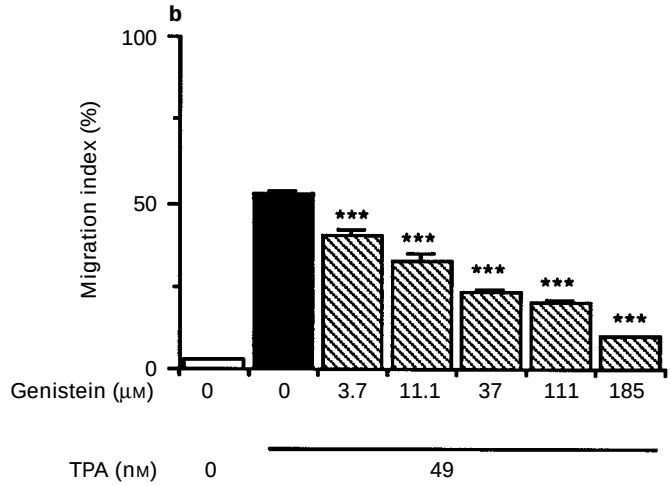


Figure 8 Effects of genistein on staurosporine- and TPA-induced chemotactic factor production by neutrophils. Peritoneal neutrophils (2×10^7 cells) were incubated for 4 h at 37°C in 2 ml of medium containing the indicated concentrations of genistein in the presence and absence of staurosporine (SS, 64 nM) (a), or TPA (49 nM) (b). Neutrophil chemotactic activity in the conditioned medium was determined after 10 fold dilution. Values are the means from 4 samples with s.e.mean shown by vertical lines. Statistical significance: $**P<0.01$, $***P<0.001$ vs corresponding control. The results were confirmed in three independent experiments.

inhibitors, H-7 or calphostin C, inhibited the production of staurosporine-induced neutrophil chemotactic factor at 4 h in a concentration-dependent manner (Figure 7a). Production of TPA-induced neutrophil chemotactic factor was also suppressed by H-7 or calphostin C (Figure 7b). Another PKC inhibitor, Ro 31-8425 at 10 μM inhibited the production of both staurosporine- and TPA-induced neutrophil chemotactic factor (migration index (%): staurosporine (64 nM), 90.2 ± 1.4; staurosporine (64 nM) plus Ro 31-8425 (10 μM), 68.3 ± 1.8, $P<0.001$; TPA (49 nM), 64.2 ± 2.4; TPA (49 nM) plus Ro 31-8425 (10 μM), 44.4 ± 1.1, $P<0.001$. means $\pm$ s.e.mean from four samples).

Incubation with the tyrosine kinase inhibitor genistein suppressed the production of staurosporine-induced neutrophil chemotactic factor (Figure 8a) and TPA-induced neutrophil chemotactic factor in a concentration-dependent manner (Figure 8b).

Effects of protein kinase inhibitors on CINC-3 mRNA levels in staurosporine- and TPA-stimulated neutrophils

Incubation of neutrophils in medium containing staurosporine (64 nM) induced the accumulation of CINC-3 mRNA at 2 h. The protein kinase C inhibitor, H-7 and the tyrosine kinase inhibitor, genistein decreased the level of CINC-3 mRNA, but had no effect on the level of GAPDH mRNA (Figure 9a). Treatment with H-7 and genistein reduced the density ratio (CINC-3 mRNA/GAPDH mRNA), as shown in Figure 9b. The TPA-induced accumulation of CINC-3 mRNA at 2 h was suppressed by H-7 and genistein in a concentration-dependent manner. Levels of GAPDH mRNA were not affected (Figure 10).

Discussion

Staurosporine, regarded as a potent nonselective inhibitor of PKC (Tamaoki *et al*., 1986), inhibits PKC at nanomolar doses *in vitro* by interacting with its catalytic domain (Nakadate *et al*., 1988; Gross *et al*., 1990). However, results of the present study suggest that staurosporine acts as a PKC agonist. Namely, staurosporine enhanced the production of neutrophil chemotactic factors in rat peritoneal neutrophils as did TPA. The PKC inhibitor H-7 suppressed the production of both staurosporine-induced and TPA-induced neutrophil chemotactic factor. However, H-7 is a poorly selective PKC inhibitor (Wilkinson & Hallam, 1994). Therefore, we examined the effects of the more specific PKC inhibitors calphostin C and Ro 31-8425, and found that they also suppressed the production of staurosporine-induced and TPA-induced neutrophil chemotactic factor. In addition, it has been shown that both staurosporine and TPA elicit changes in cell morphology, elevation of transglutaminase activity, induction of ornithine decarboxylase activity, and induction of the formation of cornified envelopes in mouse cultured keratinocytes (Sako *et al*., 1988; Dulgosz & Yuspa, 1991). Furthermore, like TPA, staurosporine at low concentrations stimulates prostaglandin E_2 production in rat peritoneal macrophages (Watanabe *et al*., 1990) and in human synovial fibroblasts (Taylor *et al*., 1990). Thus, staurosporine appears to function primarily as a PKC

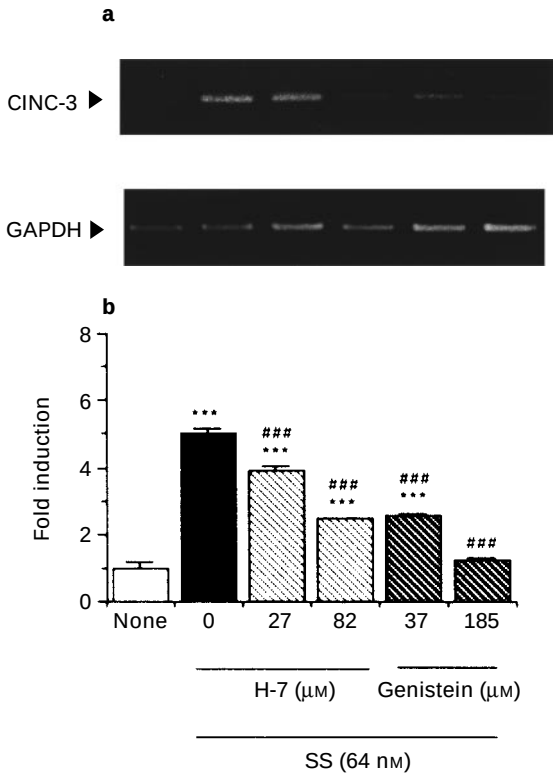


Figure 9 Effects of protein kinase inhibitors on staurosporine-induced CINC-3 mRNA accumulation in neutrophils. Peritoneal neutrophils (8×10^7 cells) were incubated for 2 h at 37°C in 8 ml of medium containing the indicated concentrations of each drug. Total RNA was extracted and RT-PCR for CINC-3 mRNA and GAPDH mRNA was performed (a) as described in Methods. The ratio of CINC-3 mRNA density to GAPDH mRNA density is shown in (b). The ratio of the mean value of the non-treated group (None) is expressed as 1.0. Histograms are the means $\pm$ s.e.mean of three separate experiments. Statistical signficiance: $^{***}P<0.001$ vs None. $^{###}P<0.001$ vs SS control.

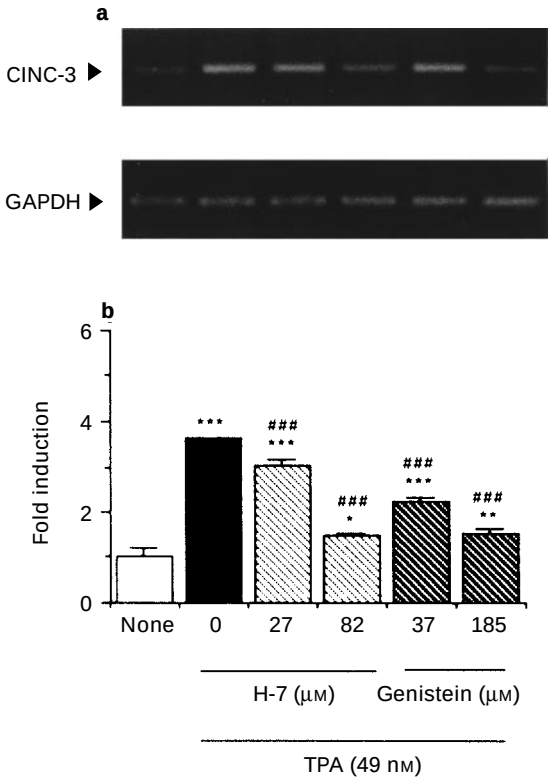


Figure 10 Effects of protein kinase inhibitors on TPA-induced CINC-3 mRNA accumulation in neutrophils. Peritoneal neutrophils (2×10^7 cells) were incubated for 2 h at 37°C in 8 ml of medium containing the indicated concentrations of each drug. Total RNA was extracted and RT-PCR for CINC-3 mRNA and GAPDH mRNA was performed (a) as described in Methods. The ratio of CINC-3 mRNA density to GAPDH mRNA density is shown in (b). The ratio of the mean value of the non-treated group (None) is expressed as 1.0. Histograms are the means $\pm$ s.e.mean of three separate experiments. Statistical significance: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs None. $^{###}P<0.001$ vs TPA control.

agonist, although it has been found that staurosporine inhibits several classes of protein kinases with IC_{50}s ranging from 3 to 61 nM (O'Brian & Ward, 1990). In rat peritoneal neutrophils, differences in the effects of staurosporine and TPA were observed in the separation profile of the neutrophil chemotactic activity in the conditioned medium by isoelectric focusing (Figure 4). Compared to staurosporine, TPA strongly induced the production of the acidic (pI 5) chemoattractant, probably MIP-1α. Studies are currently under way to determine whether altered expression or function of PKC isozymes could account for the different responsiveness of rat peritoneal neutrophils to staurosporine and TPA.

Alonso *et al*. (1996) showed that CINC-1 production in rat peritoneal macrophages induced by immune complexes is not dependent on PKC activation, but rather involves protein tyrosine phosphorylation reactions. Therefore, stimulation by immune complexes might directly activate protein tyrosine kinases. In rat peritoneal neutrophils, staurosporine enhances the production of neutrophil chemotactic factor presumably by activating the protein tyrosine kinases through PKC activation, because the tyrosine kinase inhibitor genistein and the PKC inhibitors H-7, calphostin C and Ro 31-8425, all inhibited the neutrophil chemotactic factor production. Recently, Jordan *et al*. (1996) showed that staurosporine enhances IL-8 production in IL-1α- or TNFα-stimulated human synovial fibroblasts, but does not affect basal secretion of IL-8 in unstimulated cells. However, they did not examine the possibility that staurosporine-induced IL-8 production is inhibited by the PKC inhibitors. The present study demonstrated that staurosporine enhances the production of CINC-3 and CINC-1 in the absence of IL-1α or TNF-α in rat peritoneal neutrophils. However, it is possible that the rat peritoneal neutrophils we used had been stimulated by casein injected intraperitoneally.

The ability of staurosporine to mimic the action of TPA can be explained in two ways. Firstly, if staurosporine inhibits diacylglycerol kinase, levels of the endogenous PKC activator diacylglycerol increase, thus staurosporine mimics the response of TPA. Secondly, as shown by Wolf & Baggiolini (1988), staurosporine directly activates PKC, viz staurosporine translocates a PKC isozyme not inhibited by staurosporine and this activated isozyme leads to chemokine production. It is also possible that staurosporine selectively inhibits an unknown PKC isozyme which negatively regulates chemokine production. The mechanism by which staurosporine induces production of neutrophil chemotactic factors associated with PKC activation or with selective PKC inhibition remains to be elucidated.

Staurosporine most affected CINC-3 production, which showed a marked increase, followed by CINC-1 and CINC-2α. CINC-2β levels were below the detectable amount. Similar results were obtained following treatment with TPA (Figure 5). It has been found that the neutrophil chemotactic activity of each CINC is similar, but CINC-3 is more potent than other CINCs in increasing intracellular $[Ca^{2+}]$ (Shibata *et al*., 1995). In the air pouch-type allergic inflammation model in rats, we demonstrated that CINC-3 (rat MIP-2) plays a much more significant role in neutrophil infiltration than CINC-1 (Tanabe *et al*., 1995). The present study also demonstrated that CINC-3 is the major CINC produced by rat peritoneal neutrophils in response to staurosporine or TPA. Therefore, among the CINCs, it seems likely that CINC-3 is the most important chemoattractant in rats.

In conclusion, staurosporine enhances the production of neutrophil chemotactic factors in rat peritoneal neutrophils. This potency is shared with TPA, an activator of PKC. Therefore, it must be stressed that careful interpretation is necessary when staurosporine is used as a PKC inhibitor. Finally, in rat peritoneal neutrophils, CINC-3 (rat MIP-2) is dominantly produced by treatment with staurosporine or TPA.

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