British Journal of Pharmacology (1999) 127, 1309–1316
http://www.stockton-press.co.uk/bjp

Participation of protein kinases in staurosporine-induced interleukin-6 production by rat peritoneal macrophages

[1]Kouya Yamaki & *,[1]Kazuo Ohuchi

[1]Department of Pathophysiological Biochemistry, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba Aramaki, Aoba-ku, Sendai, Miyagi 980-8578, Japan

1 The incubation of rat peritoneal macrophages in the presence of staurosporine, a non-specific protein kinase inhibitor, induced interleukin-6 (IL-6) production in a time- and concentration-dependent manner at 6.3–63 nM, but at 210 nM, the stimulant effect on IL-6 production was reduced.

2 The levels of IL-6 mRNA as determined by a reverse transcription-polymerase chain reaction were also increased by staurosporine in parallel with the ability to induce IL-6 production.

3 Compounds structurally related to staurosporine including K-252a (non-specific protein kinase inhibitor) and KT-5720 (inhibitor of cyclic AMP-dependent protein kinase, PKA), did not increase IL-6 production by peritoneal macrophages.

4 Staurosporine-induced increases in IL-6 production and expression of IL-6 mRNA were decreased by the PKC inhibitors, H-7 (2.7–27 μM), Ro 31-8425 (1–10 μM) and calphostin C (0.3–3 μM) and by the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor LY294002 (30–100 μM), but were further increased by the protein tyrosine kinase (PTK) inhibitor, genistein (12–37 μM).

5 The staurosporine-induced increase in IL-6 production was not affected by the PKA inhibitor, H-89 (0.1–3 μM).

6 These findings suggest that the induction of IL-6 production by staurosporine is secondary to elevation of IL-6 mRNA level, which, in turn, is positively regulated by the activation of PKC and PI 3-kinase and negatively regulated by the activation of PTK. PKA does not appear to play a significant role.

Keywords: Interleukin-6; staurosporine; protein kinase C; phosphatidylinositol 3-kinase; protein kinase A; protein tyrosine kinase; rat peritoneal macrophages

Abbreviations: DMSO, dimethylsulphoxide; ELISA, enzyme-linked immunosorbent assay; EMEM, Eagle's minimum essential medium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-1, interleukin-1; IL-6, interleukin-6; MIP-2, macrophage inflammatory protein-2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PCR, polymerase chain reaction; PI 3-kinase, phosphatidylinositol 3-kinase; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; PLD, phospholipase D; PTK protein tyrosine kinase; RT, reverse transcription; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

Introduction

Protein kinase C (PKC) is involved in a variety of cellular functions (Nishizuka, 1986). For example, at inflammatory sites, macrophages produce several kinds of inflammatory cytokines, such as interleukin-1 (IL-1), IL-6 and tumour necrosis factor-α (Retzlaff *et al*., 1996; Bost & Mason, 1995; Yamada *et al*., 1998), *via* the activation of PKC (Retzlaff *et al*., 1996; Gross *et al*., 1993; Picot *et al*., 1994).

In an attempt to clarify roles of PKC in various cellular functions, the effects of the PKC activators 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and phorbol 12, 13-dibutyrate, as well as PKC inhibitors such as H-7 (Hidaka *et al*., 1984), calphostin C (Kobayashi *et al*., 1989) and staurosporine (Tamaoki *et al*., 1986) have all been examined. Among the PKC inhibitors, staurosporine seems to exert biological activities which are unrelated to inhibition of PKC. For example, staurosporine induces neurite outgrowth on PC12 cells (Hashimoto & Hagino, 1989), activates phospholipase D (PLD) in human polymorphonuclear leucocytes (Périanin *et al*., 1993) and increases production of prostaglandin E_2 (PGE_2) in rat peritoneal macrophages (Watanabe *et al*., 1990) and macrophage inflammatory protein-2 (MIP-2) in rat peritoneal neutrophils (Edamatsu *et al*., 1997).

Phosphatidylinositol 3-kinase (PI 3-kinase) is another important kinase for the expression of cellular functions. It is activated by growth factors such as platelet-derived growth factor (Jackson *et al*., 1992) and nerve growth factor (Kimura *et al*., 1994) and produces phosphatidylinositol (3,4,5)-triphosphate which activates several types of PKC (Toker *et al*., 1994; Herrera-Velit *et al*., 1997) and other kinases such as Rac (Hawkins *et al*., 1995). The activation of PI 3-kinase causes PC12 cell growth (Kimura *et al*., 1994) and activates PLD in human neutrophils (Reinhold *et al*., 1990). Interestingly, both of these effects are also observed with staurosporine (Hashimoto & Hagino, 1989; Périanin *et al*., 1993).

Although mechanisms for activation of these kinases have been studied (Nakanishi *et al*., 1995; Newton, 1995), the role(s) of these kinases in the production of cytokines remain to be elucidated. Previously, we reported (Edamatsu *et al*., 1997) that staurosporine induces production of MIP-2 in rat peritoneal neutrophils which effect is inhibited by the PKC inhibitors, H-7 and calphostin C and by the protein tyrosine kinase (PTK) inhibitor, genistein. Recently, we found that staurosporine also induces IL-6 production in rat peritoneal macrophages. In the present study, we analysed pharmacolo-

* Author for correspondence.

gically the possible participation of protein kinases in staurosporine-induced IL-6 production by rat peritoneal macrophages.

Methods

Preparation of rat peritoneal macrophages

A solution containing soluble starch (Wako Pure Chemicals, Osaka, Japan) and bacto peptone (Difco Laboratories, Detroit, MI, U.S.A.) (5% each) was injected i.p. into male Sprague-Dawley rats (400–600 g, specific pathogen-free, Charles River Japan, Kanagawa, Japan) at a dose of 5 ml per 100 g body weight. Four days after the injection, the rats were sacrificed and peritoneal cells were harvested according to the procedure described by Ohuchi *et al*. (1985). The rats were treated in accordance with procedures approved by the Animal Ethics Committee of the Faculty of Pharmaceutical Sciences, Tohoku University, Japan.

Macrophage culture

The peritoneal cells were suspended in Eagle's minimum essential medium (EMEM) (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% (v v^{-1}) calf serum (Dainippon Pharmaceutical, Osaka, Japan), penicillin G potassium (18 μg ml^{-1}) and streptomycin sulphate (50 μg ml^{-1}) (Meiji Seika, Tokyo, Japan). The peritoneal cells (1.5×10^6 cells) were seeded in each well of a 12-well plastic tissue culture plate (Tissue Culture Cluster 12, Corning Costar, Cambridge, MA, U.S.A.) and incubated for 2 h at 37°C in 1 ml of medium. The wells were washed three times to remove non-adherent cells and the adherent cells were then further incubated for 20 h at 37°C, after which cells were used for the experiments.

Viability assay

After treatment with drugs, the viability of the macrophages was examined by the ability of mitochondrial succinate dehydrogenase to cleave 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to the blue compound formazan (Kobayashi *et al*., 1994). The peritoneal macrophages (1.5×10^5 cells) were incubated for the periods indicated in 100 μl of medium containing 10% (v v^{-1}) calf serum in the presence or absence of drugs. Thereafter, 10 μl of MTT solution (5 mg ml^{-1}) in phosphate-buffered saline (mM): NaCl 137, Na_2HPO_4 8.1, KCl 2.68, KH_2PO_4 1.47, pH 7.4 was added to each well and the cells were further incubated for 4 h at 37°C. After removal of the medium by aspiration, the resultant coloured product was dissolved in 200 μl of dimethylsulphoxide (DMSO) directly added to each well, and read on a Microplate Reader (Bio-Rad, Richmond, CA, U.S.A.) at 570 nm.

Measurement of IL-6 levels in the conditioned medium

After incubation at 37°C for 20 h, the wells were washed three times with medium and the adherent cells were further incubated in 1 ml of EMEM containing 10% (v v^{-1}) calf serum for the periods indicated in the presence and absence of drugs. Concentrations of IL-6 in the conditioned medium were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Cytoscreen Immunoassay Kit Rat IL-6, BioSource International, Camarillo, CA, U.S.A.). In brief, 100 μl of the conditioned medium were incubated at 37°C for 3 h in each well of 96-well microtitre plates precoated with an antibody specific for rat IL-6. After washing, 100 μl of a biotinylated antibody specific for rat IL-6 was added to each well and incubated at room temperature for 1 h. Plates were then washed, 100 μl of Streptavidin-Peroxidase solution added to each well and incubated at room temperature for 30 min. After incubation, the plates were washed again and 100 μl of the substrate solution containing tetramethylbenzidine were added to each well and incubated for 30 min at room temperature in the dark. Thereafter, 100 μl of the stop solution was added to each well and the absorbance at 450 nm was determined. The intensity of the coloured product is directly proportional to the concentration of rat IL-6 present in the original samples.

Coefficient of variation in intra-assay precision and inter-assay precision of this assay kit is 4.5–6.3% and 8.3–9.3%, respectively.

Semiquantification of IL-6 mRNA levels by reverse transcription-polymerase chain reaction (RT–PCR)

Rat peritoneal macrophages (1.5×10^7 cells) were plated in 100 mm Tissue Culture Dish (Corning Costar) and incubated at 37°C for the periods indicated in 10 ml of EMEM containing 10% (v v^{-1}) calf serum in the presence or absence of drugs. After incubation, total RNA was prepared from each sample by acid guanidinium-phenol-chloroform extraction (Chomczynski & Sacchi, 1987) 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 (mM): Tris-HCl 50, KCl 75 and $MgCl_2$ 3, pH 8.3, containing 5 μM of random hexamer oligonucleotides (Gibco BRL, Gaithersburg, MD, U.S.A.), 200 units of the reverse transcriptase from moloney murine leukaemia virus (Gibco BRL), 0.5 mM deoxyribonucleotide triphosphates (dNTP, Pharmacia Biotech, Uppsala, Sweden) and 10 mM dithiothreitol. The PCR primers for IL-6 were designed according to Nadeau *et al*. (1995); (forward) 5′-CAAGAGACTTCCAGCCAGTTGC-3′ and (reverse) 5′-TTGCCGAGTAGACCTCATAGTGACC-3′, which amplify a 614 base pair IL-6 fragment. PCR was performed for 27 cycles in 50 μl of PCR buffer (mM): Tris-HCl 2.5, KCl 50 and $MgCl_2$ 1.5, pH 8.3, containing 0.1 μl of the reverse-transcribed RNA solution, 0.25 μM of each primer, 170 μM dNTP and 1.25 units Taq polymerase (Takara Shuzo, 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 57°C, and 2 min extension at 72°C.

The rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (a housekeeping gene) was used as an internal standard gene. PCR primers for rat GAPDH were designed by Robbins & McKinney (1992); these primers used here were (forward) 5′-TGATGACAAGAAGGTGGTGAAG-3′ and (reverse) 5′-TCCTTGGAGGCCATGTAGGCCAT-3′, which amplify a 249 base pair GAPDH fragment. PCR was performed for 27 cycles; 30 s denaturation at 94°C, 1 min annealing at 57°C, and 2 min extension at 72°C. The other conditions were the same as those used for IL-6.

After the PCR, 10 μl of the PCR reaction mixture was loaded onto a 1.5% agarose minigel and the PCR products were visualized by ethidium bromide staining after electrophoresis.

Drug treatment

Staurosporine (Kyowa Medex, Tokyo, Japan), K-252a ((8*R**,9*S**,11*S**) - (−) - 9 - hydroxy - 9 -methoxycarbonyl-8-

methyl - 2,3,9,10 - tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11*a*-triazadibenzo[*a*,*g*]cycloocta[*a*,*d*,*e*]-trinden-1-one) (BIOMOL Research Laboratories, Plymouth Meeting, PA, U.S.A.), KT-5720 (BIOMOL Research Laboratories), Ro 31-8425 (3-[8 - (aminomethyl - 6,7,8,9 - tetrahydropyridol [1,2-a]-indol-10-yl] - 4 - (1 - methyl-3-indolyl)-1*H*-pyrrole-2,5-dione hydrochloride) (a gift from Dr Kohji Yamada at Eisai Tsukuba Research Institute, Tsukuba, Japan), 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) (BIOMOL Research Laboratories), LY294002 (2-(4-morpholinyl) - 8 - phenyl - 4*H*-1-benzopyran-4-one) (BIOMOL Research Laboratories) and genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one) (Wako Pure Chemicals) were dissolved in DMSO (Wako Pure Chemicals). H-7 (1 - (5 - isoquinolinesulphonyl) - 2 - methylpiperazine dihydrochloride) (Seikagaku Kogyo, Tokyo, Japan) was dissolved in ethanol. H-89 (N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride) (BIOMOL Research Laboratories) was dissolved in ethanol/water (1 : 1, v v^{-1}). An aliquot of each solution was added to the medium and the final concentration of each vehicle in the medium was adjusted to 0.1% (v v^{-1}). The control medium contained the same amount of each vehicle.

Statistical analysis

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

Results

Time-course of IL-6 production in the presence of staurosporine

When rat peritoneal macrophages were incubated in the presence of 63 nM staurosporine, IL-6 concentrations in the conditioned medium began to increase at 4 h, and thereafter increased time-dependently up to 24 h (Figure 1a). In the absence of staurosporine, macrophages did not produce a significant amount of IL-6; concentrations of IL-6 in the conditioned medium were far below the detection limit of the assay kit (<31 pg ml^{-1}) up to 24 h (Figure 1a). The RT–PCR analysis revealed that staurosporine at 63 nM increased the IL-6 mRNA levels at 1 h; the levels reached a maximum at 4 h and decreased at 8 and 24 h (Figure 1b). The ratio of IL-6 mRNA density to GAPDH mRNA density in staurosporine (63 nM)-treated macrophages is shown in Figure 1c.

Effects of various concentrations of staurosporine on IL-6 production

Rat peritoneal macrophages were incubated for 8 h in the presence of various concentrations of staurosporine. The IL-6 concentrations in the conditioned medium were increased by staurosporine in a concentration-dependent manner up to 63 nM (Figure 2a). However, at 210 nM of staurosporine, the IL-6 concentration in the conditioned medium was decreased compared with that at 63 nM staurosporine (Figure 2a). The decrease in IL-6 concentrations in the conditioned medium by 210 nM staurosporine was not due to cytotoxicity, as examined by MTT tests (data not shown). At higher concentrations than 210 nM

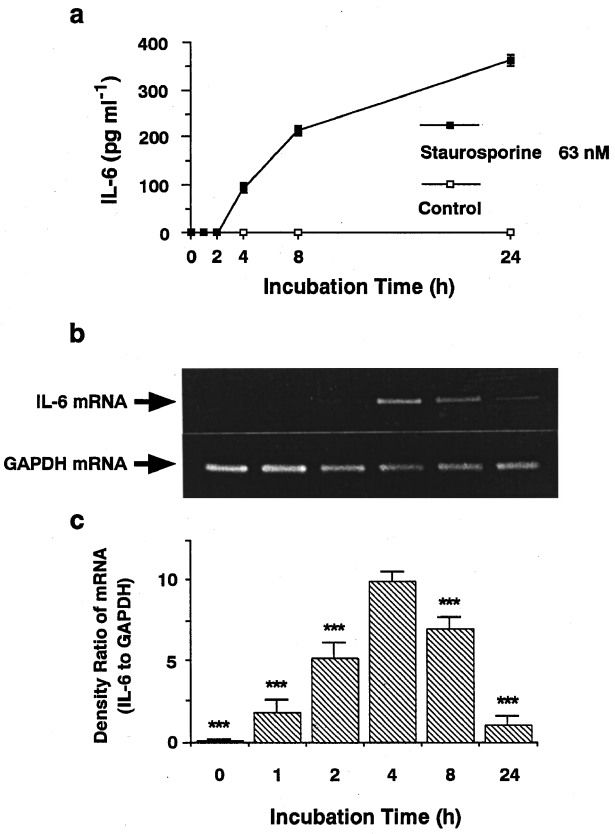

Figure 1 Time-course of IL-6 production and IL-6 mRNA expression in staurosporine-treated macrophages. Rat peritoneal macrophages (1.5×10^6 cells) were incubated for the periods indicated at 37°C in 1 ml of medium in the presence or absence of staurosporine (63 nM). Levels of IL-6 protein in the conditioned medium were determined by ELISA (a). Concentrations of IL-6 protein in the conditioned medium at 0, 1 and 2 h in the presence of staurosporine, and at 0, 1, 2, 4, 8 and 24 h in the absence of staurosporine were not detectable, and the extrapolated values are far less than the detection limit of the assay kit (<31 pg ml^{-1}). To determine levels of IL-6 mRNA, rat peritoneal macrophages (1.5×10^7 cells) were incubated for the periods indicated at 37°C in 10 ml of medium containing 63 nM staurosporine. Total RNA was extracted by the AGPC method and levels of mRNA for IL-6 and GAPDH were determined by RT–PCR (b). To facilitate comparison, the ratios of the density of mRNA for IL-6 to that for GAPDH were determined, and the density ratio in the group incubated for 4 h is set as 10 (c). Statistical significance: $***P<0.001$ vs the group at 4 h. Values are the means of quadruplicate determinations from a single experiment representative of three (a), and the means of three independent experiments (c); vertical lines show s.e.mean.

such as 630 nM, 1.36 μM and 2.1 μM, staurosporine further decreased IL-6 concentrations in the conditioned medium, but induced a dose-dependent cytotoxicity (data not shown). Levels of IL-6 mRNA at 4 h were also increased by staurosporine in a concentration-dependent manner up to 63 nM, but decreased at 210 nM (Figure 2b). Levels of mRNA for GAPDH were not changed by treatment with staurosporine at concentrations up to 210 nM (Figure 2b). The ratio of IL-6 mRNA density to GAPDH mRNA density paralleled the changes of IL-6 production (Figure 2c).

Effects of staurosporine derivatives on IL-6 production

In an attempt to investigate the specificity of staurosporine, we also compared the effect of staurosporine on IL-6 production

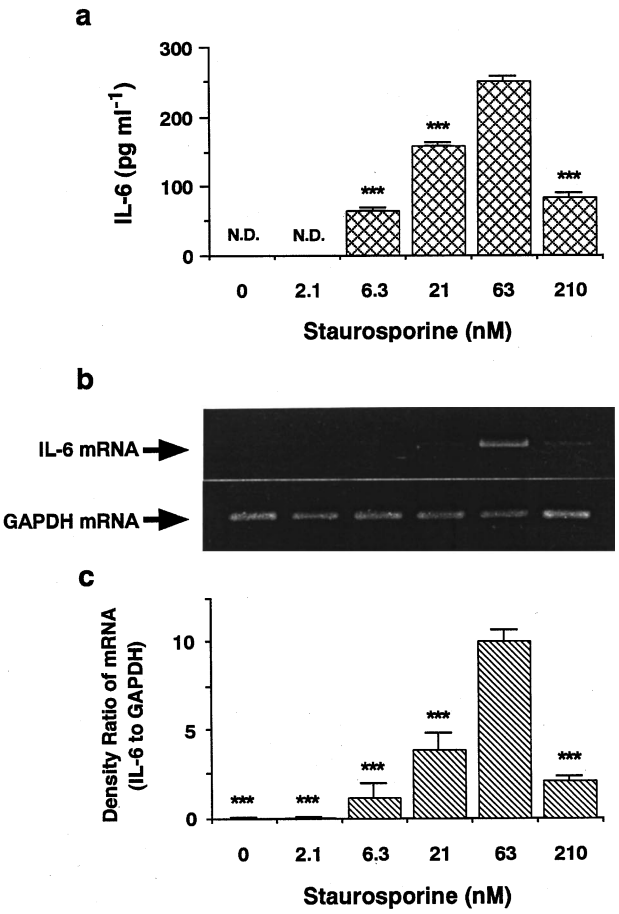

Figure 2 Effects of various concentrations of staurosporine on IL-6 production and expression of IL-6 mRNA. Rat peritoneal macrophages (1.5×10^6 cells) were incubated for 8 h at 37°C in 1 ml of medium containing the indicated concentrations of staurosporine. Levels of IL-6 protein in the conditioned medium were determined by ELISA (a). N.D. means not detectable, and the extrapolated values are far less than the detection limit of the assay kit (<31 pg ml^{-1}). To determine levels of IL-6 mRNA, rat peritoneal macrophages (1.5×10^7 cells) were incubated for 4 h at 37°C in 10 ml of medium containing the indicated concentrations of staurosporine. Total RNA was extracted by the AGPC method and levels of mRNA for IL-6 and GAPDH were determined by RT–PCR (b). To facilitate comparison, the ratios of the density of mRNA for IL-6 to that for GAPDH were determined, and the density ratio in the group incubated in the presence of 63 nM staurosporine is set as 10 (c). Statistical significance: $***P<0.001$ vs staurosporine 63 nM. Values are the means of quadruplicate determinations from a single experiment representative of three (a), and the means of three independent experiments (c); vertical lines show s.e.mean.

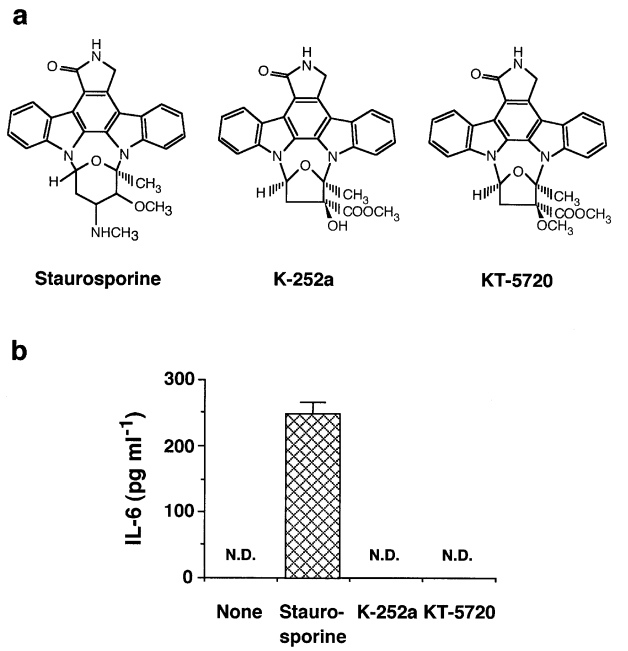

Figure 3 Chemical structures of staurosporine and its derivatives K-252a and KT-5720 (a), and the effects of these drugs on IL-6 production (b). Rat peritoneal macrophages (1.5×10^6 cells) were incubated for 8 h at 37°C in 1 ml of medium in the presence or absence of each drug (63 nM). Levels of IL-6 protein in the conditioned medium were determined by ELISA (b). N.D. means not detectable, and the extrapolated values are far less than the detection limit of the assay kit (<31 pg ml^{-1}). Values are the means of quadruplicate determinations from a single experiment representative of three; vertical lines show s.e.mean.

by rat peritoneal macrophages with its two derivatives (Figure 3a), K-252a, a non-specific protein kinase inhibitor, and KT-5720, an inhibitor of cyclic AMP-dependent protein kinase (PKA). Of the three compounds at a concentration of 63 nM, only staurosporine increased IL-6 production when measured 8 h after incubation (Figure 3b). The treatment with drugs did not induce significant changes in viability of the cells (data not shown).

Effects of PKC inhibitors on staurosporine-induced increase in IL-6 production and levels of IL-6 mRNA

Rat peritoneal macrophages were incubated in medium containing staurosporine (63 nM) and various concentrations of the PKC inhibitor. Staurosporine-induced IL-6 production at 8 h was decreased by the PKC inhibitor, H-7 or Ro 31-8425, which inhibits PKC activity by antagonizing the ability of ATP to bind to the ATP binding domain of PKC, in a concentration-dependent manner (Figure 4a and b). The staurosporine-induced IL-6 production at 8 h was also inhibited by another type of PKC inhibitor, calphostin C (Figure 4c), which inhibits PKC by antagonizing the binding of diacylglycerol to its binding domain in PKC. In parallel with the suppression of staurosporine-induced IL-6 production at 8 h, the staurosporine-induced increase in levels of IL-6 mRNA was also decreased by H-7 (27 μM), Ro 31-8425 (10 μM) and calphostin C (3 μM) (Figure 4d). Treatment with these drugs did not induce significant changes in viability of the cells (data not shown).

Effects of LY294002 and genistein on staurosporine-induced increase in IL-6 production and levels of IL-6 mRNA

Staurosporine (63 nM)-induced IL-6 production at 8 h was decreased by the PI 3-kinase inhibitor, LY294002, in a concentration-dependent manner (Figure 5a). In parallel with the decrease in IL-6 production, staurosporine (63 nM)-induced increase in the levels of IL-6 mRNA was also decreased by LY294002 in a concentration-dependent manner (Figure 5b and c).

On the other hand, staurosporine (63 nM)-induced IL-6 production at 8 h was further increased by the protein tyrosine kinase inhibitor, genistein, again in a concentration-dependent manner (Figure 6a). In parallel with the augmentation of IL-6 production, staurosporine (63 nM)-induced increase in the levels of IL-6 mRNA was also further increased by genistein in a concentration-dependent manner (Figure 6b and c).

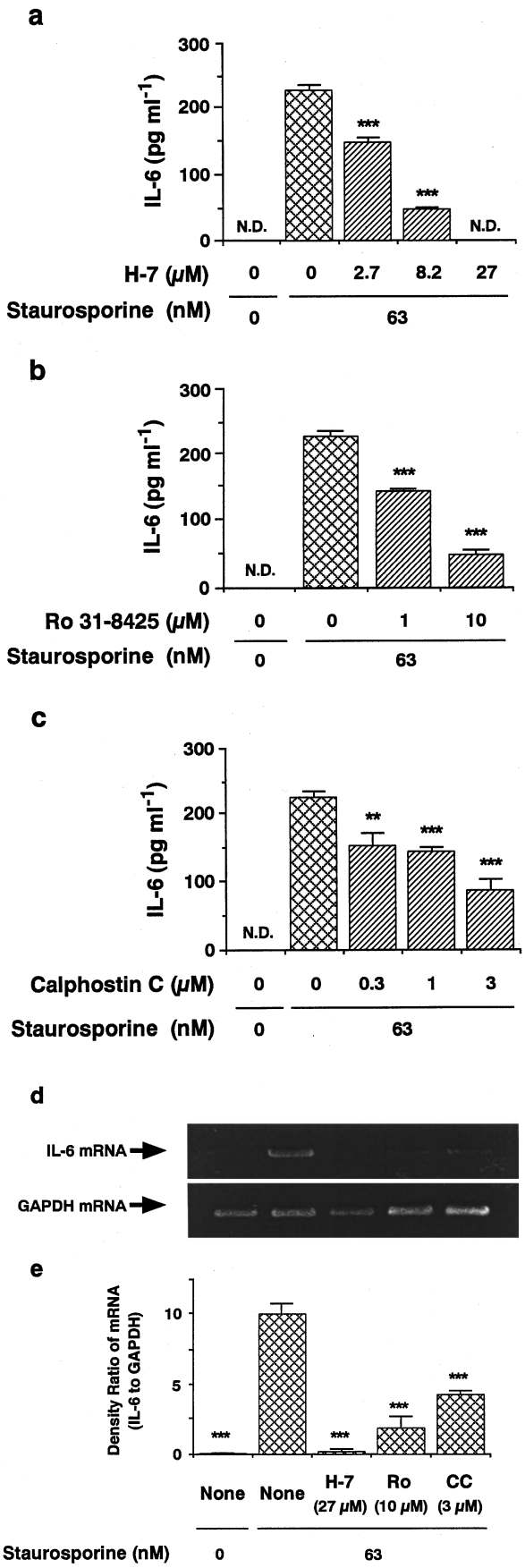

Figure 4 Effects of the PKC inhibitors H-7, Ro 31-8425 and calphostin C on staurosporine-induced increase in IL-6 production and expression of IL-6 mRNA. Rat peritoneal macrophages (1.5×10^6 cells) were incubated for 8 h at 37°C in 1 ml of medium containing staurosporine (63 nM) and the indicated concentrations of H-7, Ro 31-8425 or calphostin C. Levels of IL-6 protein in the conditioned medium were determined by ELISA (a, b, c). N.D. means not detectable, and the extrapolated values are far less than the detection limit of the assay kit (<31 pg ml^{-1}). To determine levels of IL-6 mRNA, rat peritoneal macrophages (1.5×10^7 cells) were incubated for 4 h at 37°C in 10 ml of medium containing the indicated concentration of each drug. Total RNA was extracted by the AGPC method and levels of mRNA for IL-6 and GAPDH were determined by RT–PCR (d). To facilitate comparison, the ratios of the density of mRNA for IL-6 to that for GAPDH were determined, and the density ratio in the group incubated in the presence of staurosporine (63 nM) alone is set as 10 (e). Statistical significance: $^{**}P<0.01$, $^{***}P<0.001$ vs staurosporine (63 nM) alone. Values are the means of quadruplicate determinations from a single experiment representative of three (a, b, c), and the means of three independent experiments (e); vertical lines show s.e.mean.

However genistein by itself did not increase IL-6 production at such concentrations (data not shown). The treatment with drugs did not induce significant changes in viability of the cells (data not shown).

Effects of H-89, a PKA inhibitor, on staurosporine-induced increase in IL-6 production

Staurosporine (63 nM)-induced increase in IL-6 production at 8 h was not affected by treatment with the PKA inhibitor, H-89, at 0.1 to 3 μM (Figure 7). The treatment with drugs did not induce significant changes in viability of the cells (data not shown).

Discussion

The present study was conducted to clarify a signal transduction pathway for IL-6 production induced by staurosporine in rat peritoneal macrophages. IL-6 production was increased by staurosporine at 6.3–63 nM in a concentration-dependent manner, but at the higher concentration (210 nM), the stimulant effect of staurosporine was reduced (Figure 2). Similar bell-shaped effects of staurosporine have also been reported for PGE_2 production by rat peritoneal macrophages (Watanabe *et al*., 1990), chemokine production in human synovial fibroblasts (Jordan *et al*., 1996) and nitric oxide synthase expression in vascular smooth muscle cells (Hecker *et al*., 1997). It is reported that staurosporine inhibits several subtypes of PKC with IC_{50}s ranging from 2 to 1086 nM (Tamaoki *et al*., 1986; Geiges *et al*., 1997). Therefore, it is possible that staurosporine at lower concentrations does not inhibit, but activates, some PKCs which effect is responsible for the increase in IL-6 production, whilst at higher concentrations (e.g. 210 nM), staurosporine inhibits some PKCs responsible for the increase in IL-6 production. Clearly, the mechanism of action of staurosporine on IL-6 production has yet to be fully elucidated, but it is conceivable that the bell-shaped concentration effect may be due to difference in the inhibitory effect of staurosporine on PKC subtypes.

As shown in Figure 4, the staurosporine-induced increase in IL-6 production was suppressed by the PKC inhibitors H-7 (Hidaka *et al*., 1984), Ro 31-8425 (Muid *et al*., 1991) and calphostin C (Kobayashi *et al*., 1989). Therefore, it is reasonable to suggest that the staurosporine-induced IL-6 production is regulated by PKC. In human monocytes and fibroblasts, the level of IL-6 mRNA is increased *via* a PKC-independent pathway (Gross *et al*., 1993; Zhang *et al*., 1988). However, in the human epidermoid carcinoma cell line HEp-2,

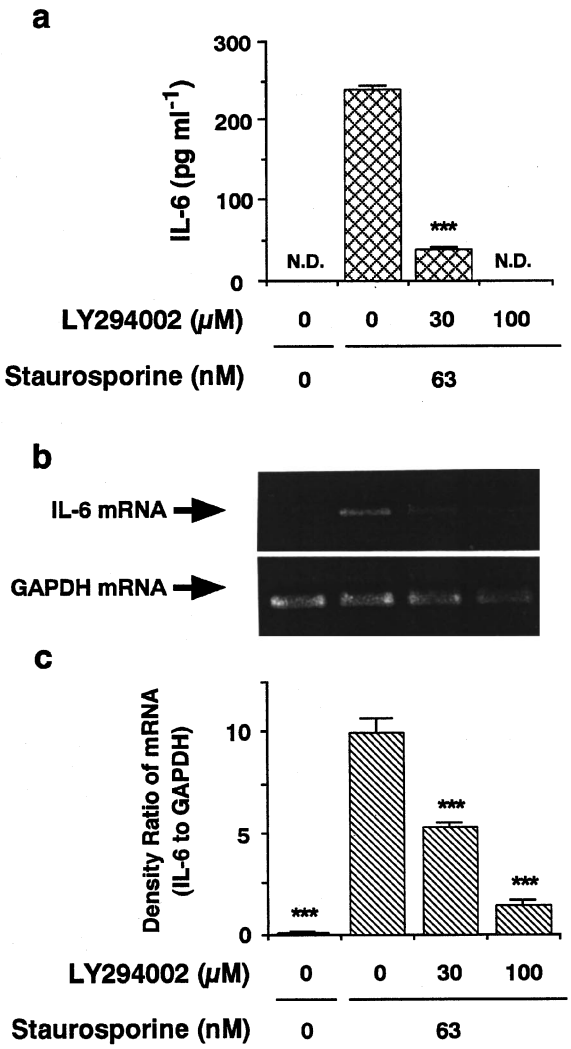

Figure 5 Effects of the PI 3-kinase inhibitor LY294002 on staurosporine-induced increases in IL-6 production and expression of IL-6 mRNA. Rat peritoneal macrophages (1.5×10^6 cells) were incubated for 8 h at 37°C in 1 ml of medium containing staurosporine (63 nM) and the indicated concentrations of LY294002. Levels of IL-6 protein in the conditioned medium were determined by ELISA (a). N.D. means not detectable, and the extrapolated values are far less than the detection limit of the assay kit (<31 pg ml^{-1}). To determine levels of IL-6 mRNA, rat peritoneal macrophages (1.5×10^7 cells) were incubated for 4 h at 37°C in 10 ml of medium containing staurosporine (63 nM) and the indicated concentrations of LY294002. Total RNA was extracted by the AGPC method and levels of mRNA for IL-6 and GAPDH were determined by RT–PCR (b). To facilitate comparison, the ratios of the density of mRNA for IL-6 to that for GAPDH were determined, and the density ratio in the group incubated in the presence of staurosporine (63 nM) alone is set as 10 (c). Statistical significance: ***$P<0.001$ vs staurosporine (63 nM) alone. Values are the means of quadruplicate determinations from a single experiment representative of three (a), and the means of three independent experiments (c); vertical lines show s.e.mean.

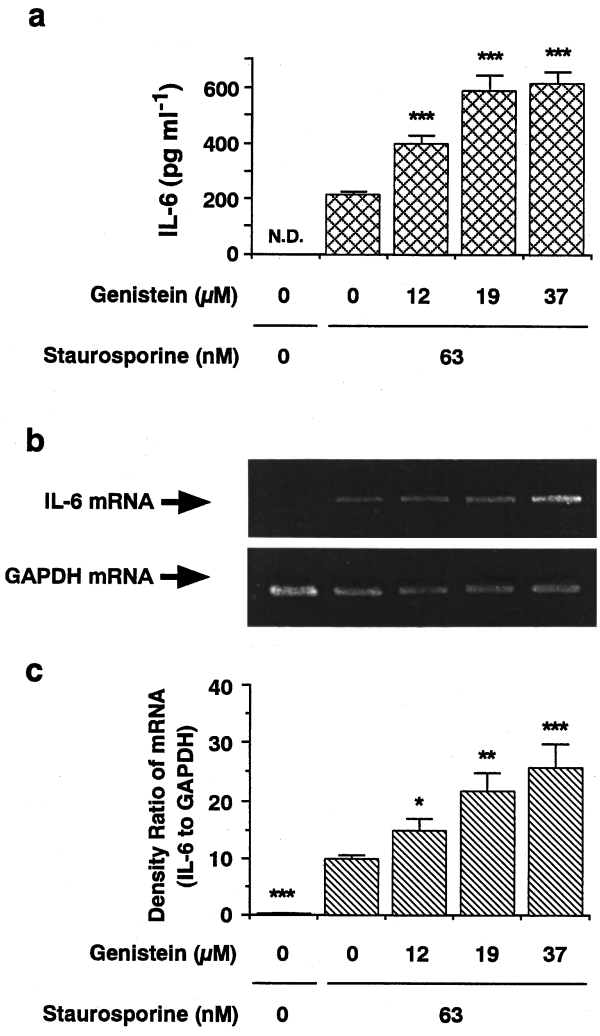

Figure 6 Effects of the PTK inhibitor genistein on staurosporine-induced increases in IL-6 production and expression of IL-6 mRNA. Rat peritoneal macrophages (1.5×10^6 cells) were incubated for 8 h at 37°C in 1 ml of medium containing staurosporine (63 nM) and the indicated concentrations of genistein. Levels of IL-6 protein in the conditioned medium were determined by ELISA (a). N.D. means not detectable, and the extrapolated values are far less than the detection limit of the assay kit (<31 pg ml^{-1}). To determine levels of IL-6 mRNA, rat peritoneal macrophages (1.5×10^7 cells) were incubated for 4 h at 37°C in 10 ml of medium containing staurosporine (63 nM) and the indicated concentrations of genistein. Total RNA was extracted by the AGPC method and levels of mRNA for IL-6 and GAPDH were determined by RT–PCR (b). To facilitate comparison, the ratios of the density of mRNA for IL-6 to that for GAPDH were determined, and the density ratio in the group incubated in the presence of staurosporine (63 nM) alone is set as 10 (c). Statistical significance: *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs staurosporine (63 nM) alone. Values are the means of quadruplicate determinations from a single experiment representative of three (a), and the means of three independent experiments (c); vertical lines show s.e.mean.

PKC activation increases the level of IL-6 mRNA (Gross *et al.*, 1993). Thus, the role of PKC in IL-6 production seems to vary with different types of cells. Because the PKC activator TPA stimulates macropinocytosis (Swanson, 1989), tumour necrosis factor-α production (Hambleton *et al.*, 1995), and arachidonic acid metabolism (Ohuchi *et al.*, 1985; 1988), it is suggested that PKC is one of the responsible molecules for macrophage activation.

It has also been reported that tumour necrosis factor-stimulated IL-6 mRNA induction is mediated by PKA activation but not by PKC activation in human fibroblasts (Zhang *et al.*, 1988), while in the human epidermoid carcinoma cell line HEp-2 IL-6 mRNA is inducible by PKC activation (Gross *et al.*, 1993). In the present study, the PKA inhibitor H-89 did not inhibit staurosporine-induced IL-6 production (Figure 7). Therefore, it appears that staurosporine does not activate PKA in rat peritoneal macrophages. Although there are two pathways for IL-6 production (a PKA pathway, Zhang *et al.*, 1988 and a PKC pathway, Gross *et al.*, 1993), staurosporine might activate only the PKC pathway to induce IL-6 production.

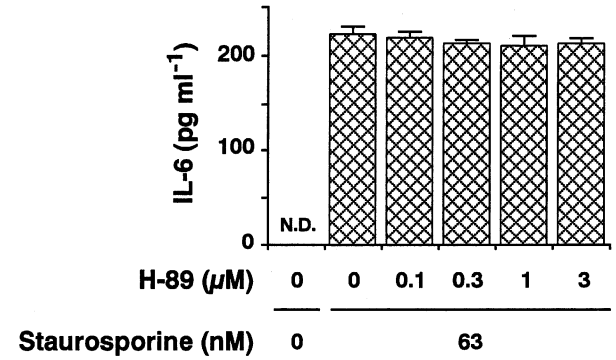

Figure 7 Effects of the PKA inhibitor H-89 on staurosporine-induced increase in IL-6 production. Rat peritoneal macrophages (1.5×10^6 cells) were incubated for 8 h at 37°C in 1 ml of medium containing staurosporine (63 nM) and the indicated concentrations of H-89. Levels of IL-6 protein in the conditioned medium were determined by ELISA. N.D. means not detectable, and the extrapolated values are far less than the detection limit of the assay kit (<31 pg ml^{-1}). Values are the means of quadruplicate determinations from a single experiment representative of three; vertical lines show s.e.mean.

It is also possible that PI 3-kinase plays a significant role in staurosporine-induced IL-6 production, in that staurosporine-induced IL-6 production was inhibited by the PI 3-kinase inhibitor LY294002 (Figure 5). The requirement of PI 3-kinase activation for the completion of macropinocytosis by macrophages (Araki *et al*., 1996) also suggests that PI 3-kinase is involved in signal transduction for macrophage activation. However, the IL-6 production induced by the cross-linking of IgE receptors in murine bone marrow-derived mast cells is not prevented by pretreatment with the PI 3-kinase inhibitor wortmannin (Marquardt *et al*., 1996), suggesting that activation of PI 3-kinase is not critical for IL-6 production. Taken together, these findings indicate that the mechanism for regulating IL-6 production might differ between different types of cells and stimuli.

As to the role of PTK in staurosporine-induced IL-6 production, it was suggested that PTK negatively regulates IL-6 production, because the PTK inhibitor genistein augmented staurosporine-induced IL-6 production (Figure 6). In fact, staurosporine induces tyrosine phosphorylation of a 145 kDa protein, which causes neurite outgrowth of PC12 cells (Rasouly & Lazarovici, 1994). We also reported a significant role of PTK in staurosporine-induced MIP-2 production (Edamatsu *et al*., 1997). These findings suggest that staurosporine may well modulate PTK activity in various situations. In addition, in IL-1β-stimulated human astrocytoma cells, PTK activity is essential for the induction of IL-6 mRNA (Lieb *et al*., 1996) and production of IL-6 (Carlson & Aschmies, 1995). In contrast, in the present study, staurosporine-induced increases in IL-6 production and IL-6 mRNA levels were further increased by the PTK inhibitor genistein. This discrepancy might be due to the difference in types of cells and stimuli.

Staurosporine is used as a PKC inhibitor in cell culture systems (Gross *et al*., 1993; García-Sáinz *et al*., 1988). The staurosporine derivative K-252a also inhibits PKC non-specifically (Kase *et al*., 1987), but it did not induce IL-6 production in these experiments (Figure 3). The PKC inhibitors, Ro 31-8425 and calphostin C, also did not stimulate IL-6 production by themselves (data not shown). Therefore, IL-6 production induced by staurosporine is not due to inhibition of PKC.

In conclusion, it is suggested that the induction of IL-6 production by staurosporine is positively regulated by the activation of PKC and PI 3-kinase and negatively regulated by the activation of PTK in rat peritoneal macrophages.

References

ARAKI, N., JOHNSON, M.T. & SWANSON, J.A. (1996). A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J. Cell Biol.,* **135,** 1249 – 1260.

BOST, K.L. & MASON, M.J. (1995). Thapsigargin and cyclopiazonic acid initiate rapid and dramatic increases of IL-6 mRNA expression and IL-6 secretion in murine peritoneal macrophages. *J. Immunol.,* **155,** 285 – 296.

CARLSON, R.O. & ASCHMIES, S.H. (1995). Tyrosine kinase activity is essential for interleukin-1β-stimulated production of interleukin-6 in U373 human astrocytoma cells. *J. Neurochem.,* **65,** 2491 – 2499.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, *Anal. Biochem.,* **162,** 156 – 159.

EDAMATSU, T., XIAO, Y.-Q., TANABE, J., MUE, S. & OHUCHI, K. (1997). Induction of neutrophil chemotactic factor production by staurosporine in rat peritoneal neutrophils. *Br. J. Pharmacol.,* **121,** 1651 – 1658.

GARCÍA-SÁINZ, J.A., ALCÁNTARA-HERNÁNDEZ, R. & VÁZQUEZ-PRADO, J. (1998). α1-Adrenoceptor subtype activation increases proto-oncogene mRNA levels. Role of protein kinase C. *Eur. J. Pharmacol.,* **342,** 311 – 317.

GEIGES, D., MEYER, T., MARTE, B., VANEK, M., WEISSGERBER, G., STABEL, S., PFEILSCHIFTER, J., FABBRO, D. & HUWILER, A. (1997). Activation of protein kinase C subtypes α, γ, δ, ε, ζ, and η by tumor-promoting agents. *Biochem. Pharmacol.,* **53,** 865 – 875.

GROSS, V., ZHANG, B., GENG, Y., VILLIGER, P.M. & LOTZ, M. (1993). Regulation of interleukin-6 (IL-6) expression: Evidence for a tissue-specific role of protein kinase C. *J. Clin. Immunol.,* **13,** 310 – 320.

HAMBLETON, J., MCMAHON, M. & DEFRANCO, A.L. (1995). Activation of Raf-1 and mitogen-activated protein kinase in murine macrophages partially mimics lipopolysaccharide-induced signalling events. *J. Exp. Med.,* **182,** 147 – 154.

HASHIMOTO, S. & HAGINO, A. (1989). Staurosporine-induced neurite outgrowth in PC12h cells. *Exp. Cell Res.,* **184,** 351 – 359.

HAWKINS, P.T., EGUINOA, A., QIU, R.-G., STOKOE, D., COOKE, F.T., WALTERS, R., WENNSTRÖM, S., CLAESSON-WELSH, L., EVANS, T., SYMONS, M. & STEPHENS, L. (1995). PDGF stimulates an increase in GTP-Rac via activation of phosphoinositide 3-kinase. *Curr. Biol.,* **5,** 393 – 403.

HECKER, M., PREIß, C. & SCHINI-KERTH, V.B. (1997). Induction by staurosporine of nitric oxide synthase expression in vascular smooth muscle cells: role of NF-κB, CREB and C/EBPβ. *Br. J. Pharmacol.,* **120,** 1067 – 1074.

HERRERA-VELIT, P., KNUTSON, K.L. & REINER, N.E. (1997). Phosphatidylinositol 3-kinase-dependent activation of protein kinase C-ζ in bacterial lipopolysaccharide-treated human monocyte. *J. Biol. Chem.,* **272,** 16445 – 16452.

HIDAKA, H., INAGAKI, M., KAWAMOTO, S. & SASAKI, Y. (1984). Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry,* **23,** 5036 – 5041.

JACKSON, T.R., STEPHENS, L.R. & HAWKINS, P.T. (1992). Receptor specificity of growth factor-stimulated synthesis of 3-phosphorylated inositol lipids in Swiss 3T3 cells. *J. Biol. Chem.,* **267,** 16627 – 16636.

JORDAN, N.J., WATSON, M.L., YOSHIMURA, T. & WESTWICK, J. (1996). Differential effects of protein kinase C inhibitors on chemokine production in human synovial fibroblasts. *Br. J. Pharmacol.,* **117,** 1245 – 1253.

KASE, H., IWAHASHI, K., NAKANISHI, S., MATSUDA, Y., YAMADA, K., TAKAHASHI, M., MURAKATA, C., SATO, A. & KANEKO, M. (1987). K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem. Biophys. Res. Commun.,* **142,** 436 – 440.

KIMURA, K., HATTORI, S., KABUYAMA, Y., SHIZAWA, Y., TAKAYANAGI, J., NAKAMURA, S., TOKI, S., MATSUDA, Y., ONODERA, K. & FUKUI, Y. (1994). Neurite outgrowth of PC12 cells is suppressed by wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase. *J. Biol. Chem.,* **269,** 18961 – 18967.

KOBAYASHI, E., NAKANO, H., MORIMOTO, M. & TAMAOKI, T. (1989). Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.,* **159,** 548 – 553.

KOBAYASHI, Y., SAHEKI, T. & SHINOZAWA, T. (1994). Induction of PC12 cell death, apoptosis, by a sialoglycopeptide from bovine brain. *Biochem. Biophys. Res. Commun.,* **203,** 1554 – 1559.

LIEB, K., KALTSCHMIDT, C., KALTSCHMIDT, B., BAEUERLE, P.A., BERGER, M., BAUER, J. & FIEBICH, B.J. (1996). Interleukin-1β uses common and distinct signaling pathways for induction of the interleukin-6 and tumor necrosis factor-α genes in the human astrocytoma cell line U373. *J. Neurochem.,* **66,** 1496 – 1503.

MARQUARDT, D.L., ALONGI, J.L. & WALKER, L.L. (1996). The phosphatidylinositol 3-kinase inhibitor wortmannin blocks mast cell exocytosis but not IL-6 production. *J. Immunol.,* **156,** 1942 – 1945.

MUID, R.E., DALE, M.M., DAVIS, P.D., ELLIOTT, L.H., HILL, C.H., KUMAR, H., LAWTON, G., TWOMEY, B.M., WADSWORTH, J., WILKINSON, S.E. & NIXON, J.S. (1991). A novel conformationally restricted protein kinase C inhibitor, Ro 31-8425, inhibits human neutrophil superoxide generation by soluble, particulate and post-receptor stimuli. *FEBS Lett.,* **293,** 169 – 172.

NADEAU, K.C., AZUMA, H. & TILNEY, N.L. (1995). Sequential cytokine dynamics in chronic rejection of rat renal allografts: Role for cytokines RANTES and MCP-1. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 8729 – 8733.

NAKANISHI, S., YANO, H. & MATSUDA, Y. (1995). Novel functions of phosphatidylinositol 3-kinase in terminally differentiated cells. *Cell. Signal.,* **7,** 545 – 557.

NEWTON, A.C. (1995). Protein kinase C: Structure, function, and regulation. *J. Biol. Chem.,* **270,** 28495 – 28498.

NISHIZUKA, Y. (1986). Studies and perspectives of protein kinase C. *Science,* **233,** 305 – 312.

OHUCHI, K., SUGAWARA, T., WATANABE, M., HIRASAWA, N., TSURUFUJI, S., FUJIKI, H., CHRISTENSEN, S.B. & SUGIMURA, T. (1988). Analysis of the stimulative effect of thapsigargin, a non-TPA-type tumour promoter, on arachidonic acid metabolism in rat peritoneal macrophages. *Br. J. Pharmacol.,* **94,** 917 – 923.

OHUCHI, K., WATANABE, M., YOSHIZAWA, K., TSURUFUJI, S., FUJIKI, H., SUGANUMA, M., SUGIMURA, T. & LEVINE, L. (1985). Stimulation of prostaglandin E_2 production by 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-type and non-TPA-type tumor promoters in macrophages and its inhibition by cycloheximide. *Biochim. Biophys. Acta,* **834,** 42 – 47.

PÉRIANIN, A., COMBADIÉRE, C., PEDRUZZI, E., DJERDJOURI, B. & HAKIM, J. (1993). Staurosporine stimulates phospholipase D activation in human polymorphonuclear leukocytes. *FEBS Lett.,* **315,** 33 – 37.

PICOT, S., SHEICK, I., SYLVI, A., DONADILLE, A. & AMBROISE-THOMAS, P. (1994). Signal transduction pathways involved in tumour necrosis factor secretion by *Plasmodium falciparum*-stimulated human monocytes. *Immunology,* **83,** 70 – 74.

RASOULY, D. & LAZAROVICI, P. (1994). Staurosporine induces tyrosine phosphorylation of a 145 kDa protein but does not activate $gp140^{trk}$ in PC12 cells. *Eur. J. Pharmacol.,* **269,** 255 – 264.

REINHOLD, S.L., PRESCOTT, S.M., ZIMMERMAN, G.A. & MCINTYRE, T.M. (1990). Activation of human neutrophil phospholipase D by three separable mechanisms. *FASEB J.,* **4,** 208 – 214.

RETZLAFF, C., YAMAMOTO, Y., OKUBO, S., HOFFMAN, P.S., FRIEDMAN, H. & KLEIN, T.W. (1996). *Legionella pneumophila* heat-shock protein-induced increase of interleukin-1β mRNA involves protein kinase C signalling in macrophages. *Immunology,* **89,** 281 – 288.

ROBBINS, M. & MCKINNEY, M. (1992). Transcriptional regulation of neuromodulin (GAP-43) in mouse neuroblastoma clone N1E-115 as evaluated by the RT/PCR method. *Brain Res.,* **13,** 82 – 92.

SWANSON, J.A. (1989). Phorbol esters stimulates macropinocytosis and solute flow through macrophages. *J. Cell Sci.,* **94,** 135 – 142.

TAMAOKI, T., NOMOTO, H., TAKAHASHI, I., KATO, Y., MORIMOTO, M. & TOMITA, F. (1986). Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. *Biochem. Biophys. Res. Commun.,* **135,** 397 – 402.

TOKER, A., MEYER, M., REDDY, K.K., FALCK, J.R., ANEJA, R., ANEJA, S., PARRA, A., BURNS, D.J., BALLAS, L.M. & CANTLEY, L.C. (1994). Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3. *J. Biol. Chem.,* **269,** 32358 – 32367.

WATANABE, M., TAMURA, T., OHASHI, M., HIRASAWA, N., OZEKI, T., TSURUFUJI, S., FUJIKI, H. & OHUCHI, K. (1990). Dual effects of staurosporine on arachidonic acid metabolism in rat peritoneal macrophages. *Biochim. Biophys. Acta,* **1047,** 141 – 147.

YAMADA, Y., ICHINOWATARI, G., TANIMOTO, A., YAGINUMA, H. & OHUCHI, K. (1998). Inhibition of tumor necrosis factor-α production by SK&F 98625, a CoA-independent transacylase inhibitor, in cultured rat peritoneal macrophages. *Life sciences,* **62,** 297 – 302.

ZHANG, Y., LIN, J.-X. & VILCEK, J. (1988). Synthesis of interleukin-6 (interferon-β_2/B cell stimulatory factor 2) in human fibroblasts is triggered by an increase in intercellular cyclic AMP. *J. Biol. Chem.,* **263,** 6177 – 6182.

(Received November 13, 1998
Revised April 7, 1999
Accepted April 19, 1999)