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Cellular localization of the inhibitory action of abruquinone A against respiratory burst in rat neutrophils

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1 The possible mechanisms of action of the inhibitory effect of abruquinone A on the respiratory burst in rat neutrophils *in vitro* was investigated.

2 Abruquinone A caused an irreversible and a concentration-dependent inhibition of formylmethionyl-leucyl-phenylalanine (fMLP) plus dihydrocytochalasin B (CB)- and phorbol 12-myristate 13-acetate (PMA)-induced superoxide anion ($O_2^{\cdot -}$) generation with IC_{50} values of 0.33 ± 0.05 μg ml^{-1} and 0.49 ± 0.04 μg ml^{-1}, respectively.

3 Abruquinone A also inhibited O_2 consumption in neutrophils in response to fMLP/CB and PMA. However, abruquinone A did not scavenge the generated $O_2^{\cdot -}$ in xanthine-xanthine oxidase system and during dihydroxyfumaric acid (DHF) autoxidation.

4 Abruquinone A inhibited both the transient elevation of $[Ca^{2+}]_i$ in the absence of $[Ca^{2+}]_o$ (IC_{50} 7.8 ± 0.2 μg ml^{-1}) and the generation of inositol trisphosphate (IP_3) (IC_{50} 10.6 ± 2.0 μg ml^{-1}) in response to fMLP.

5 Abruquinone A did not affect the enzyme activities of neutrophil cytosolic protein kinase C (PKC) and porcine heart protein kinase A (PKA).

6 Abruquinone A had no effect on intracellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels but decreased the adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels.

7 The cellular formation of phosphatidic acid (PA) and phosphatidylethanol (PEt) induced by fMLP/CB was inhibited by abruquinone A with IC_{50} values of 2.2 ± 0.6 μg ml^{-1} and 2.5 ± 0.3 μg ml^{-1}, respectively. Abruquinone A did not inhibit the fMLP/CB-induced protein tyrosine phosphorylation but induced additional phosphotyrosine accumulation on proteins of 73–78 kDa in activated neutrophils.

8 Abruquinone A inhibited both the $O_2^{\cdot -}$ generation in PMA-activated neutrophil particulate NADPH oxidase (IC_{50} 0.6 ± 0.1 μg ml^{-1}) and the iodonitrotetrazolium violet (INT) reduction in arachidonic acid (AA)-activated cell-free system (IC_{50} 1.5 ± 0.2 μg ml^{-1}).

9 Collectively, these results indicate that the inhibition of respiratory burst in rat neutrophils by abruquinone A is mediated partly by the blockade of phospholipase C (PLC) and phospholipase D (PLD) pathways, and by suppressing the function of NADPH oxidase through the interruption of electron transport.

Keywords: Abruquinone A; rat neutrophil; superoxide anion; inositol phosphate; protein kinase C; cyclic AMP; phospholipase D; tyrosine phosphorylation; NADPH oxidase; electron transport

Introduction

Neutrophils play an important role in the body's defence against bacterial infection. In order to perform this function, neutrophils migrate from the circulation to sites of tissue damage or inflammation under the influence of chemoattractant factors produced by various humoral or cellular immunological processes at these sites. Upon reaching the site of infection or inflammation, neutrophils are exposed to higher concentrations of soluble stimuli, phagocytoze microorganisms or damaged tissue, increase their O_2 uptake from the surrounding medium and concomitantly generate large amounts of superoxide anion ($O_2^{\cdot -}$), which subsequently leads to the formation of other toxic O_2 metabolites (Badwey & Karnovsky, 1980). This non-mitochondrial O_2 consumption process is known as the respiratory burst. Reactive O_2 species produced during the respiratory burst are believed to serve as bactericidal agents, as evinced by the susceptibility of patients with chronic granulomatous disease to serve recurrent infections (Smith & Curnutte, 1991). Under certain circumstances, the excessive or inappropriate release of these highly reactive O_2 species can result in undesirable tissue damage. This is probably involved in the pathogenesis of many diseases (Halliwell & Gutteridge, 1990). Therefore, a drug that would inhibit the generation of toxic O_2 metabolites could terminate this tissue damage.

Multiple mechanisms of neutrophil activation have been described. Stimulation of neutrophils by receptor-binding ligands can activate phospholipase C (PLC) with the formation of inositol trisphosphate (IP_3), which increases intracellular Ca^{2+}, and diacylglycerol, which activates protein kinase C (PKC) (Berridge, 1987). These two second messengers act synergistically for $O_2^{\cdot -}$ generation (Robinson *et al*., 1984). Phospholipase D (PLD) is also activated by certain agonists in neutrophils and appears to be functionally linked to $O_2^{\cdot -}$ generation (Bonser *et al*., 1989). PLD acts upon phosphatidylcholine (PC) to release phosphatidic acid (PA), which can then convert into diradylglycerol with consequent activation of PKC (Billah *et al*., 1989). Upon cell activation, the cytosolic components of the NADPH oxidase (mainly $p47^{phox}$ and $p67^{phox}$) migrate to the membrane and associate with the flavocytochrome b_{558} to form a functionally active complex which catalyses the reduction of O_2 to $O_2^{\cdot -}$ with NADPH as the electron donor (Segal & Abo, 1993).

Abruquinone A, a natural isoflavanquinone, was originally isolated from the roots of *Abrus precatorius* L. (*Leguminosae*) (Alessandro *et al*., 1979). The roots of *Abrus precatorius* have

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been used as a folk medicine for diuresis and for the treatment of fever, sore throat, bronchitis and hepatitis in the Far East. Recently, abruquinone A was found to inhibit platelet aggregation (Kuo *et al*., 1995), mediator release from mast cells *in vitro* and to suppress plasma extravasation caused by inflammatory mediators *in vivo* (Wang *et al*., 1995a). In this study, we investigated the potential inhibitory effect of abruquinone A on the respiratory burst in rat peripheral neutrophils.

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-Hypaque (Wang *et al*., 1995b). Purified neutrophils containing >95% viable cell were normally resuspended in Hank's balanced salt solution (HBSS) containing 10 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid] (HEPES), pH 7.4, and 4 mM $NaHCO_3$, and kept in ice bath before use.

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

The generation of $O_2^{\cdot -}$ in neutrophil suspensions or in xanthine-xanthine oxidase system was determined by the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c* as described previously (Wang *et al*., 1994, 1995b). The $O_2^{\cdot -}$ generation during dihydroxyfumaric acid (DHF) autoxidation was determined by the reduction of nitroblue tetrazolium (NBT) as previously described (Goldbert & Stern, 1977). Absorbance changes of the reduction of ferricytochrome *c* and NBT were monitored continuously in a double-beam spectrophotometer (Hitachi, U-3210). Whole cell O_2 consumption was measured continuously with a Clark-type oxygen electrode by 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 the stimulation with formylmethionyl-leucyl-phenylalanine (fMLP), reactions were 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 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 as described in detail previously (Downes & Michell, 1981).

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

Neutrophils (1×10^7 cells ml^{-1}) were suspended in HEPES buffer A (composition in 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-2AM (Molecular Probes) as described previously (Wang *et al*., 1995b). After being washed, cells were resuspended in HEPES buffer A with 0.05% bovine serum albumin (BSA). The fluorescence was monitored by a double-wavelength fluorescence spectrophotometer (PTI, Deltascan 4000) at 510 nm with excitation 340 and 360 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).

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 partially purified PKC (see Wang *et al*., 1995b for details). Enzyme activity of neutrophil cytosolic PKC was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]-ATP (Amersham) into peptide substrate by 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 by use of PKA assay kit (Life Technologies), based on the method described previously by Roskoski (1983).

Determination of cyclic AMP and cyclic GMP levels

The adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) content was determined as described by Simchowitz *et al*. (1980). Neutrophils (2×10^6 cells ml^{-1} for cyclic AMP, or 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-l-methylxanthine (IBMX) for cyclic AMP assay or 0.05 mM M&B 22948 for cyclic GMP assay. In some experiments, cells were incubated with forskolin or sodium nitroprusside for 10 min at 37°C without addition of fMLP. After being boiled for 5 min, the suspension was kept in ice, sonicated and followed by sedimentation. The supernatant were acetylated by the addition of 0.025 volume of triethylamine:acetic anhydride (2:1, v/v). The cyclic AMP and cyclic GMP content in aliquots of the acetylated samples were assayed by using enzyme immunoassay kits (Amersham).

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 mixture containing test drugs, 1 mM $CaCl_2$ with or without 0.5% ethanol, were incubated for 3 min at 37°C before the reaction was initiated 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 by Billah *et al*. (1989) with certain modifications. Thus the plates were developed halfway by using the solvent system consisting of hexane:diethyl ether:methanol: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 ^{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 the reaction was initiated by adding fMLP/CB. One minute later, reactions were quenched by adding stop solution (20% trichloroacetic acid, 1 mM phenylmethylsulphonyl fluoride (PMSF), 7 μg ml^{-1} of aprotinin and pepstatin, 2 mM N-ethylmaleimide, 100 mM NaF, 5 mM diisopropyl fluorophosphate (DFP) (Berkow, 1992). Protein pellets were washed with ice-cold acetone, and boiled in Laemmli sample buffer. The samples were subjected to SDS-PAGE, transferred to polyvinylidene membrane (Millipore), and blocked with 5% non-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 with the enhanced chemiluminescence system (Amersham). The approximate molecular mass of the phosphotyrosine-containing proteins was interpolated from a standard curve constructed with standard proteins of known molecular weight.

Measurements of NADPH oxidase activity

Particulate NADPH oxidase was isolated as described by Wang *et al*. (1994). NADPH oxidase activity was measured spectrophotometrically at 28°C by detecting the superoxide dismutase (SOD)-inhibitable ferricytochrome *c* reduction as described above. The assay mixture contained 0.04% sodium deoxycholate, 12.5 μM FAD, 0.5 mg ml^{-1} of ferricytochrome *c*, 0.2 ml of particulate protein solution and 50 μM NADPH in a final volume of 1.6 ml. The reduction of ferricytochrome *c* was continuously monitored at 550 nm in a double-beam spectrophotometer (Hitachi, U-3210).

Determination of iodonitrotetrazolium violet (INT) reduction

Neutrophils (2×10^8 cells ml^{-1}) were treated with 2.5 mM DFP for 15 min at 4°C, disrupted in incubation buffer (composition in mM: KCl 100, NaCl 3, $MgCl_2$ 3.5, ATP 1 and 1,4-piperazinediethanesulphonic acid (PIPES) 10, pH 7.3) with 1 mM PMSF, 10 mM benzamidine, 1 μg ml^{-1} leupeptin, antipain and pepstatin, and 1 mM NaN_3 by sonication, and then centrifuged to remove the unbroken cells. Supernatants were placed on discontinuous percoll gradient (Borregaard *et al*., 1983) and centrifugation was carried out at 4°C for 20 min at 48,000 *g*. Membrane and cytosol fractions were collected and stored at −70°C as previously described (Umeki, 1990). Before the reaction, membrane fractions were thawed in solubilization buffer (1 mM NaN_3, 1.7 μM $CaCl_2$, 20 mM glycine, pH 8.0, 50% glycerol and 1% n-octyl-β-D-glucopyranoside) and vortexed briefly. After centrifugation at 350,000 *g* for 30 min at 4°C, supernatants were pooled for use. The assay mixture contained 6.7 μg ml^{-1} SOD, 3.3 μg ml^{-1} GTPγS, 10 μM FAD, membrane and cytosol fractions (1×10^7 cells eq.), 100 μM arachidonic acid (AA) and 53 μM INT at 25°C. Reactions were initiated by the addition of 0.1 mM NADPH. INT reduction was followed as an increase in absorbance at 500 mM (Cross *et al*., 1994).

Drugs

Abruquinone A was isolated and purified from *Abrus precatorius* as previously described (Kuo *et al*., 1995). 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); Hank's 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]-1H-pyrrole-2,5-dione, Biomol Research Lab. Inc., Plymouth Meeting, U.S.A.); diphenylene iodonium (Research Biochemicals International, Natick, U.S.A.); M&B 22948 (2-O-propoxyphenyl-8-azapurin-6-one, 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). Means are represented as mean ± s.e.mean.

Results

Effect of abruquinone A on $O_2^{\cdot-}$ generation and O_2 consumption

The method of SOD-inhibitable ferricytochrome *c* reduction was utilized to investigate the $O_2^{\cdot-}$ generation. Addition of 0.3 μM fMLP plus 5 μg ml^{-1} CB into neutrophil suspensions

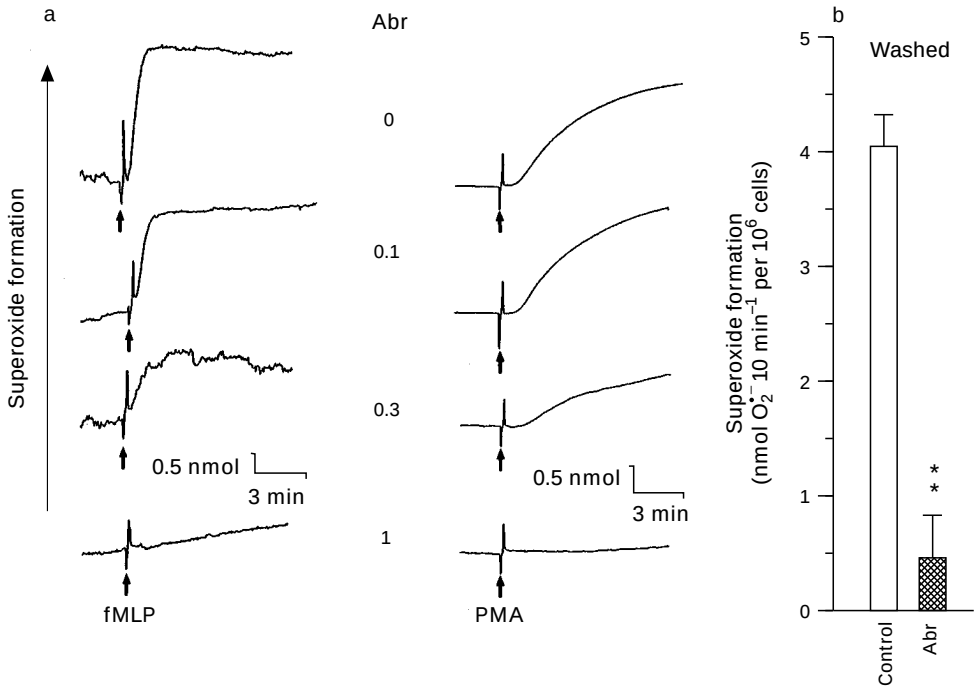


Figure 1 Effect of abruquinone A (Abr) on formylmethionyl-leucyl-phenylalanine (fMLP)- and phorbol 12-myristate 13-acetate (PMA)-induced $O_2^{\cdot-}$ generation in rat neutrophils. (a) Neutrophils (1×10^6 cells ml^{-1}, at 37°C) were preincubated with various concentrations of abruquinone A for 3 min before the addition of 0.3 μM fMLP plus 5 μg ml^{-1} cytochalasin B (CB), or 3 nM PMA. The results shown are representative of 4 separate experiments. (b) Neutrophils were preincubated with DMSO (control) or 1 μg ml^{-1} abruquinone A for 3 min, washed twice and then stimulated with fMLP/CB. Results are expressed as mean ± s.e.mean of 4–5 separate experiments. $^{**}P < 0.01$ compared to the control value.

induced a rapid and transient generation of $O_2^{\cdot -}$, whilst a slow onset but long lasting response was evoked by the addition of 3 nM PMA. Abruquinone A cauesd a concentration-dependent inhibition of both fMLP/CB- and PMA-induced $O_2^{\cdot -}$ generation with IC_{50} values of 0.33 ± 0.05 μg ml^{-1} (95% CI, 1.3–0.5 μM) and 0.49 ± 0.04 μg ml^{-1} (95% CI, 1.7–1.0 μM), respectively (Figure 1a). Significant inhibition ($P<0.01$) was observed at concentrations of abruquinone A $\geqslant 0.3$ μg ml^{-1} for both fMLP/CB- and PMA-induced responses. After washing the abruquinone A (1 μg ml^{-1})-pretreated neutrophils twice with HBSS, significant inhibition ($\sim$90%) of fMLP/CB-induced $O_2^{\cdot -}$ generation still remained (Figure 1b). More than 95% viability was observed with trypan blue exclusion in cells treated with 10 μg ml^{-1} of abruquinone A for 3 min. Unlike SOD, which greatly reduced ($P<0.01$) the $O_2^{\cdot -}$ generation in xanthine (0.15 mM)-xanthine oxidase (1 mu ml^{-1}) system and during DHF (0.89 mM) autoxidation, abruquinone A (0.3–3 μg ml^{-1}) was inactive (Table 1). Addition of 0.3 μM fMLP plus 5 μg ml^{-1} CB, or 10 nM PMA to the neutrophil suspension in the presence of 1 mM NaN_3 evoked non-mitochondrial O_2 consumption. Both fMLP/CB- and PMA-induced responses were suppressed by abruquinone A in a concentration-dependent manner (Figure 2); approximately 48 and 41% inhibition, respectively, was observed at 3 μg ml^{-1} abruquinone A.

Effect of abruquinone A on $[Ca^{2+}]_i$ and inositol phosphate formation

Addition of 0.3 μM fMLP to *myo*-[^{3}H]-inositol-loaded neutrophils, promoted a significant generation of IP_2 and IP_3 (both $P<0.01$); the IP content was unchanged. As expected, a PLC inhibitor, U73122 (Smith *et al*., 1990), at 30 μM greatly reduced the IP_2 and IP_3 formation in neutrophils in response to fMLP. Abruquinone A also inhibited fMLP-induced IP_2 and IP_3 formation in a concentration-dependent manner (Figure 3) with estimated IC_{50} of 10.6 ± 2.0 μg ml^{-1} (95% CI, 45.0–13.8 μM) for IP_3 formation. In the presence of 1 mM EDTA, to remove the extracellular Ca^{2+} in cell suspensions, 0.1 μM fMLP induced a rapid and transient elevation of $[Ca^{2+}]_i$ (about 44.5 ± 4.2 nM increase from the resting level of 165.2 ± 3.8 nM) in neutrophils. Abruquinone A inhibited the fMLP-induced $[Ca^{2+}]_i$ elevation in a concentration-dependent manner with an IC_{50} of 7.8 ± 0.2 μg ml^{-1} (95% CI, 23.4–19.8 μM), significant inhibition was also observed at concentrations of abruquinone A $\geqslant 5$ μg ml^{-1} (29.0 ± 4.1% inhibition at 5 μg ml^{-1} abruquinone A, $P<0.05$).

Effect of abruquinone A on PKC and PKA activities, and intracellular cyclic nucleotide levels

To evaluate whether abruquinone A had an inhibitory effect on PKC, neutrophil cytosolic PKC was prepared to study the incorporation of ^{32}P from [γ-^{32}P]-ATP into peptide substrate in the presence of Ca^{2+}, phosphatidylserine (PS) and PMA. Unlike the PKC inhibitor staurosporine (1 nM) (Tamaoki *et al*., 1986), which effectively attenuated ($P<0.01$) the PKC activity, abruquinone A (up to 30 μg ml^{-1}) failed to affect the enzyme activity of neutrophil cytosolic PKC (Figure 4a).

In the presence of cyclic AMP, porcine heart PKA was activated as evidenced by the increased incorporation of ^{32}P from [γ-^{32}P]-ATP into kemptide. Both staurosporine (30 nM) and a PKA inhibitor, KT5720 ((8R,9S,11S)-(−)-9-hydroxy-9-hydroxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7,6,11a-triazadibenzo[a,g]cycloocta[cde]trinden-1-one, 30 μM) (Kase *et al*., 1987), suppressed the PKA activity, but abruquinone A (30 μg ml^{-1}) was inactive (Figure 4b). Moreover, abruquinone A alone exerted no PKA stimulating activity in the absence of cyclic AMP. Analysis of cyclic nucleotide levels in neutrophils showed that forskolin (10 μM) and sodium nitroprusside (300 μM) significantly increased ($P<0.01$) cyclic AMP and cyclic GMP levels, respectively. In addition, the cyclic AMP and cyclic GMP levels of fMLP (1 μM)-stimulated neutrophils were increased in the presence of a nonselective phosphodiesterase inhibitor isobutyl-methylxanthine (IBMX, 300 μM) and a cyclic GMP-specific phosphodiesterase inhibitor M&B22948 (100 μM) (Beavo & Reifsnyder, 1990), respectively (Table 2). Abruquinone A (3 μg ml^{-1}) did not affect the cellular cyclic GMP content of rat neutrophils. However, it significantly decreased ($P<0.01$) the level of cyclic AMP.

Effect of abruquinone A on PLD activity and protein tyrosine phosphorylation

Addition of 1 μM fMLP plus 5 μg ml^{-1} CB to [^{3}H]-alkyl-lysoPC-loaded neutrophils, significantly increased ($P<0.01$)

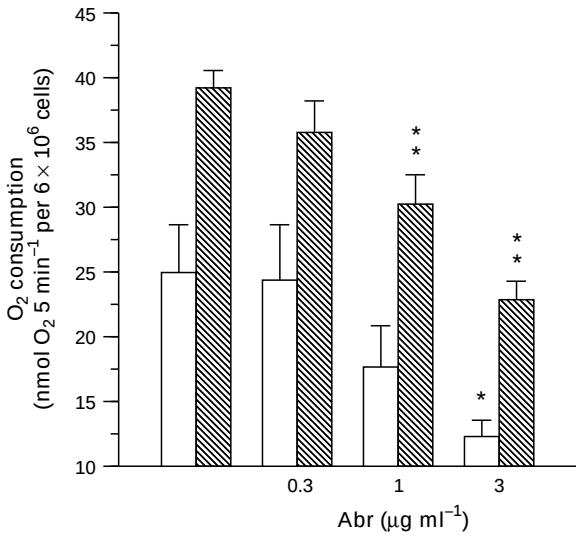


Figure 2 Effect of abruquinone A (Abr) on formylmethionyl-leucyl-phenylalanine (fMLP)- and phorbol 12-myristate 13-acetate (PMA)-induced O_2 consumption in rat neutrophils. Neutrophils (2×10^6 cells ml^{-1}, at 37°C) were preincubated with DMSO (control) or various concentrations of abruquinone A for 3 min before the addition of 0.3 μM fMLP plus 5 μg ml^{-1} CB (open columns), or 10 nM PMA (hatched columns). Results 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 abruquinone A and superoxide dismutase (SOD) on $O_2^{\cdot -}$ generation in dihydroxyfumaric acid (DHF) autoxidation and xanthine (0.15 mM)-xanthine oxidase (1 mu ml^{-1}) system

		DHF ΔA_{560}	*Xanthine-xanthine oxidase* $O_2^{\cdot -}$ (nmol 10 min^{-1})
Control		0.080 ± 0.007	3.36 ± 0.20
Abruquinone A	0.3 μg ml^{-1}	0.082 ± 0.008	3.53 ± 0.27
	1 μg ml^{-1}	0.080 ± 0.009	3.14 ± 0.16
	3 μg ml^{-1}	0.084 ± 0.011	2.84 ± 0.15
SOD	3 μg ml^{-1}	0.010 ± 0.003**	0.30 ± 0.05**

Values are expressed as mean ± s.e.mean of 5–6 separate experiments. **$P<0.01$ compared to corresponding control values.

PA formation, and phosphatidylethanol (PEt) mass if 0.5% ethanol was present in the cell suspension (Figure 5a). PA and PEt formation in response to fMLP/CB were reduced in parallel by abruquinone A and a tyrosine kinase inhibitor genistein (Akiyama *et al*., 1987). Significant inhibition ($P<0.01$) of both PA and PEt formation was observed at concentrations of abruquinone A $\geqslant 3$ μg ml^{-1} with IC_{50} values of 2.2 ± 0.6 μg ml^{-1} (95% CI, 10.8–1.4 μM) and 2.5 ± 0.3 μg ml^{-1} (95% CI, 9.4–4.9 μM), respectively (Figure 5b). To determine whether protein tyrosine phosphorylation was affected by abruquinone A, the tyrosine-phosphorylated proteins of activated neutrophils were established by immunoblotting using a monoclonal anti-phosphotyrosine antibody. Several proteins were labelled to variable extents in the resting cells (Figure 6, lane 1) and addition of 0.1 μM fMLP plus 5 μg ml^{-1} CB to neutrophil suspension induced a substantial phosphotyrosine accumulation on a protein of 62 kDa (cf lanes 1–2, arrow) which was inhibited by genistein (10 μg ml^{-1}). In contrast, abruquinone A (0.1–3 μg ml^{-1}) did not reduce the labelled intensity of the 62 kDa protein but rather induced additional tyrosine phosphorylation of 73–78 kDa proteins (lanes 4–7).

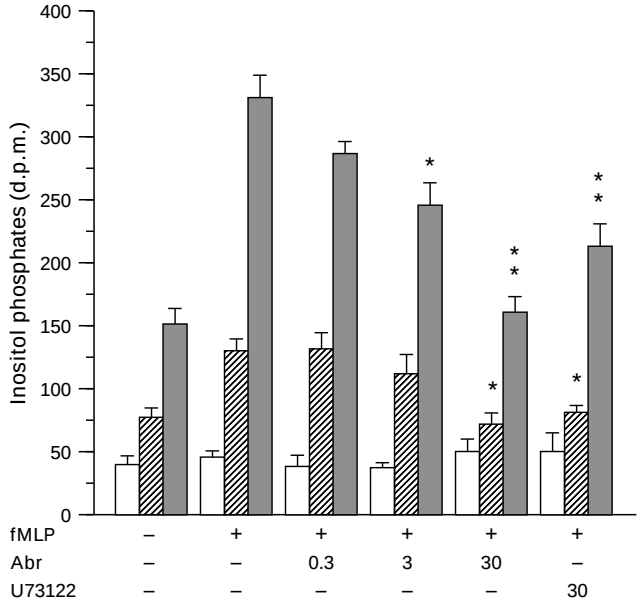


Figure 3 Effect of abruquinone A (Abr) on formylmethionyl-leucyl-phenylalanine (fMLP)-induced inositol phosphate generation in rat neutrophils. DMSO (control), abruquinone A (0.3–30 μg ml^{-1}) or 30 μM U73122 was added to the myo-[^{3}H]-inositol-loaded cell suspension in the presence of 10 mM LiCl at 37°C for 3 min before addition of 0.3 μM fMLP to start the reaction. Values of IP (open columns), IP_2 (hatched columns) and IP_3 (cross-hatched columns) are expressed as mean ± s.e.mean of 4–6 separate experiments. *$P<0.05$, **$P<0.01$ compared to the corresponding control values.

Effect of abruquinone A on NADPH oxidase activity

Abruquinone A (0.1–1 μg ml^{-1}), like a NADPH oxidase inhibitor trifluoperazine (TFP) (10–60 μM) (Bellavite *et al*., 1983), inhibited the generation of $O_2^{\cdot-}$ in PMA-activated particulate NADPH oxidase in a concentration-dependent manner (Figure 7). Significant inhibition was observed at concentrations of abruquinone A $\geqslant 0.1$ μg ml^{-1} and the IC_{50} value was estimated to be 0.6 ± 0.1 μg ml^{-1} (95% CI, 2.6–0.9 μM). In AA (100 μM)-activated cell-free system, INT reduction was observed at the expense of NADPH oxidation. The INT reduction was inhibited by abruquinone A in a concentration-dependent manner with an IC_{50} value of 1.5 ± 0.2 μg ml^{-1} (95% CI, 5.9–2.8 μM) (Figure 8a). As

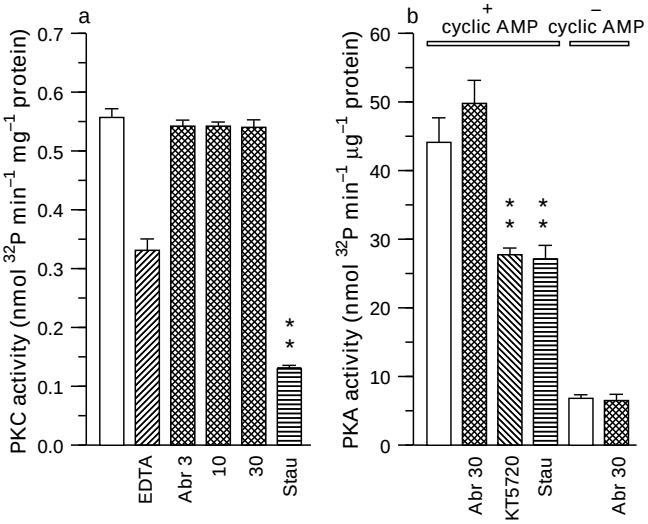
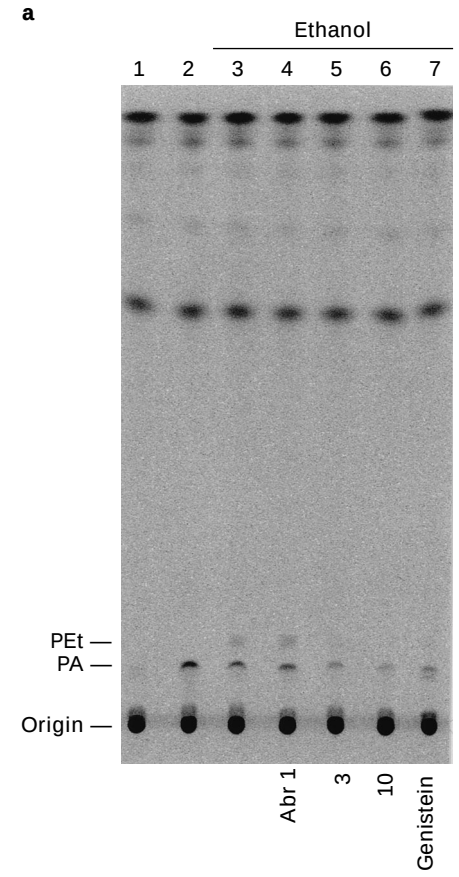


Figure 4 Effect of abruquinone A (Abr) on the enzyme activities of neutrophil cytosolic PKC and porcine heart PKA. (a) Neutrophil cytosolic PKC was preincubated with DMSO (control, open column), abruquinone A (3–30 μg ml^{-1}) or 1 nM staurosporine at 25°C for 3 min before the addition of 1 mM $CaCl_2$, 6 mM PS, 2 μg ml^{-1} of PMA, 50 μM ATP (0.2 μCi [γ-^{32}P]-ATP per tube) and 75 μM PKC substrate. In some experiments, 3 mM EDTA replaced $CaCl_2$ in the reaction mixture. Reactions were terminated after 15 min by the addition of stop reagent. Values are expressed as mean ± s.e.mean of 4–6 separate experiments. **$P<0.01$ compared to the control value. (b) Porcine heart PKA was preincubated with DMSO (control, 1st open column), 30 μg ml^{-1} abruquinone A, 30 μM KT5720 or 30 nM staurosporine at 30°C for 3 min before the addition of 100 μM ATP (0.3 μCi [γ-^{32}P]-ATP per tube), 50 μM kemptide and 10 μM cyclic AMP. In some experiments, PKA was incubated with DMSO (control, 2nd open column) or 30 μg ml^{-1} abruquinone A in the absence of cyclic AMP. Reactions were terminated after incubation for 5 min by the addition of stop reagent. Values are expressed as mean ± s.e.mean of 4–5 separate experiments. **$P<0.01$ compared to the corresponding control values.

Table 2 Effect of abruquinone A, 3-isobutyl 1-methylxanthine (IBMX), forskolin, M&B22948 and sodium nitroprusside on the cyclic AMP and cyclic GMP levels of neutrophils

		Cyclic AMP (pmol per 2×10^6 cells)	*Cyclic GMP* (pmol per 2×10^7 cells)
Control		0.37 ± 0.04	0.85 ± 0.14
fMLP	1 μM	0.55 ± 0.05	0.80 ± 0.10
+ abruquinone A	3 μg ml^{-1}	0.18 ± 0.02**	0.73 ± 0.15
+ IBMX	300 μM	0.99 ± 0.14**	–
+ M&B22948	100 μM	–	3.89 ± 0.47**
Forskolin	10 μM	1.89 ± 0.23†	–
Sodium nitroprusside	300 μM	–	2.84 ± 0.38†

Values are expressed as mean ± s.e.mean of 4–5 separate experiments. **$P<0.01$ compared to the corresponding fMLP alone treated group. †$P<0.01$ compared to the corresponding control values. –, not determined.



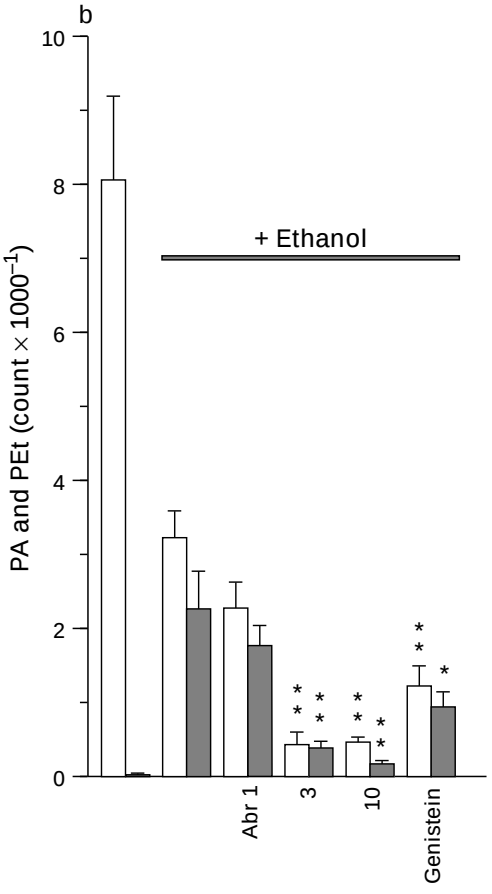


Figure 5 Effect of abruquinone A (Abr) on PLD activity of rat neutrophils. (a) [^{3}H]-alkyl-lysoPC-loaded cells were preincubated with DMSO (lanes 1–3), 1–10 μg ml^{-1} abruquinone A (lanes 4–6), or 30 μg ml^{-1} genistein (lane 7) for 3 min at 37°C in the presence (lanes 3–7) or absence (lanes 1–2) of 0.5% ethanol before addition of DMSO (lane 1) or 1 μM fMLP plus 5 μg ml^{-1} CB (lanes 2–7). The lipids were extracted from the reaction mixture, separated on silica gel 60 plates, and the ^{3}H products were visualized and quantified by phosphor screen autoradiography. The location of phosphatidic acid (PA) and phosphatidylethanol (PEt) are indicated. The results shown are representative of 4 separate experiments. (b) The volumes of PA (open columns) and PEt (cross-hatched columns) in accordance with (a), lanes 2–7, were calculated and expressed as mean ± s.e.mean of 4 separate experiments. $^*P<0.05$, $^{**}P<0.01$ compared to the corresponding control values (the 2nd group column in accordance with (a), lane 3).

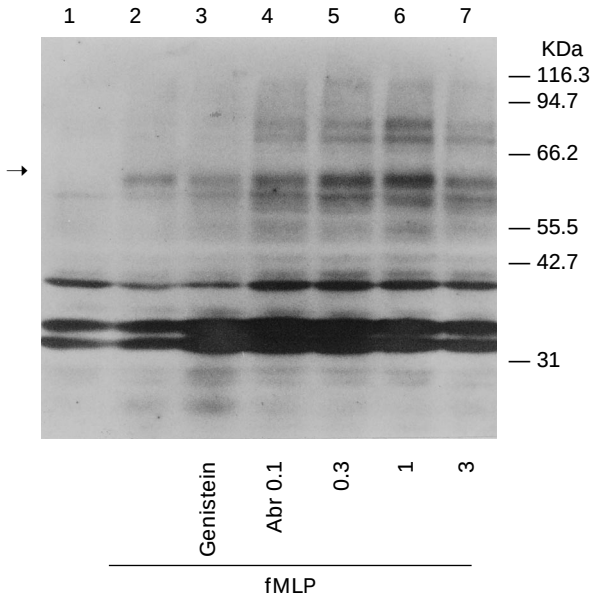


Figure 6 Effect of abruquinone A (Abr) on protein tyrosine phosphorylation. Neutrophils were preincubated with DMSO (lanes 1–2), 10 μg ml^{-1} genistein (lane 3), or 0.1–3 μg ml^{-1} abruquinone A (lanes 4–7) for 5 min at 37°C before addition of DMSO (lane 1) or 0.1 μM fMLP plus 5 μg ml^{-1} CB (lanes 2–7). One minute later, cells were rapidly sedimented, boiled in Laemmli sample buffer, and subjected to SDS-PAGE. Analysis was performed by immunoblotting with a monoclonal antibody to phosphotyrosine. The arrow points to the protein of 62 kDa. The results shown are representative of 3 separate experiments.

expected, a NADPH oxidase inhibitor, diphenylene iodonium (DPI) (Cross & Jones, 1986), inhibited the INT reduction in the presence of NADPH (Figure 8b).

Discussion

Abruquinone A, a natural isoflavanquinone, inhibited neutrophil $O_2^{\cdot -}$ generation and O_2 consumption in response to fMLP/CB and PMA. Since abruquinone A did not reduce the $O_2^{\cdot -}$ generation in xanthine-xanthine oxidase system or during DHF autoxidation, these data preclude the possibility that abruquinone A acted simply as an $O_2^{\cdot -}$ scavenger and suggest an interaction with certain signal transduction steps that follows neutrophil activation.

It is well established that fMLP and PMA elicit a respiratory burst in neutrophils by activating the same NADPH oxidase, but that they utilize a different transduction mechanism and are regulated differently (Segal & Abo, 1993). fMLP-activates neutrophils by binding to G protein-linked receptor on the membrane (Ohta *et al*., 1985), which in turn activates PLC with the formation of IP_3, which increases the $[Ca^{2+}]_i$ and diacylglycerol, which stimulates PKC (Berridge, 1987). In contrast, PMA bypasses the membrane receptor and directly activates PKC (Castagna *et al*., 1982). fMLP/CB- but not PMA-induced $O_2^{\cdot -}$ generation is a Ca^{2+}-dependent pro-

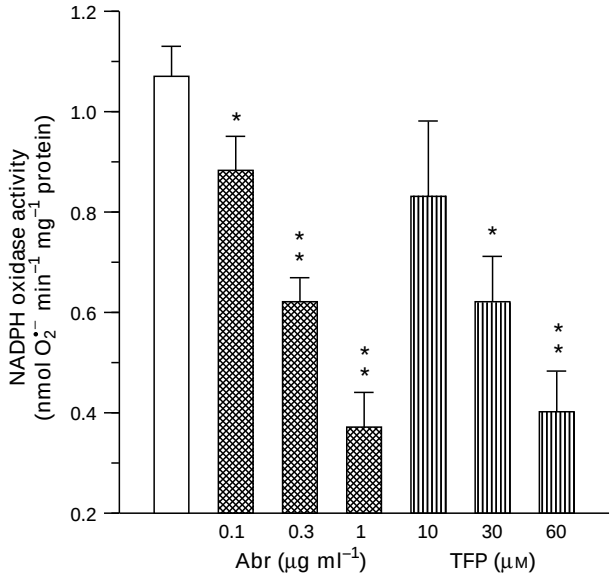


Figure 7 Effect of abruquinone A (Abr) on NADPH oxidase activity. Phorbol 12-myristate 13-acetate (PMA)-activated particulate NADPH oxidase was incubated with DMSO (control, open column), 0.1–1 μg ml^{-1} abruquinone A, or 10–60 μM trifluoperazine (TFP) at 28°C for 3 min before addition of 50 μM NADPH to start the reaction. NADPH oxidase activity was measured by continuously monitoring the absorbance change 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 control value.

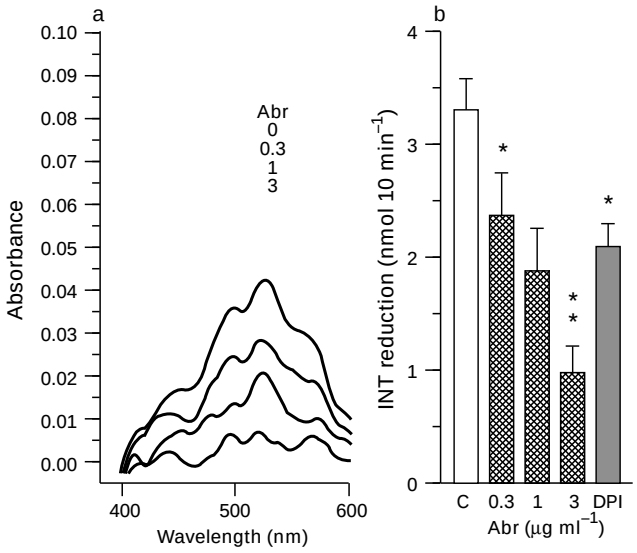


Figure 8 Effect of abruquinone A (Abr) on the NADPH oxidase-dependent reduction of INT. Neutrophil cytosol and membrane fractions (1×10^7 cells eq.) were preincubated with 6.7 μg ml^{-1} SOD, 3.3 μg ml^{-1} GTPγS, 10 μM FAD and 100 μM AA at 25°C for 6 min, then reacted with DMSO (control), 0.3–3 μg ml^{-1} abruquinone A or 1 μM diphenylene iodonium (DPI) for 2 min. Two minutes after the addition of 53 μM INT to the assay mixture, 0.1 μM NADPH was added to start the reaction. (a) Absorption spectra from 400–600 nm of the control and the sample, in the presence of various concentrations of abruquinone A, preparations were recorded 10 min after induction of the reaction. The results shown are representative of 4 separate experiments. (b) INT reduction was calculated in nmol per 10 min reaction time. Values are expressed as mean ± s.e.mean of 4–5 separate experiments. $^*P<0.05$, $^{**}P<0.01$ compared to the control value (c, open column).

cess (Lehmeyer *et al*., 1979). Elevation of $[Ca^{2+}]_i$ in response to chemoattractants results in the release of Ca^{2+} from intracellular stores and from the influx of extracellular Ca^{2+}. Thus in the absence of extracellular Ca^{2+} the increase of $[Ca^{2+}]_i$ in response to chemoattractant results mainly from the mobilization of Ca^{2+} from IP_3-sensitive intracellular stores. The observations that the rise in $[Ca^{2+}]_i$ as well as the generation of IP_3 in response to fMLP were inhibited in parallel by abruquinone A indicate that the PLC pathway is a target for this drug.

Mammalian PKCs consist of at least 12 different isoforms (Dekker & Parker, 1994). Major isoforms of PKC that are present in human neutrophils include conventional (α, β_1 and β_2), novel (δ and η), and atypical (ζ) (Lopez *et al*., 1995). The translocatable, Ca^{2+}-dependent isoforms of PKC such as PKC-β may play a role in the phosphorylation of membrane associated $p47^{phox}$ and the assembly or maintenance of an active NADPH oxidase (Majumbar *et al*., 1993). The inability of abruquinone A to suppress neutrophil cytosolic PKC activity in the presence of Ca^{2+}, PS and PMA indicates that the inhibition of PMA-induced respiratory burst in neutrophils by abruquinone A is not attributable to the suppression of PKC activity. However, with evidence that abruquinone A inhibits the PLC pathway, there is a reason to believe that a lower cellular PKC activity might be observed in fMLP-activated neutrophils due to a reduction in diacylglycerol production.

One recognized means to down-regulating $O_2^{\cdot-}$ generation is to increase intracellular cyclic AMP pharmacologically (Fantone & Kinnes, 1983). However, there is no clear consensus about the role of cyclic GMP in the regulation of respiratory burst in neutrophils. Rap1A appears to be the major PKA substrate in human neutrophils (Bokoch *et al*., 1991), and phosphorylation of Rap1A prevents it binding to flavocytochrome b_{558} which could contribute to the inhibition of NADPH oxidase activity by cyclic AMP-elevating agents. In the present study, abruquinone A had no effect on the cellular cyclic GMP level, whilst it decreased the cyclic AMP level in fMLP-activated neutrophils. The findings that porcine heart PKA activity was unaffected by abruquinone A whether the cyclic AMP was present or not indicate that abruquinone A probably has no ability to activate PKA directly. Moreover, the cellular PKA activity is assumed to be decreased rather than increased because of the marked reduction of cellular cyclic AMP by abruquinone A. Thus, there is no indication that inhibition of respiratory burst by abruquinone A is acting via the changes of intracellular cyclic nucleotides levels or the PKA pathway.

It has been shown that PLD activation is functionally linked to the $O_2^{\cdot-}$ generation in neutrophils (Bonser *et al*., 1989). Existence of at least two different PLD isozymes, the RhoA- and ARF-responsive enyzmes, in HL60 cells has been demonstrated (Siddiqi *et al*., 1995). PLD catalyses the hydrolysis primarily of PC to produce PA. In the presence of ethanol PA, via a transphosphatidylation reaction, yields PEt. PA could act on the respiratory burst through the activation of PKC or NADPH oxidase (Bellavite *et al*., 1988). The observations that abruquinone A suppressed the PA and PEt formation in response to fMLP/CB suggests that the PLD pathway is blocked during neutrophil activation. The mechanisms by which PLD is activated in neutrophils are not fully defined, but pathways linked to Ca^{2+}/CaM (Takahashi *et al*., 1996), PKC (Conricode *et al*., 1992) and tyrosine kinase (TK) (Uings *et al*., 1992) have all been implicated. In human neutrophils, the fMLP-activated PLD was entirely Ca^{2+} and TK dependent, but PKC independent (Planat *et al*., 1996). Ca^{2+}/CaM stimulates the PLD activity in concert with ARF (Takahashi *et al*., 1996). Whilst, PMA-activated PLD was PKC dependent, but Ca^{2+} and TK independent (Planat *et al*., 1996). It has been demonstrated that abruquinone A inhibited the mobilization of Ca^{2+} and this effect might contribute to the inhibition of the PLD pathway in fMLP-activated neutrophils. The finding that genistein inhibited the formation of PLD-derived products reconciles earlier observations on the other TK inhibitors (Uings *et al*., 1992). Since PLD is a downstream effector of fMLP-induced TK activation that leads to activation of NADPH oxidase (Yasui *et al*., 1994), the effect of abruquinone A on the protein tyrosine phosphorylation was determined.

Tyrosine phosphorylation was assessed by immunoblotting with the use of monoclonal anti-phosphotyrosine antibody. At least five TKs, $p53/56^{lyn}$, $p56/59^{hck}$, $p59^{fgr}$, $p72^{syk}$ and $p77^{btk}$ are present in human neutrophils (Brumell *et al*., 1996). Among these, $p53/56^{lyn}$ and $p59^{fgr}$ are involved in the receptor-mediated generation of reactive oxygen intermediates (Hamada *et al*., 1993; Stephens *et al*., 1993). It has been shown that fMLP phosphorylates 40 and 42 kDa proteins in human neutrophils that probably represent mitogen-activated protein (MAP) kinases and could play a regulatory role in the signal transduction pathway leading to the respiratory burst (Torres *et al*., 1993). In this study, we did not observe phosphorylation of 40–42 kDa proteins on tyrosine residues. The most prominent phosphotyrosine-containing protein was 62 kDa after stimulation with fMLP/CB. Abruquinone A did not reduce the tyrosine phosphorylation on 62 kDa, but increased the labelled intensity of 73–78 kDa proteins, indicating that abruquinone A may enhance certain TKs activities or alternatively suppress the tyrosine phosphatase in activated neutrophils. These observations suggest that the TK pathway is probably not involved in the inhibition by abruquinone A of the PLD pathway in fMLP-activated rat neutrophils.

The $O_2^{\cdot-}$ -generating NADPH oxidase complex in neutrophils constitutes a heterodimeric flavocytochrome b_{558} and cytosolic factors, mainly $p47^{phox}$ and $p67^{phox}$ (Segal & Abo, 1993). Upon activation, $p47^{phox}$ is phosphorylated and its polyproline motif is rendered accessible to the C-terminal SH3 domain of $p67^{phox}$. This interaction changes the overall structure of the complex and makes it able to recognize the membrane flavocytochrome b_{558}, favourable to electron transport, and therefore the univalent reduction of O_2 (Segal & Abo, 1993). In the PMA-activated NADPH oxidase preparation, abruquinone A inhibited the $O_2^{\cdot-}$ generation with an IC_{50} value over the same concentration range as that required to suppress the fMLP/CB- and PMA-induced $O_2^{\cdot-}$ generation in neutrophils. However, the IC_{50} for respiratory burst is clearly lower than that for PLC and PLD activities. These findings suggest that the inhibition of NADPH oxidase by abruquinone A may be the major site of action. So far, we do not have evidence to evaluate the mode of action of abruquinone A on PLC and PLD, but it is assumed to be different from that on NADPH oxidase. Two redox centres are present in NADPH oxidase, the FAD centre transfers from the electron donor, NADPH, to the heme centre, the latter serves as the terminal electron donor to O_2 (Segal & Abo, 1993). Recently, an INT diaphorase activity associated with NADPH oxidase has been demonstrated (Cross *et al*., 1994), and electrons are transferred to the dye at the level of the FAD redox centre (Cross & Curnutte, 1995). In this study, the finding that NADPH oxidase inhibitor diphenylene iodonium (DPI) inhibited the INT reduction is in agreement with previous data (Cross *et al*., 1994). The demonstration that INT reduction was suppressed by abruquinone A in a concentration-dependent manner indicates that inhibition of NADPH oxidase by abruquinone A could be due to the suppression of electron flow at the FAD centre.

In conclusion, abruquinone A inhibited the $O_2^{\cdot-}$ generation and O_2 consumption in rat neutrophils activated by fMLP/CB and PMA. This effect may be attributable to blockade of PLC and PLD pathways, although the suppression of NADPH oxidase activity through the interruption of electrons transport at the FAD redox centre is likely to represent the principal mechanism of action.

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