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Inhibition by HAJ11 of respiratory burst in neutrophils and the involvement of protein tyrosine phosphorylation and phospholipase D activation

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1 The possible mechanisms of the inhibitory effect of ethyl 2-(3-hydroxyanilino)-4-oxo-4,5-dihydrofuran-3-carboxylate (HAJ11) on the respiratory burst of rat neutrophils *in vitro* was investigated.

2 HAJ11 caused a reversible and a concentration-dependent inhibition of formyl-Met-Leu-Phe (fMLP)-induced superoxide anion ($O_2^{\cdot-}$) generation (IC_{50} 4.9 ± 0.7 μM) and O_2 consumption (IC_{50} 4.9 ± 1.5 μM). Concanavalin A (Con A)- and NaF-induced $O_2^{\cdot-}$ generation were also suppressed by HAJ11. However, HAJ11 was a weak inhibitor of the phorbol 12-myristate 13-acetate (PMA)-induced responses.

3 HAJ11 did not scavenge the $O_2^{\cdot-}$ generation in the xanthine-xanthine oxidase system and dihydroxyfumaric acid (DHF) autoxidation.

4 HAJ11 showed no activity on fMLP-induced inositol phosphates formation and $[Ca^{2+}]_i$ elevation in intact neutrophils. In addition, HAJ11 had no effect on neutrophil cytosolic phospholipase C (PLC) activity.

5 HAJ11 reduced fMLP-induced phosphatidic acid (PA) (IC_{50} 29.1 ± 6.5 μM) and phosphatidylethanol (PEt) (IC_{50} 22.6 ± 1.9 μM) formation in a concentration-dependent manner. HAJ11 also reduced protein tyrosine phosphorylation in neutrophils stimulated by fMLP.

6 HAJ11 was a weak inhibitor of neutrophil cytosolic protein kinase C (PKC) activity, and had a negligible effect on brain PKC. Cellular cyclic nucleotides levels were not altered by HAJ11. In addition, HAJ11 did not affect protein kinase A (PKA) activity.

7 HAJ11 had no effect on the $O_2^{\cdot-}$ generation of PMA-activated and arachidonic acid (AA)-activated NADPH oxidase preparations.

8 Taken together these results indicate that the inhibition of respiratory burst by HAJ11 probably mainly occurs through inhibition of protein tyrosine phosphorylation and phospholipase D (PLD) activity.

Keywords: HAJ11; neutrophil; superoxide anion; oxygen consumption; phospholipase C; intracellular calcium concentration; phospholipase D; tyrosine phosphorylation; protein kinase C; cyclic nucleotides; NADPH oxidase

Introduction

Peripheral blood neutrophils play a crucial role in the elimination of invading microorganisms. For this purpose, they are equipped for chemotaxis, phagocytosis, degranulation and generation of toxic O_2 metabolites (Borregaard, 1988). When neutrophils ingest particles or are exposed to soluble stimuli, they markedly increase O_2 consumption and produce a variety of highly reactive O_2 species, including superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide, hydroxyl radical, hypochlorous acid and peroxynitrite ion (Tauber & Babior, 1985; Ischiropoulos 1992). It has been suggested that an initial biochemical event in the production of active O_2 species is a conversion of O_2 to $O_2^{\cdot-}$ through a one-electron reduction catalysed by a membrane-bound enzyme system, NADPH oxidase (Segal & Abo, 1993). The other active O_2 species are assumed to be derived secondarily from $O_2^{\cdot-}$. This non-mitochondrial O_2 consumption process is known as respiratory burst, the active O_2 species produced are believed to serve as microbicidal agents and have potent inflammatory effects. Therefore, defects in the generation of active O_2 species is often associated with an increased susceptibility to infections. In contrast, extensive production of active O_2 species from neutrophils may deleteriously affect the adjacent normal cells or structural matrix components of tissue. This is probably involved in the pathogenesis of many diseases, including emphysema, glomerulonephritis, rheumatoid arthritis and tissue damage in ischaemia-reperfusion states (Halliwell & Gutteridge, 1990). Drugs that inhibit the formation of $O_2^{\cdot-}$ and other active O_2 species are proposed to exert an anti-inflammatory effect and to prevent the oxygen radical-induced tissue damage (Flohé *et al.*, 1985).

In the study of the anti-inflammatory and antiallergic activities of *N*-alkyl-2,3,4,9-tetrahydrofuro[2,3-*b*]quinoline-3,4-diones derivatives (Kuo *et al.*, 1991), compound ethyl 2-(3-hydroxyanilino)-4-oxo-4,5-dihydrofuran-3-carboxylate (HAJ11) (Figure 1) was found through preliminary *in vitro* tests, to inhibit the degranulation of rat peritoneal mast cells, reduce $O_2^{\cdot-}$ generation and lysosomal enzyme release from rat neutrophils. In this study, we investigated the inhibitory effect of HAJ11 on respiratory burst in rat peripheral neutrophils and assessed the underlying mechanism. The present data provide evidence that the attenuation of the respiratory burst by HAJ11 is mediated, probably, mainly through the suppression of protein tyrosine phosphorylation and phospholipase D (PLD) activity.

Methods

Isolation of neutrophils

Rat blood was collected from the abdominal aorta and the neutrophils were purified by dextran sedimentation, hypotonic lysis of erythrocytes and centrifugation through Ficoll-Hypa-

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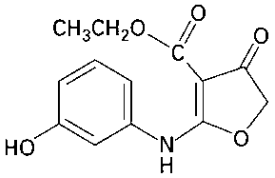

Figure 1 Chemical structure of HAJ11.

que (Wang *et al*., 1995). Purified neutrophils containing >95% viable cells were normally resuspended in Hanks' balanced salt solution containing 10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid] (HEPES), pH 7.4, and 4 mM $NaHCO_3$ (HBSS) to a final concentration of 2×10^6 cells ml^{-1}, and kept in an ice bath before use.

Measurement of $O_2^{\cdot-}$ generation and O_2 consumption

Whole cell $O_2^{\cdot-}$generation, and the $O_2^{\cdot-}$ generation in xanthine-xanthine oxidase system were determined by the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c* as described previously (Wang *et al*., 1994; 1995). The $O_2^{\cdot-}$ generation during dihydroxyfumaric acid (DHF) autoxidation was determined by the reduction of nitroblue tetrazolium (NBT) as previously described (Goldberg & Stern, 1977). Absorbance changes of the reduction of ferricytochrome *c* and NBT were continuously monitored in a double-beam spectrophotometer (Hitachi, U-3210). Whole cell O_2 consumption was continuously measured with a Clark-type oxygen electrode with a YSI biological oxygen monitor (Model 5300) (Ingraham *et al*., 1982).

Determination of inositol phosphate levels

Neutrophils (3×10^7 cells ml^{-1}) were loaded with *myo*-[^{3}H]inositol (83 Ci $mmol^{-1}$, Amersham) at 37°C for 2 h (Wang *et al*, 1994). Ten seconds after stimulation with formyl-Met-Leu-Phe (fMLP), the reaction was stopped by adding $CHCl_3$: CH_3OH (1:1, v/v) mixture and 2.4 M HCl. The aqueous phase was removed and neutralized by 0.4 M NaOH, and then applied to AG 1-X8 resin (formate) column (Bio-Rad). Inositol phosphate (IP), inositol bisphosphate (IP_2) and inositol trisphosphate (IP_3) were eluted sequentially by using 0.2, 0.4, and 1.0 M ammonium formate, respectively, in 0.1 M formic acid as eluents, and then counted in d.p.m. as described in detail previously (Downes & Michell, 1981).

Measurement of cytosolic PLC activity

Neutrophils (4×10^7 cells ml^{-1}) were disrupted in relaxing buffer, (mM: KCl 115, KH_2PO_4 5, EGTA 2, $MgSO_4$ 0.91 and HEPES 10, pH 7.4) with 0.1 mM dithiothreitol, 1 μM leupeptin and aprotinin, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 3 mM benzamidine, by sonication. The lysate was centrifuged at $100{,}000 \times g$ for 60 min at 4°C, and the supernatant fluid was retained for PLC assays. Substrate stock was prepared by mixing L-α-phosphatidyl-D-*myo*-inositol-4,5-bisphosphate (PIP_2) (Boehringer) and 5 μCi L-3-phosphatidyl[2-^{3}H]-inositol 4,5-bisphosphate ([^{3}H]-PIP_2) (1 Ci $mmol^{-1}$, Amersham) in solvent mixture (20% sodium cholate, 250 mM 2-mercaptoethanol, 1 M piperazine-*N*-*N*′-bis[2-ethanesulphonic acid (PIPES), pH 6.8, 2.5 M NaCl) to produce a 0.361 mM PIP_2 solution, sonicated on ice, and then stored at −20°C. PLC activity was assayed by measuring the hydrolysis of PIP_2 into inositol phosphates (Cockcroft *et al*., 1994).

Measurement of $[Ca^{2+}]_i$

Neutrophils (1×10^7 cells ml^{-1}) were suspended in HEPES buffer A (mM: NaCl 124, KCl 4, Na_2HPO_4 0.64, KH_2PO_4 0.66, $NaHCO_3$ 15.2, dextrose 5.56 and HEPES 10, pH 7.4), and loaded with 5 μM fura 2-AM (Molecular Probes) as described previously (Wang *et al*., 1995). After washing, cells were resuspended in HEPES buffer A with 0.05% bovine serum albumin (BSA) in the presence of 1 mM $CaCl_2$ (or 1 mM EDTA). The flourescence was monitored by a double-wavelength fluorescence spectrophotometer (PTI, Deltascan 4000) at 510 nm with excitation 340 and 380 nm in the ratio mode. Calibration of the excitation ratio in terms of Ca^{2+} concentration was performed as previously described (Grynkiewicz *et al*., 1985).

Measurement of PLD activity

Neutrophils (5×10^7 cells ml^{-1}) were suspended in HEPES buffer A and loaded with 10 μCi 1-*O*-[^{3}H]-octadecyl-*sn*-glycero-3-phosphocholine (150 Ci $mmol^{-1}$, Amersham) at 37°C for 75 min, then washed and resuspended in HEPES buffer A with 0.05% BSA. The assay mixtures containing test drugs, 1 mM $CaCl_2$, with or without 0.5% ethanol, were incubated for 3 min at 37°C before initiating the reaction by adding fMLP and dihydrocytochalasin B (CB). Thirty seconds later, the lipids in the reaction mixture were extracted (Bligh & Dyer, 1959), dried and separated as described (Billah *et al*., 1989) with modifications. The plates were developed halfway by using the solvent system consisting of hexane:diethyl ether:CH_3OH:acetic acid (90:20:3:2, v/v/v/v). The plates were dried and developed again to the top with the upper phase of the solvent system consisting of ethylacetate:isooctane:acetic acid:water (110:50:20:100, v/v/v/v). The lipids were located by staining with iodine vapour. The radioactivity of the ^{3}H products were directly quantified with a PhosphorImager (Molecular Dynamics 445 SI) by use of ImageQuaNT software.

Detection of neutrophil proteins phosphorylated on tyrosine residues

Neutrophils (1×10^7 cells ml^{-1}) in HBSS were preincubated with dimethylsulphoxide (DMSO) or test drugs at 37°C for 5 min before addition of 0.1 μM fMLP plus 5 μg ml^{-1} CB to start the reaction. One minute later, the reaction was quenched by adding stop solution (20% trichloroacetic acid (TCA), 1 mM PMSF, 7 μg ml^{-1} of aprotinin and pepstatin, 2 mM *N*-ethylmaleimide, 100 mM NaF, 5 mM diisopropylfluorophosphate (DFP)) (Berkow, 1992). Protein pellets were washed with ice-cold acetone, and boiled in Laemmli sample buffer (Laemmli, 1970). The samples were subjected to SDS-PAGE, transferred to polyvinylidene fluoride membrane (Millipore), and blocked with 5% non-fat milk in TST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20). The proteins were then probed with mouse monoclonal anti-phosphotyrosine antibody (PY-20, Transduction) (1:1000 dilution in TST buffer with 0.5% non-fat milk). Detection was made by use of the enhanced chemiluminescence system (Amersham). The approximate molecular mass of the phosphotyrosine-containing proteins was determined by a log plot of the migration of molecular weight standards.

PKC and PKA assays

For the preparation of cytosolic PKC, neutrophils were disrupted by sonication. After centrifugation, the supernatant was subjected to DE-52 cellulose column (Whatman) to obtain PKC. The method has been described in detail previously (Wang *et al*, 1995). The enzyme activities of neutrophil cytosolic PKC and rat brain PKC (Boehringer) were assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]-ATP (Amersham) into peptide substrate by use of a PKC assay kit (Amersham), based on the mixed micelle method as previously described (Hannun *et al*., 1986). Porcine heart protein kinase A (PKA) activity was assayed by measuring the incorporation of ^{32}P into kemptide in the presence of cyclic AMP with a PKA

assay kit (Life Technologies), based on the method previously described (Roskoski, 1983).

Determination of cyclic AMP and cyclic GMP levels

Cyclic AMP and cyclic GMP contents were determined as described by Simchowitz *et al.* (1980). Neutrophils (2×10^6 cells ml^{-1} for cyclic AMP, 9×10^7 cells ml^{-1} for cyclic GMP) in HBSS were preincubated with test drugs for 9.5 min at 37°C. Thirty seconds after addition of fMLP, the reaction mixture was immediately added to 1.0 ml of 0.05 M acetate buffer, pH 6.2, containing 0.05 mM 3-isobutyl-1-methylxanthine for cyclic AMP assay or 0.05 mM M&B 22948 for cyclic GMP assay. In some experiments, cells were reacted with forskolin or sodium nitroprusside for 10 min at 37°C, without addition of fMLP. After boiling for 5 min, the suspension was kept in ice, sonicated and followed by sedimentation. The supernatants were acetylated by the addition of 0.025 volume of triethylamine:acetic anhydride (2:1. v/v). The cyclic AMP and cyclic GMP contents of the aliquots were assayed by using enzyme immunoassay kits (Amersham).

Measurement of NADPH oxidase activity

For the arachidonic acid (AA)-induced NADPH oxidase activation, the subcellular fractions of neutrophils were prepared as described by Bellavite *et al.* (1983) with modifications. Neutrophils (1×10^8 cells ml^{-1}) were treated with 2.5 mM DFP for 30 min at 4°C, disrupted in Tris buffer (0.34 M sucrose, 10 mM Tris-HCl, pH 7.0, 10 mM benzamidine, and 2 mM PMSF) by sonication, and then centrifuged at $48{,}000\times g$ for 45 min at 4°C. Supernatants were pooled as cytosol fraction, and pellets were collected and resuspended in Tris buffer as membrane fraction. The reaction mixture contained cytosol and membrane fractions, 10 μM flavin adenine dinucleotide (FAD), 3 μM GTPγS, 0.25 mg ml^{-1} of ferricytochrome *c*, 50 μM NADPH and 100 μM AA in phosphate buffer (0.17 M sucrose 2 mM NaN_3, 1 mM $MgCl_2$, 1 mM EGTA, 65 mM KH_2PO_4-NaOH, pH 7.0). PMA-activated NADPH oxidase was isolated and determined the activity as described previously (Wang *et al.*, 1994). The assay mixture contained 0.04% sodium deoxycholate, 12.5 μM FAD, 0.25 mg ml^{-1} of ferricytochrome *c*, particulate protein solution and 62.5 μM NADPH in a final volume of 1.6 ml. Superoxide dismutase (SOD, 6.6 μg ml^{-1}) was also present during the reference cuvettes of both assays (AA-induced NADPH oxidase activation and PMA-activated NADPH oxidase). NADPH oxidase activity was measured spectrophotometrically by continuously detecting the absorbance changes of the reduction of ferricytochrome *c*.

Drugs

HAJ11 (ethyl2-(3-hydroxyanilino)-4-oxo-4,5-dihydrofuran-3-carboxylate) was synthesized as the described previously (Kuo *et al.*, 1991). All chemicals were purchased from Sigma Chemical Co., St. Louis, U.S.A. except for the following: dextran T-500 (Pharmacia Biotech Ltd., Uppsala, Sweden); Hanks' balanced salt solution (Life Technologies Gibco BRL Co., Gaithersburg, U.S.A.); U73122 (1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1**H**-pyrrole-2,5-dione, Biomol Research Lab. Inc., Plymouth Meeting, PA. U.S.A.); 2,5-dihydroxymethylcinnamate (Research Biochemicals International, Natick, MA. U.S.A.); M&B 22948 (2-**O**-propoxyphenyl-8-azapurin-6-one) was supplied by Rhône-Poulenc Rorer Ltd., Essex, U.K.

Statistical analysis

Statistical analyses were performed by the Bonferroni *t* test method after analysis of variance. A *P* value less than 0.05 was considered significant for all tests. Analysis of the regression line test was used to calculate IC_{50} values with 95% confidence interval (CI). Data are presented as means ± s.e.mean.

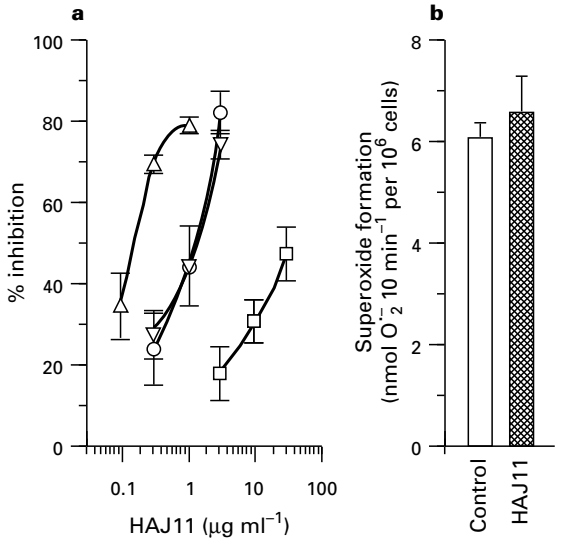

Figure 2 Effect of HAJ11 on formyl-Met-Leu-Phe (fMLP, ○)-, concanavalin A (Con A, △)-, NaF(▽)- and phorbol 12-myristate 13-acetate (PMA, □)-induced $O_2^{\cdot-}$ generation by rat neutrophils. (a) Neutrophils (1×10^6 cells ml^{-1}, at 37°C) were preincubated with various concentrations of HAJ11 for 3 min before the addition of 0.3 μM fMLP plus 5 μg ml^{-1} dihydrocytochalasin B (CB), 300 μg ml^{-1} Con A plus 5 μg ml^{-1} CB, 10 mM NaF or 1 nM PMA. Responses were calculated as % inhibition of control (DMSO vehicle-treated) values. (b) Neutrophils were preincubated with vehicle (control) or 3 μg ml^{-1} of HAJ11 for 3 min, washed twice and then stimulated with fMLP plus CB. Results are expressed as mean ± s.e.mean of 4–6 separate experiments.

Results

Effect of HAJ11 on $O_2^{\cdot-}$ generation and O_2 consumption of neutrophils

When fMLP (0.3 μM) plus 5 μg ml^{-1} of CB, NaF (10 mM), Con A (300 μg ml^{-1}) plus 5 μg ml^{-1} of CB, or PMA (1 nM) was added to rat neutrophil suspension, $O_2^{\cdot-}$ generation was measured and found to be 7.1 ± 0.5, 3.5 ± 0.5, 6.5 ± 0.6, and 20.6 ± 3.0 nmol $O_2^{\cdot-}$ $10\ min^{-1}$ per 2×10^6 cells, respectively, by detecting the reduction of ferricytochrome *c* in the reaction mixture. HAJ11 caused a concentration-dependent inhibition of the responses of all four stimulants (Figure 2a). Significant inhibition ($P<0.05$) was observed at concentrations of HAJ11 $\geqslant$0.3 μg ml^{-1} for fMLP-, $\geqslant$1 μg ml^{-1} for NaF-, $\geqslant$0.1 μg ml^{-1} for Con A-, and $\geqslant$3 μg ml^{-1} for PMA-induced responses. The IC_{50} values of HAJ11 for the inhibition of fMLP-, NaF- and Con A-induced $O_2^{\cdot-}$ generation were estimated to be 1.3 ± 0.2 μg ml^{-1} (95% CI, 6.8–2.6 μM), 1.3 ± 0.1 μg ml^{-1} (95% CI, 6.5–3.0 μM), and 0.2 ± 0.1 μg ml^{-1} (95% CI, 1.1–0.3 μM), respectively. HAJ11 was found to be a weak inhibitor of the PMA (1 nM)-induced response. HAJ11, up to 30 μg ml^{-1}, had no effect on $O_2^{\cdot-}$ generation in response to 3 nM PMA (data not shown). After washing the HAJ11-pretreated neutrophils, the inhibition by HAJ11 of the fMLP-induced $O_2^{\cdot-}$ generation was completely reversed (Figure 2b). In the xanthine (0.15 mM)-xanthine oxidase (0.33 mu) system, $O_2^{\cdot-}$ generation was reduced by SOD 10 μg ml^{-1} (1.0 ± 0.1 *vs.* 6.4 ± 0.5 nmol $O_2^{\cdot-}$ $10\ min^{-1}$, $P<0.01$). The $O_2^{\cdot-}$ generation during DHF (0.891 mM) autoxidation was also inhibited by SOD 10 μg ml^{-1} (0.011 ± 0.002 *vs.* 0.137 ± 0.014 ΔOD_{560}, $P<0.01$) as detected by the reduction of NBT. However, HAJ11 had no effect on $O_2^{\cdot-}$ generation in these two cell-free systems.

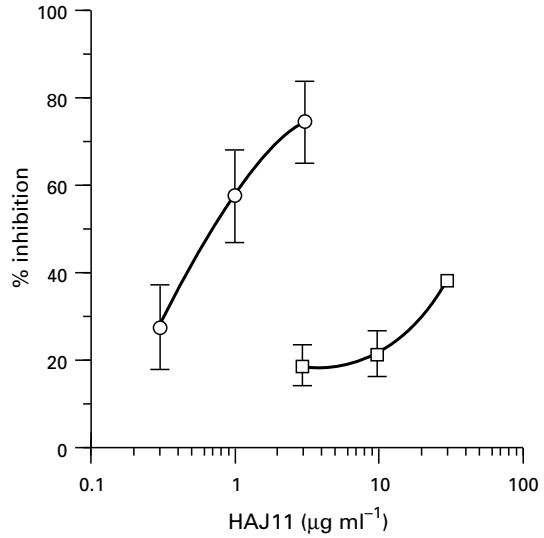

Figure 3 Effect of HAJ11 on formyl-Met-Leu-Phe (fMLP, ○)- and phorbol 12-myristate 13-acetate (PMA, □)-induced O_2 consumption by rat neutrophils. Neutrophils (2×10^6 cells ml^{-1}, at 37°C) were preincubated with various concentrations of HAJ11 for 3 min before the addition of 0.1 μM fMLP plus 5 $\mu g\,ml^{-1}$ dihydrocytochalasin B (CB), or 10 nM PMA. Responses were calculated as the % inhibition of control (DMSO vehicle-treated) values. Results are expressed as mean ± s.e.mean of 3–4 separate experiments.

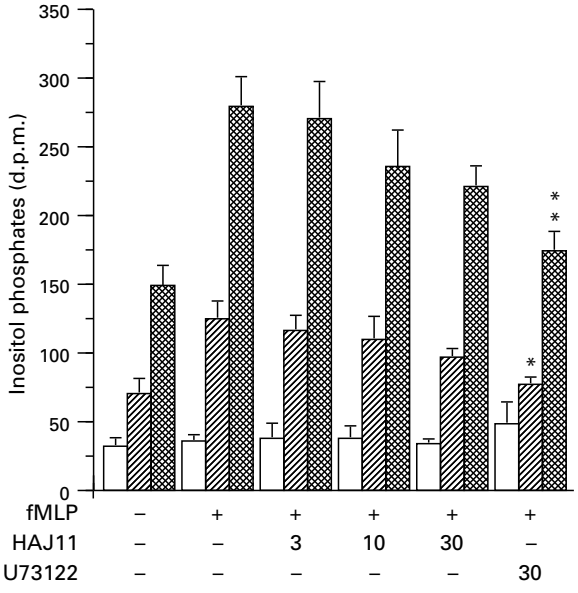

Figure 4 Effect of HAJ11 on formyl-Met-Leu-Phe (fMLP)-induced inositol phosphates generation in rat neutrophils. HAJ11 3–30 $\mu g\,ml^{-1}$ or U73122 30 μM was added to the *myo*-[^{3}H]-inositol-loaded cell suspension in the presence of 10 mM LiCl at 37°C for 3 min before addition of 0.3 μM fMLP to start the reaction. After extraction and separation, the levels of inositol phosphates were counted in d.p.m. Open columns, IP; hatched columns, IP_2; cross-hatched columns, IP_3. Values are expressed as mean ± s.e.mean of 5 separate experiments. $^*P<0.05$, $^{**}P<0.01$ compared to the corresponding values in the group treated with fMLP alone.

Addition of fMLP (0.1 μM) plus 5 $\mu g\,ml^{-1}$ of CB or PMA (10 nM) to the neutrophil suspensions in the presence of 1 mM NaN_3, induced non-mitochondrial O_2 consumption to about 9.7 ± 0.5 and 64.1 ± 4.0 nmol O_2 5 min^{-1} per 6×10^6 cells, respectively. A comparable inhibitory effect, as demonstrated in the $O_2^{\cdot-}$ generation, was also observed in the O_2 consumption of neutrophils pretreated with HAJ11 (Figure 3). Significant

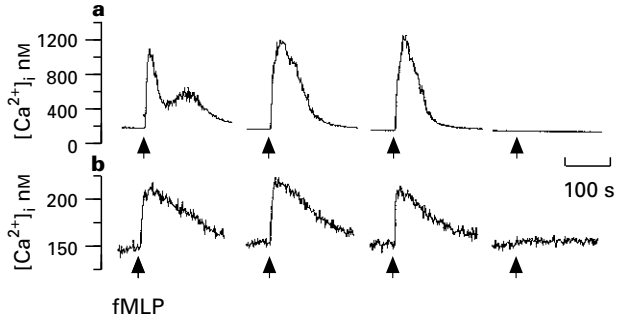

Figure 5 Effect of HAJ11 on $[Ca^{2+}]_i$ in neutrophils challenged by formyl-Met-Leu-Phe (fMLP) in (a) the presence of 1 mM $CaCl_2$ or (b) 1 mM EDTA in medium. The fura 2-loaded cell suspension was preincubated with DMSO (vehicle control), HAJ11 30 and 50 $\mu g\,ml^{-1}$, or U73122 1 μM at 37°C for 3 min before addition of 0.1 μM fMLP. $[Ca^{2+}]_i$ levels were monitored in the excitation ratio 340/380 nm mode with emission wavelength at 510 nm. The results shown are representative of 3–4 separated experiments.

inhibition ($P<0.01$) was shown at the concentrations of HAJ11 ⩾ 1 $\mu g\,ml^{-1}$ (IC_{50} value 1.3 ± 0.4 $\mu g\,ml^{-1}$ with 95% CI, 8.8–1.1 μM) for fMLP- and ⩾ 3 $\mu g\,ml^{-1}$ for PMA-induced responses. The IC_{50} value of HAJ11 for the inhibition of O_2 consumption is quite in accord with that estimated from the inhibition of $O_2^{\cdot-}$ generation. Again, HAJ11 was demonstrated to be a poor inhibitor of the PMA-induced response.

Effect of HAJ11 on inositol phosphates formation and cytosolic PLC activity

Addition of fMLP (0.3 μM) to the *myo*-[^{3}H]-inositol-loaded neutrophil suspensions, significantly increased IP_2 ($P<0.05$) and IP_3 ($P<0.01$), but not IP, levels (Figure 4). U73122, a PLC inhibitor (Smith *et al*., 1990), at 30 μM greatly inhibited IP_2 and IP_3 formation. In contrast, HAJ11, even up to the concentration of 30 $\mu g\,ml^{-1}$, had no effect on the inositol phosphate levels. In neutrophil cytosolic PLC preparation, PLC was activated by the addition of Ca^{2+} to reaction mixture, and then catalysed the PIP_2 hydrolysis reaction at the rate of 568.8 ± 63.9 pmol $min^{-1}\,mg^{-1}$ protein. In good agreement with the results on inositol phosphates formation, PLC activity was inhibited by U73122 (73.4 ± 5.9% inhibition at 30 μM U73122, $P<0.01$), but not affected by HAJ11 (3–30 $\mu g\,ml^{-1}$).

Effect of HAJ11 on $[Ca^{2+}]_i$ of neutrophils

In fura 2-loaded neutrophils, 0.1 μM fMLP induced a rapid and transient elevation of $[Ca^{2+}]_i$ in the Ca^{2+}-containing medium; however, much less extensive $[Ca^{2+}]_i$ changes were observed in the Ca^{2+}-free medium. Pretreatment of cells with U73122 (1 μM) abolished the fMLP-induced $[Ca^{2+}]_i$ changes in the presence or absence of $[Ca^{2+}]_o$. In contrast, HAJ11 did not affect the $[Ca^{2+}]_i$ whether $[Ca^{2+}]_o$ was present or not (Figure 5).

Effect of HAJ11 on PLD activity and protein tyrosine phosphorylation

In order to determine whether the PLD activity was affected by HAJ11 or not, neutrophils were labelled in phosphatidylcholine (PC) by incubating the cells with [^{3}H]-alkyl-lysoPC. Addition of 1 μM fMLP with 5 $\mu g\,ml^{-1}$ of CB to these ^{3}H-labelled neutrophils induced [^{3}H]-alkyl-PA formation, and [^{3}H]-alkyl-PEt formation was also observed if the reaction medium containing 0.5% ethanol. HAJ11, as well as the tyrosine kinase inhibitor genistein (Akiyama *et al*., 1987), inhibited this [^{3}H]-alkyl-PA formation in a concentration-dependent manner (Figure 6). Moreover, a comparable effect was also observed on the [^{3}H]-alkyl-PEt formation. Significant inhibition ($P<0.01$) was obtained at concentrations of HAJ11

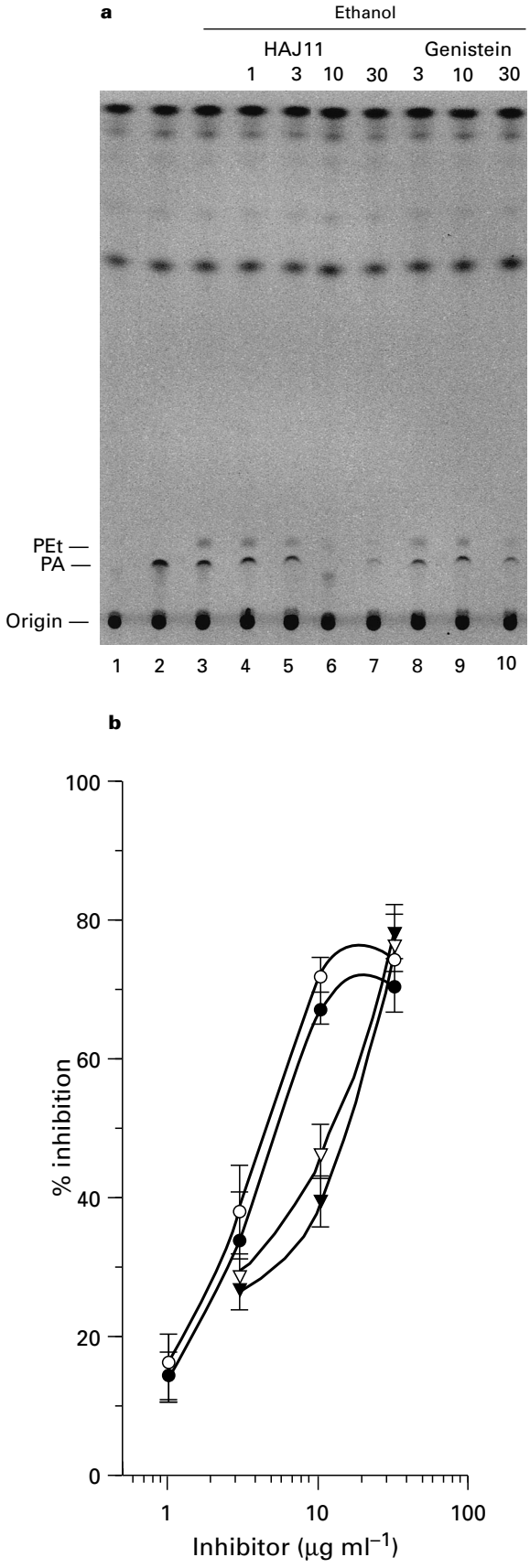

Figure 6 Effect of HAJ11 and genistein on phospholipase D (PLD) activity of rat neutrophils. (a) [^{3}H]-alkyl-PC-loaded cell suspensions were preincubated with DMSO (lanes 1–3), HAJ11 1–30 μg ml^{-1} (lanes 4–7) or genistein 3–30 μg ml^{-1} (lanes 8–9) for 3 min at 37°C in the presence (lanes 3–10) or absence (lanes 1–2) of 0.5% ethanol before addition of DMSO (lane 1) or 1 μM fMLP plus 5 μg ml^{-1} dihydrocytochalasin B (CB, lanes 2–10). The lipids were extracted from the reaction mixture and separated on silica gel 60 plates. The ^{3}H products were visualized and quantified by phosphor screen autoradiography. The location of phosphatidic acid (PA) and phosphatidylethanol (PEt) are indicated. (b) Volume reduction of the PA (○,▽) and PEt (●,▼) formation by HAJ11 (●,○) and genistein (▼,▽) was calculated as % inhibition of the corresponding control values (lane 3, PA 3996.7±776.3 count and PEt 2673.7±441.8 count). The results are expressed as mean±s.e.mean of 4–5 separate experiments.

$\geqslant$10 μg ml^{-1}. The IC_{50} values were estimated to be 7.6±1.7 μg ml^{-1} (95% CI, 50.5–7.6 μM) and 5.9±0.5 μg ml^{-1} (95% CI, 28.7–16.4 μM) for the inhibition of [^{3}H]-alkyl-PA and [^{3}H]-alkyl-PEt formation, respectively.

The effect of HAJ11 on protein tyrosine phosphorylation was studied in neutrophils stimulated with 0.1 μM fMLP plus 5 μg ml^{-1} of CB. It was noted that several proteins were labelled to variable extent by antiphosphotyrosine antibody before neutrophils activation. As shown in Figure 7, addition of fMLP and CB to neutrophil suspension induced a prominent accumulation of phosphotyrosine on proteins of 118 and about 62 kDa (*cf* lanes 1–2, arrows). Two tyrosine kinase inhibitors, genistein and a stable erbstatin analogue 2,5-dihydroxymethylcinnamate (HMC) (Umezawa *et al*., 1992), effectively attenuated the tyrosine phosphorylation produced by fMLP (lanes 3–4). Moreover, preincubation of neutrophils with HAJ11 (1–30 μg ml^{-1}) produced a concentration-dependent inhibition of protein tyrosine phosphorylation (lanes 5–8).

Effect of HAJ11 on PKC activity

In the presence of Ca^{2+}, phosphatidylserine and PMA, the incorporation of ^{32}P from [γ-^{32}P]-ATP into peptide substrate was demonstrated in the assays of neutrophil cytosolic PKC and rat brain PKC. As shown in Table 1, staurosporine, a PKC inhibitor (Tamaoki *et al*., 1986), effectively attenuated ($P<0.01$) the PKC activities of both neutrophil and brain PKC preparations. HAJ11 (30 μg ml^{-1}) was found to induce weak but significant ($P<0.05$) inhibition of neutrophil cytosolic PKC activity, but failed to affect the phosphorylation in the brain PKC assay.

Effect of HAJ11 on cyclic nucleotide levels and PKA activity

Analysis of the cyclic nucleotides in neutrophils showed that a significant increase ($P<0.01$) in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels were observed in the neutrophil suspensions treated with 10 μM forskolin (2.50±0.49 vs. 0.53±0.09 pmol cyclic AMP per 2×10^6 cells), an adenylate cyclase activator (Seamon & Daly, 1986), and 300 μM sodium nitroprusside (2.74±0.36 *vs* 0.84±0.13 pmol cyclic GMP per 2×10^7 cells), respectively. Sodium nitroprusside is thought to release NO, which in turn activates soluble guanylate cyclase (Ignarro, 1990). Whereas, treatment of the neutrophils with HAJ11 (30 μg ml^{-1}) alone failed to affect the levels of cyclic nucleotides. The cyclic GMP level in fMLP-stimulated neutrophils was greatly increased in the presence of M&B22948 (3.98±0.52 *vs* 0.79±0.09 pmol cyclic GMP per 2×10^7 cells, $P<0.01$), a cyclic GMP-specific phosphodiesterase inhibitor (Gillespie & Beavo, 1989). However, the cyclic nucleotide levels in neutrophils challenged by fMLP were unaffected by HAJ11 (30 μg ml^{-1}).

The presence of 10 μM cyclic AMP greatly stimulated the porcine heart PKA activity, measured by detecting the incorporation of ^{32}P from [γ-^{32}P]-ATP into kemptide (7.0±0.4 *vs* 44.2±3.5 pmol ^{32}P min^{-1} μg^{-1} protein, $P<0.01$). Unlike staurosporine (30 nM) and KT5720 (30 μM), a PKA inhibitor (Kase *et al*., 1987), which effectively inhibited ($P<0.01$) cyclic AMP-stimulated PKA activity to 27.0±2.0 and 27.8±1.1 pmol ^{32}P min^{-1} μg^{-1} protein, respectively, HAJ11

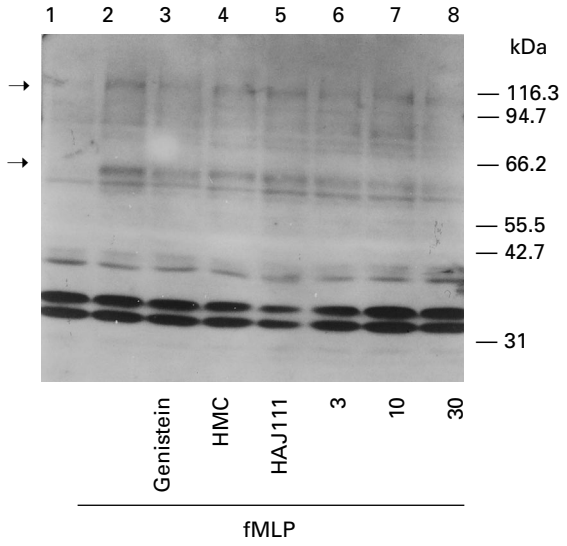

Figure 7 Effect of HAJ11 on tyrosine phosphorylation. Neutrophils were preincubated with DMSO (lanes 1–2), genistein 10 $\mu g\,ml^{-1}$ (lane 3), 2,5-dihydroxymethylcinnamate (HMC) 5 $\mu g\,ml^{-1}$ (lane 4), or HAJ11 1–30 $\mu g\,ml^{-1}$ (lanes 5–8) for 5 min at 37°C before addition of DMSO (lane 1) or 0.1 μM fMLP plus 5 $\mu g\,ml^{-1}$ dihydrocytochalasin B (CB; lanes 2–8). One minute later, cells were then rapidly sedimented, boiled in Laemmli sample buffer, and subjected to SDS-PAGE. Analysis was performed by immunoblotting with a monoclonal antibody to phosphotyrosine. The arrows point to the proteins of 118 and 62 kDa. The results shown are representative of 3 separate experiments.

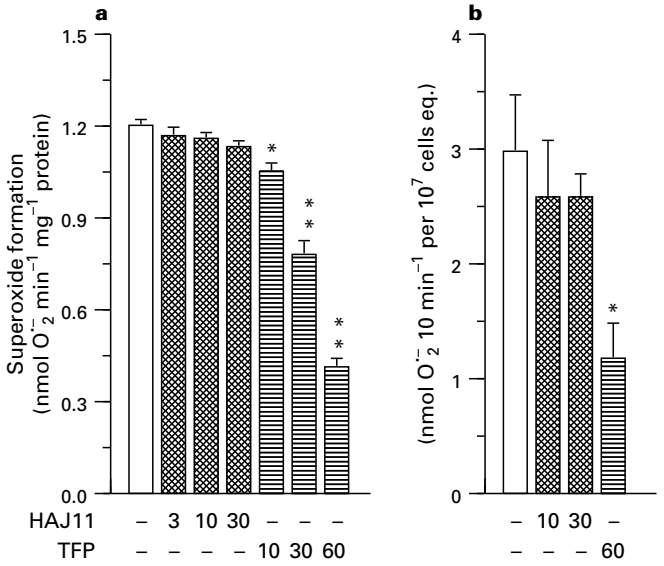

Figure 8 Effect of HAJ11 on NADPH oxidase activity. (a) Phorbol 12-myristate 13-acetate (PMA)-activated particulate NADPH oxidase was incubated with DMSO (control), HAJ11 3–30 $\mu g\,ml^{-1}$, or trifluoperazine (TFP) 10–60 μM at 28°C for 3 min before addition of 62.5 μM NADPH to start the reaction. (b) Neutrophil cytosol and membrane fractions (about 1×10^7 cells equivalent) were preincubated with DMSO (control), HAJ11 10, and 30 $\mu g\,ml^{-1}$, or TFP 60 μM, in the presence of 50 μM NADPH at 28°C for 3 min before addition of 100 μM arachidonic acid (AA) to start the reaction. NADPH oxidase activity was measured by continuously detecting the absorbance changes of ferricytochrome *c*. Values are expressed as mean ± s.e.mean of 4–5 separate experiments. $^*P<0.05$, $^{**}P<0.01$ compared to the corresponding control values.

Table 1 Effect of HAJ11, EDTA and staurosporine on the neutrophil cytosolic protein kinase C (PKC) and brain protein kinase C activities

		Neutrophil cytosolic PKC (*nmol $^{32}P\,min^{-1}$ mg^{-1} protein*)	*Brain PKC* (*nmol $^{32}P\,min^{-1}$ μg^{-1} protein*)
Control		0.557 ± 0.015	3.38 ± 0.22
EDTA	3 mM	0.332 ± 0.020**	1.24 ± 0.11**
HAJ11	3 $\mu g\,ml^{-1}$	0.517 ± 0.017	3.51 ± 0.26
	10 $\mu g\,ml^{-1}$	0.503 ± 0.015	3.66 ± 0.32
	30 $\mu g\,ml^{-1}$	0.483 ± 0.014*	3.63 ± 0.11
Staurosporine	3 nM	0.070 ± 0.002**	ND
	10 nM	ND	1.53 ± 0.25**

Values are expressed as mean ± s.e.mean of 4–6 separate experiments. $^*P<0.05$, $^{**}P<0.01$ compared to corresponding control values. ND, not determined.

(30 $\mu g\,ml^{-1}$) failed to affect PKA activity. Moreover, HAJ11 (30 $\mu g\,ml^{-1}$) alone did not stimulate PKA activity in the absence of cyclic AMP.

Effect of HAJ11 on NADPH oxidase activity

In PMA-activated neutrophil particulate NADPH oxidase preparation, addition of NADPH induced $O_2^{\cdot-}$ generation by detecting the reduction of ferricytochrome *c*. As shown in Figure 8a, trifluoperazine (TFP, 10–60 μM), a NADPH oxidase inhibitor (Bellavite *et al*., 1983), caused a concentration-dependent inhibition of NADPH oxidase activity. In an AA-activated cell-free system, addition of AA induced the assembly of the components of NADPH oxidase from the cytosol and membrane fractions and then generation of $O_2^{\cdot-}$ in the presence of NADPH. A significant inhibition by TFP (60 μM) of $O_2^{\cdot-}$ generation was also observed in an AA-activated cell-free system (Figure 8b). However, HAJ11 (3–30 $\mu g\,ml^{-1}$) had negligible effects on $O_2^{\cdot-}$ generation of either system.

Discussion

It is well established that neutrophils generate $O_2^{\cdot-}$ by activation of NADPH oxidase in response to a wide range of stimuli through different transduction mechanisms (Segal & Abo, 1993). In this study, we found that HAJ11 strongly inhibited fMLP-, Con A-, and NaF-induced $O_2^{\cdot-}$ generation, but showed little activity in the PMA-induced response. fMLP activates neutrophils by binding to G protein linked receptor on the membrane (Ohta *et al*, 1985), whilst NaF and PMA bypass the membrane receptor and directly activate the G protein and PKC (Castagna *et al*., 1982; Hartfield & Robinson, 1990), respectively, followed by a cascade of events that leads to activation of NADPH oxidase. A common feature of the activation of receptors and G proteins consists of activation of PLC that catalyses the hydrolysis of PIP_2 to generate two second messengers, IP_3 and diacylglycerol. IP_3 mobilizes Ca^{2+} from intracellular stores leading to a transient rise in $[Ca^{2+}]_i$, whereas diacylglycerol stimulates PKC (Rana & Hokin, 1990). In the presence of CB, Con A binds to saccharide moieties on the surface of neutrophils and induces $O_2^{\cdot-}$ production (Cohen *et al*., 1982). It is debatable whether PLC activation is involved in Con A-induced $O_2^{\cdot-}$ generation, and pertussis toxin-sensitive G protein is probably not involved in the Con A-induced $[Ca^{2+}]_i$ elevation and $O_2^{\cdot-}$ generation (Liang *et al*., 1990). The finding that HAJ11 did not inhibit $O_2^{\cdot-}$ generation either in xanthine-xanthine oxidase system or during DHF autoxidation but reduced the fMLP-induced O_2 consumption in neutrophils excludes the possibility that HAJ11 poses as a $O_2^{\cdot-}$ scavenger. Furthermore, the observation that HAJ11 caused a parallel inhibition of fMLP-induced $O_2^{\cdot-}$ generation and O_2

consumption, and exerted a much less activity on PMA- than fMLP-induced responses indicates that HAJ11 may interfere with the transduction steps between the activation of G protein and PKC.

Since the respiratory burst evoked by receptor-binding ligand is a Ca^{2+}-dependent process, the effect of HAJ11 on the PLC activity and $[Ca^{2+}]_i$ level of neutrophils were also determined. The finding that U73122, an aminosteroid PLC inhibitor, inhibited fMLP-induced IP_3 formation and $[Ca^{2+}]_i$ elevation in neutrophils agrees with results from a previous study (Smith *et al*., 1990). However, HAJ11 had no effect on the IP_3 formation and $[Ca^{2+}]_i$ elevation in fMLP-activated neutrophils. In addition, HAJ11 was incapable of interfering with the neutrophil cytosolic PLC activity, which was effectively reduced by U73122. These results indicate that the inhibition by HAJ11 of respiratory burst can probably not be attributed to its interference with PLC activity and $[Ca^{2+}]_i$ concentration of neutrophils.

It has been shown that PLD activation is functionally linked to $O_2^{\cdot-}$ production in neutrophils (Bonser *et al*., 1989). PLD utilizes diradyl-PC as the major substrate to produce PA and, in the presence of ethanol, PEt (Billah *et al*., 1989; Mullmann *et al*., 1991). PA could act on the respiratory burst through PKC or directly activate the NADPH oxidase (Bellavite *et al*., 1988). Unlike PLC, which can be fully activated by fMLP in the absence of CB or $[Ca^{2+}]_o$, activation of PLD by fMLP requires the simultaneous presence of both Ca^{2+} and CB (Mullmann *et al*., 1993). In this study, HAJ11 caused a concentration-dependent inhibition of PA as well as of PEt formation in fMLP-activated neutrophils. These results indicate that HAJ11 inhibits PLD activity and this inhibitory effect might contribute to its suppressive effect of respiratory burst. The fMLP receptor activates PLD by a Ca^{2+}-dependent, PKC-dependent and a wortmannin-inhibitable route (Reinhold *et al*., 1990). However, the role of PKC in receptor-mediated PLD activation remains controversial (Uings *et al*., 1992; Kessels *et al*., 1993). It has been already demonstrated that HAJ11 does not inhibit the level of $[Ca^{2+}]_i$, therefore, the exact mechanism of action of HAJ11 on the inhibition of PLD needs further investigation. The finding that genistein inhibited the PLD activity agrees with data on the effect of other tyrosine kinase inhibitors (Uings *et al*., 1992). Since tyrosine kinase activity is involved in receptor coupling to PLD (Uings *et al*., 1992), the effect of HAJ11 on tyrosine kinase was, thereafter, determined.

It has been demonstrated that the tyrosine kinase inhibitors suppress fMLP-induced $O_2^{\cdot-}$ production, and proposed that PLD is a downstream effector of fMLP-induced tyrosine kinase activation that leads to activation of NADPH oxidase (Yasui *et al*., 1994). At least five tyrosine kinases, $p53/56^{lyn}$, $p56/59^{hck}$, $p59^{fgr}$, $p72^{syk}$, and $p77^{btk}$, are present in human neutrophils (Brumell *et al*., 1996). fMLP-induced tyrosine kinases phosphorylate several proteins on tyrosine residues (Torres *et al*., 1993), and one of them may be linked to the activation of PLD. However, at present, the nature of these proteins is virtually unknown. Torres *et al*. (1993) proposed that the fMLP phosphorylated 40 and 42 kDa proteins in human neutrophils, these been possible MAP kinases, may play a regulatory role in the signal transduction pathway leading to the respiratory burst induced by fMLP. In this study, we did not find that the proteins of 40–42 kDa increased phosphotyrosine immunoreactivity. The most prominent phosphotyrosine-containing proteins were of 118 kDa and about 62 kDa after stimulation with fMLP. HAJ11, as well as two tyrosine kinase inhibitors genistein and HMC, reduced the tyrosine phosphorylation on these proteins. These results concomitant with previous findings that tyrosine kinase inhibitors did not inhibit IP_3 generation or increase $[Ca^{2+}]_i$ and had little effect on PMA-induced $O_2^{\cdot-}$ production (Naccache *et al*., 1990; Yasui *et al*., 1994), support the proposal that inhibition of protein tyrosine phosphorylation contributes to the effect of HAJ11. In cells tyrosine phosphorylation is regulated by a balance between tyrosine kinase activity and tyrosine phosphatase activity. It has been shown that plasma membrane tyrosine phosphatase modulates fMLP-induced $O_2^{\cdot-}$ release, and the tyrosine phosphatase inhibitor, vanadate, stimulates O_2 consumption (Cui *et al*., 1994). To determine which of the pathways, tyrosine kinase or phosphatase, is influenced by HAJ11 to account for the inhibition of tyrosine phosphorylation needs further investigation.

It has already been established that a rise in $[Ca^{2+}]_i$ *per se* is not a sufficient stimulus for $O_2^{\cdot-}$ generation (Pozzan *et al*., 1983), and that PKC activity is required together with PLD to stimulate the respiratory burst by fMLP (Kessels *et al*., 1993). PKC has been postulated to play an important role in the activation of the NADPH oxidase by phosphorylating one of the cytosolic components, $p47^{phox}$ (Segal *et al*., 1986). Moreover, activation of PKC and elevation in $[Ca^{2+}]_i$ can thereafter induce PLD to yield PA in receptor-mediated activation (Exton, 1990). At least 12 different PKC isoforms have been found, in which α, β and ξ have been identified in neutrophils (Pontremoli *et al*., 1990; Stasia *et al*., 1990). Rat brain PKC preparation contains mainly Ca^{2+}-dependent isoforms (α,β and γ isoforms) (Go *et al*., 1987). The present study showed that HAJ11 did not affect brain PKC activity, however, slightly but significantly reduced the neutrophil cytosolic PKC activity. These results suggest that the reduction of PKC activity may partly contribute to the inhibition by HAJ11 of respiratory burst.

The effector functions of cyclic AMP, as an inhibitor of chemotaxis, enzyme release and $O_2^{\cdot-}$ generation are well-established (Becker & Ward, 1980; Fantone & Kinnes, 1983). In contrast, the effector functions of cyclic GMP remain controversial. Rap1A appears to be the major PKA substrate in human neutrophils (Bokoch *et al*., 1991). It is possible that Rap1A plays a role in human neutrophils in mediating the inhibitory effects of cyclic AMP-elevating agents on chemoattractant-stimulate NADPH oxidase. The present study indicates that HAJ11 did not affect the cyclic AMP and cyclic GMP levels. Moreover, HAJ11 had no effect on the PKA activity in the presence of cyclic AMP, and did not mimic the cyclic AMP activation of PKA.

The $O_2^{\cdot-}$-generating NADPH oxidase complex in phagocytic cells is constituted of a heterodimeric flavocytochrome *b* and cytosolic factors, mainly $p67^{phox}$ and $p47^{phox}$ (Segal & Abo, 1993). Upon activation, $p47^{phox}$ is phosphorylated (El Benna *et al*., 1994), the polyproline motif of $p47^{phox}$ would then be accessible to the C-terminal SH3 domain of $p67^{phox}$. This new interaction changes the overall structure of the complex and makes it able to recognize the flavocytochrome *b*, favourable to electron transport, and thereby proceeding to the univalent reduction of O_2 (Segal & Abo, 1993). PMA activates PKC, which in turn phosphorylates $p47^{phox}$. Whereas, AA could mimic the effect of $p47^{phox}$ phosphorylation (Fuchs *et al*., 1995), and enhance the assembly of cytosolic factor and membrane component. The failure of HAJ11 to suppress the $O_2^{\cdot-}$generation of both PMA-activated and AA-activated NADPH oxidase preparations indicates that HAJ11 does not directly inhibit the oxidase activity.

Taken together, these data suggest that the inhibition by HAJ11 of respiratory burst can probably be attributed mainly to the inhibition of protein tyrosine phosphorylation and PLD activity, and, in part, a weak inhibitory effect on PKC during neutrophil activation. HAJ11 does not appear to function via the scavenging of $O_2^{\cdot-}$, or by altering the levels of cyclic nucleotides, inositol phosphates and $[Ca^{2+}]_i$.

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