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The signal transduction mechanism involved in kazinol B-stimulated superoxide anion generation in rat neutrophils

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1 In this study, the underlying mechanism of stimulation of respiratory burst by kazinol B, a natural isoprenylated flavan, in rat neutrophils *in vitro* was investigated.

2 Kazinol B concentration-dependently stimulated the superoxide anion ($O_2^{\cdot-}$) generation, with a lag but transient activation profile, in neutrophils but not in a cell-free system. The maximum response (13.2 ± 1.4 nmol $O_2^{\cdot-}$ 10 min^{-1} per 10^6 cells) was observed at 10 μM kazinol B.

3 Pretreatment of neutrophils with phorbol 12-myristate 13-acetate (PMA) or formylmethionyl-leucyl-phenylalanine (fMLP) significantly enhanced the $O_2^{\cdot-}$ generation following the subsequent stimulation of cells with kazinol B.

4 Cells pretreated with EGTA or a protein kinase inhibitor staurosporine effectively attenuated the kazinol B-induced $O_2^{\cdot-}$ generation. However, a p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 and a phosphoinositide 3-kinase (PI3K) inhibitor wortmannin had no effect on the kazinol B-induced response.

5 Kazinol B significantly stimulated $[Ca^{2+}]_i$ elevation in neutrophils, with a lag and slow rate of rise activation profile, and this response was attenuated by a phospholipase C (PLC) inhibitor U73122. Kazinol B also stimulated the inositol bis- and trisphosphate (IP_2 and IP_3) formation with a 1 min lag time.

6 The membrane-associated PKC-α and PKC-θ but not PKC-ι were increased following the stimulation of neutrophils with kazinol B. It was more rapid and sensitive in the activation of PKC-θ than PKC-α by kazinol B. Kazinol B partially inhibited the [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDB) binding to the neutrophil cytosolic PKC.

7 Neither the cellular mass of phosphatidic acid (PA) and phosphatidylethanol (PEt), in the presence of ethanol, nor the protein tyrosine phosphorylation were stimulated by kazinol B. In addition, the kazinol B-induced $O_2^{\cdot-}$ generation remained relatively unchanged in cells pretreated with ethanol or a tyrosine kinase inhibitor genistein.

8 Collectively, these results indicate that the stimulation of the respiratory burst by kazinol B is probably mediated by the synergism of PKC activation and $[Ca^{2+}]_i$ elevation in rat neutrophils.

Keywords: Kazinol B; rat neutrophil; superoxide anion; inositol phosphate; cellular free Ca^{2+} concentration; protein kinase C; translocation

Introduction

Neutrophils constitute the first line of host defence against bacterial infection. In order to achieve this function of killing microorganisms, large numbers of neutrophils are recruited 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, phagocytose 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. The importance of these reactive O_2 species produced during the respiratory burst are highlighted by the recurrence and severity of infections in patients with chronic granulomatous disease (Smith & Curnutte, 1991).

The mechanism underlying $O_2^{\cdot-}$ generation remains obscure. Stimulation of neutrophils by receptor-binding ligands can activate phospholipase C (PLC) with the formation of inositol trisphosphate (IP_3), which increases in $[Ca^{2+}]_i$, and diacylglycerol (DAG), which activates protein kinase C (PKC) (Berridge, 1987). These two second messengers act synergistically for $O_2^{\cdot-}$ generation (Robinson *et al*., 1984). Protein tyrosine phosphorylation and phospholipase D (PLD) activation appear to be functionally linked to $O_2^{\cdot-}$ generation in neutrophils stimulated with certain agonists (Bonser *et al*., 1989; Torres *et al*., 1993). PLD acts upon phosphatidylcholine to release phosphatidic acid (PA), which in turn stimulates the respiratory burst through the activation of PKC or NADPH oxidase (Bellavite *et al*., 1988). Recent advances have highlighted the involvement of phosphoinositide 3-kinase (PI3K) (Arcaro & Wymann, 1993) and p38 mitogen-activated protein kinase (MAPK) (Nick *et al*., 1997) in the neutrophil respiratory burst. 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 catalyzes the reduction of

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O_2 to $O_2^{\cdot-}$ using NADPH as the electron donor (Segal & Abo, 1993).

Phorbol ester bypasses the membrane receptor, activates PKC directly (Castagna *et al*., 1982) and thus stimulates the respiratory burst (Tauber, 1987). Phorbol ester range of natural products from plants of the families *Euphorbiaceae* and *Thymelaceae* (Evans & Taylor, 1983) contains a number of chemically related substances with similar biological activity. Daphnoretin, a bicoumarin natural product isolated from a Thymelaceous plant *Wikstroemia indica*, was also found to directly activate PKC which in turn elicited the respiratory burst in neutrophils (Wang *et al*., 1995). Kazinol B, a natural isoprenylated flavan isolated from the roots bark of *Broussonetia papyrifera* (L.) Vent. (*Moraceae*) (Ikuta *et al*., 1985), has been shown to inhibit platelet aggregation and cyclo-oxygenase activity (Lin *et al*., 1996), and was found to stimulate the respiratory burst in rat neutrophils in our recent preliminary study. In this study, we have examined the stimulatory effect of kazinol B on the respiratory burst in rat peripheral neutrophils and also investigated the underlying mechanism.

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*., 1995). Purified neutrophils containing >95% viable cell were normally resuspended in Hanks' balanced salt solution (HBSS) containing 10 mM HEPES, pH 7,4, and 4 mM $NaHCO_3$, and kept in an ice bath before use.

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

The generation of $O_2^{\cdot-}$ in neutrophil suspensions was determined by the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c* as described previously (Wang *et al*., 1995). Briefly, the assay mixture contained neutrophils (1×10^6 cells) and 0.5 mg ml^{-1} of ferricytochrome *c* in a final volume of 1.5 ml. The reference cuvette also received 6.7 μg ml^{-1} of SOD. Absorbance changes of the reduction of ferricytochrome *c* were monitored continuously at 550 nm in a double-beam spectrophotometer (Hitachi, U-3210).

Measurement of NADPH oxidase activity in a cell-free system

Neutrophils (1×10^8 cells ml^{-1}) were treated with 2.5 mM diisopropyl fluorophosphate (DFP) and disrupted in Tris buffer (composition in mM: sucrose 340, Tris-HCl 10, pH 7.0, benzamidine 10, and phenylmethylsulphonyl fluoride (PMSF) 2) by sonication, and fractionated as previously described (Wang *et al*., 1997a). The cytosol and the plasma membrane fractions were stored at −70°C until use. The plasma membrane and the cytosol fractions were mixed in 1.5 ml of assay buffer (composition in mM: sucrose 170, NaN_3 2, $MgCl_2$ 1, EGTA 1, KH_2PO_4-NaOH 65, pH 7.0) contained 10 μM FAD, 3 μM GTPγS, 0.25 mg ml^{-1} of ferricytochrome *c*, 50 μM NADPH, and activated by kazinol B or arachidonate (AA) at 37°C. The $O_2^{\cdot-}$ generation in a cell-free system was determined by the SOD-inhibitable reduction of ferricytochrome *c* as described above.

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

Neutrophils (1×10^7 cells ml^{-1}) were suspended in HEPES buffer (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 2.5 μM fura 2-AM (Molecular Probes) (Wang *et al*., 1995). After washing, cells were resuspended in HEPES buffer with 0.05% (w/v) BSA. The fluorescence 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 cellular free Ca^{2+} concentration was performed as previously described (Grynkiewicz *et al*., 1985).

Determination of inositol phosphate levels

Neutrophils (3×10^7 cells ml^{-1}) were loaded with *myo*-[^{3}H]inositol (Amersham, 83 Ci $mmol^{-1}$) at 37°C for 2 h (Wang *et al*., 1997a). After the stimulation with kazinol B or formylmethionyl-leucyl-phenylalanine (fMLP) in the presence of 10 mM LiCl, 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 monophosphate (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 (Downes & Michell, 1981).

Immunoblot analysis of the subcellular distribution of PKC

Neutrophils (4×10^7 cells ml^{-1}) were stimulated with kazinol B or phorbol 12-myristate 13-acetate (PMA) at 37°C, and then disrupted in Tris buffer with 1 mM EGTA and 10 μg ml^{-1} of leupeptin and antipain by sonication, and fractionated as previously described (Wang *et al*., 1997b). The cytosol and the particulate fractions were stored at −70°C until use. The particulate and the cytosol fractions were boiled in Laemmli sample buffer, subjected to SDS–PAGE, and then transferred to Immobilon-P membrane (Millipore). These membranes were incubated with 5% (w/v) non-fat milk in TST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% (v/v) Tween-20) and probed with a specific mouse monoclonal antibodies against PKC isoforms (Transduction, 1:500 to 1:2000 dilution in TST buffer with 0.5% (w/v) non-fat milk). Detection was made using the enhanced chemiluminescence (ECL) kit (Amersham).

[^{3}H]Phorbol 12,13-dibutyrate binding assay

For the preparation of cytosolic PKC, neutrophils were disrupted by sonication. The cytosolic fraction was subjected to DE-52 cellulose column (Whatman) to obtain partially purified PKC (Wang *et al*., 1995). Reaction mixture contained 20 mM Tris-HCl, pH 7.2, 100 mM KCl, 50 μg ml^{-1} of phosphatidylserine, 0.5 mM $CaCl_2$, 30 nM [^{3}H]phorbol 12,13-dibutyrate (PDB) (DuPont NEN, 18.6 Ci $mmol^{-1}$), cytosolic PKC and test drugs at 30°C in a total volume of 250 μl. For the determination of non-specific binding, 30 μM PDB was present in the reaction mixture. After addition of ice-cold 0.5% (v/v) dimethylsulfoxide (DMSO) solution to terminate the reaction, mixture was poured onto Whatman GF/C filter. The filter was then washed and counted (Tanaka *et al*., 1986).

Detection of neutrophil proteins phosphorylated on tyrosine residues

Neutrophils (1×10^7 cells ml^{-1}) in HBSS were stimulated with kazinol B or fMLP plus dihydrocytochalasin (CB) at 37°C. Reactions were terminated by adding stop solution (20% (w/v) trichloroacetic acid, 1 mM PMSF, 7 μg ml^{-1} of aprotinin and pepstatin, 2 mM *N*-ethylmaleimide, 100 mM NaF, 5 mM 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 Immobilon-P membrane, blocked with 5% (w/v) non-fat milk in TST buffer and probed with mouse monoclonal anti-phosphotyrosine antibody (PY-20) (Transduction, 1:1000 dilution in TST buffer with 0.5% (w/v) non-fat milk) (Wang *et al*., 1997a). Detection was made using the ECL kit.

Measurement of PLD activity

Neutrophils (5×10^7 cells ml^{-1}) were suspended in HEPES buffer and loaded with 10 μCi 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine (Amersham, 150 Ci $mmol^{-1}$) at 37°C for 75 min, then washed and resuspended in HEPES buffer with 0.05% (w/v) BSA. The assay mixture containing 1 mM $CaCl_2$, 0.5% (v/v) ethanol, and stimulated with kazinol B or fMLP/CB at 37°C. The lipids in the reaction mixture were extracted, dried, and then separated on silica gel 60 plate (Wang *et al*., 1997a). 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), and then dried and developed again to the top using the upper phase of the solvent system consisting of ethylacetate:iso-octane:acetic acid:water (110:50:20:100, v/v/v/v). The radioactivity of [^{3}H]products were directly quantified with a PhosphorImager (Molecular Dynamics 445 SI) using ImageQuaNT software.

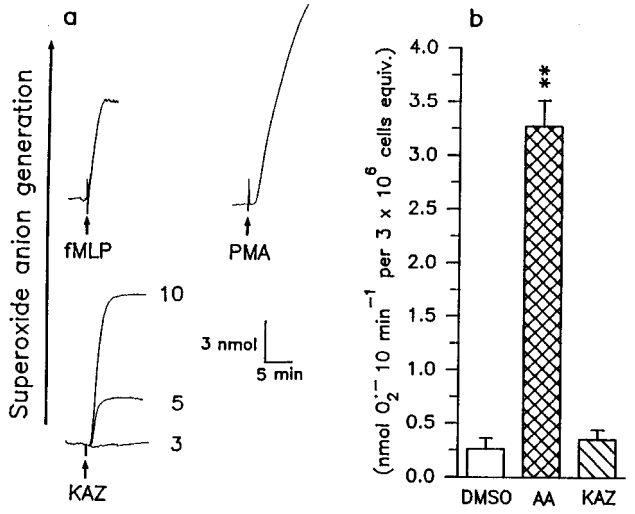

Figure 1 Stimulation of $O_2^{\cdot-}$ generation by kazinol B (KAZ) in rat neutrophil suspension but not in a cell-free system. (a) Neutrophils (1×10^6 cells ml^{-1}, at 37°C) were stimulated with 0.3 μM fMLP plus 5 μg ml^{-1} of CB, 1 nM PMA or various concentrations of kazinol B (3–10 μM). Original recordings from a representative experiment are shown. Similar results were obtained in 4 separate experiments. (b) In a cell-free system, DMSO (vehicle control), 100 μM AA or 10 μM kazinol B was added to the reaction mixture contained neutrophil cytosol and membrane fractions. $O_2^{\cdot-}$ generation was measured by continuously detecting the absorbance changes of ferricytochrome *c*. Values are expressed as means±s.e.mean of 3–5 separate experiments. $^{**}P<0.01$ compared to the control value.

Drugs

Kazinol B (7,3′-dihydroxy-2′-(3,3-dimethylallyl)-4′,5′-(2,2-dimethylchromeno)-flavan) was isolated and purified from *Broussonetia papyrifera* as previously described (Ikuta *et al*., 1985). The purity of kazinol B was identified by h.p.l.c. (>99% purity) and n.m.r. (without impurity signals). All chemicals were purchased from Sigma Chemical Co. (St. Louis, U.S.A.) except for the following: dextran T-500 (Pharmacia Biotech., Uppsala, Sweden); Hanks' balanced salt solution (Gibco BRL Co., Gaithersburg, U.S.A.); 4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)-imidazole (SB203580) (Alexis Co., Läufelfingen, Switzerland); [γ-^{32}P]ATP (Amersham International plc., Buckinghamshire, U.K.); 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122) (Biomol Research Lab. Inc., Plymouth Meeting, U.S.A.).

Statistical analysis

Statistical analyses were performed using the Bonferroni *t*-test method after analysis of variance and the Student's *t*-test. 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. Data are presented as means±s.e.mean.

Results

Kazinol B stimulated $O_2^{\cdot-}$ generation in rat neutrophils

Like fMLP and PMA, addition of kazinol B into neutrophil suspensions evoked $O_2^{\cdot-}$ generation in a concentration-dependent manner (max. 13.2±1.4 nmol $O_2^{\cdot-}$ 10 min^{-1} per 10^6 cells at 10 μM kazinol B), however, with the difference of activation profiles among the three stimulants as assessed by the detection of SOD-inhibitable ferricytochrome *c* reduction (Figure 1a). fMLP induced a rapid and transient $O_2^{\cdot-}$ generation, and PMA induced a slow onset but long lasting response, whilst a slow onset but transient $O_2^{\cdot-}$ generation profile was evoked by kazinol B. In a cell-free oxidase system, addition of 100 μM AA to the mixture of neutrophil cytosol and membrane fractions initiated $O_2^{\cdot-}$ generation. Kazinol B 10 μM, or up to 30 μM (data not shown), was without effect on a cell-free system (Figure 1b).

Cells preincubated with 0.3 μM fMLP or 1 nM PMA for 20 min in the absence of ferricytochrome *c* greatly attenuated the $O_2^{\cdot-}$ generation by the subsequent addition of the same stimulants in the presence of ferricytochrome *c*. In contrast, the $O_2^{\cdot-}$ generation caused by fMLP or PMA was enhanced in cells pretreated with PMA and fMLP, respectively (Figure 2). Pretreatment of cells with fMLP or PMA significantly enhanced the 10 μM kazinol B-induced $O_2^{\cdot-}$ generation.

In the presence of 3 mM EGTA to remove extracellular Ca^{2+}, fMLP (0.3 μM)- and kazinol B (10 μM)-induced $O_2^{\cdot-}$ generation were both reduced, but did not change the response caused by 3 nM PMA under the same conditions (Figure 3a). The protein kinase inhibitor staurosporine (Tamaoki *et al*., 1986) at the concentration (30–100 nM) which significantly inhibited the PMA- and kazinol B-induced $O_2^{\cdot-}$ generation had no effect on the fMLP-induced response (Figure 3b). Cells pretreated with a p38 MAPK inhibitor SB203580 (Cuenda *et al*., 1995) 10 μM or a PI3K inhibitor wortmannin (Arcaro & Wymann, 1993) 0.1–1 μM significantly attenuated the fMLP-induced $O_2^{\cdot-}$ generation, whilst left alone the PMA- (data not shown) and kazinol B-induced responses (Figure 3c).

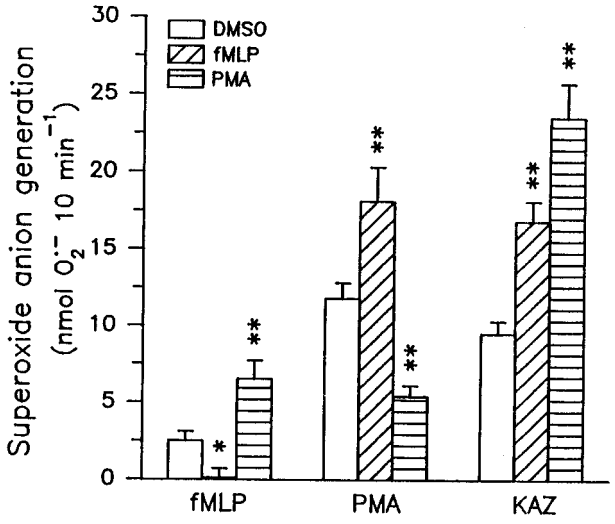

Figure 2 Effect of sequential addition of formylmethionyl-leucyl-phenylalanine (fMLP), phorbol 12-myristate 13-acetate (PMA) and kazinol B (KAZ) on $O_2^{\cdot-}$ generation in rat neutrophils. Neutrophils (1×10^6 cells ml^{-1}, at 37°C) were pretreated with DMSO (vehicle control), 0.3 μM fMLP or 1 nM PMA for 20 min in the absence of ferricytochrome *c*, and then stimulated with 0.3 μM fMLP plus 5 μg ml^{-1} of CB, 1 nM PMA or 10 μM kazinol B in the presence of 0.5 mg ml^{-1} of ferricytochrome *c*. Values are expressed as means $\pm$s.e.mean of 6–8 separate experiments. $^*P<0.05$, $^{**}P<0.01$ compared to the corresponding control values.

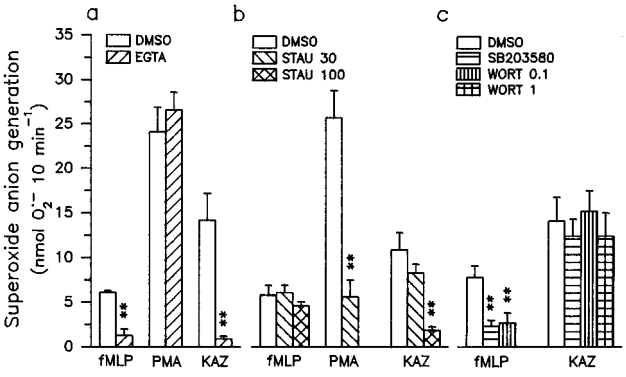

Figure 3 Effect of EGTA, staurosporine (STAU), SB203580 and wortmannin (WORT) on kazinol B (KAZ)-stimulated $O_2^{\cdot-}$ generation in rat neutrophils. Neutrophils (1×10^6 cells ml^{-1}, at 37°C) were preincubated with DMSO (vehicle control), (a) 3 mM EGTA, (b) staurosporine (30 and 100 nM), (c) 10 μM SB203580 or wortmannin (0.1 and 1 μM) for 3 min before stimulation with 0.3 μM fMLP plus 5 μg ml^{-1} of CB, 3 nM PMA or 10 μM kazinol B. Values are expressed as means $\pm$s.e.mean of 3–4 separate experiments. $^{**}P<0.01$ compared to the corresponding control values.

Kazinol B stimulated [Ca^{2+}]$_i$ elevation and IP$_3$ formation

In the Ca^{2+}-containing medium, kazinol B showed a concentration-dependent stimulation of $[Ca^{2+}]_i$ elevation in fura 2-loaded neutrophils with a lag and slow rate of rise activation profile as assessed by the increase in fura 2 fluorescence (Figure 4a). A rapid and transient elevation of $[Ca^{2+}]_i$ is generally observed after addition of 0.3 μM fMLP. In the absence of extracellular Ca^{2+}, the profiles of kazinol B-induced responses appear to be similar to those obtained in a Ca^{2+}-containing medium but with a reduced $[Ca^{2+}]_i$ change (Figure 4a,b). Cells pretreated with a PLC inhibitor U73122 (Smith *et al*., 1990) 2 μM abolished the Ca^{2+} response caused

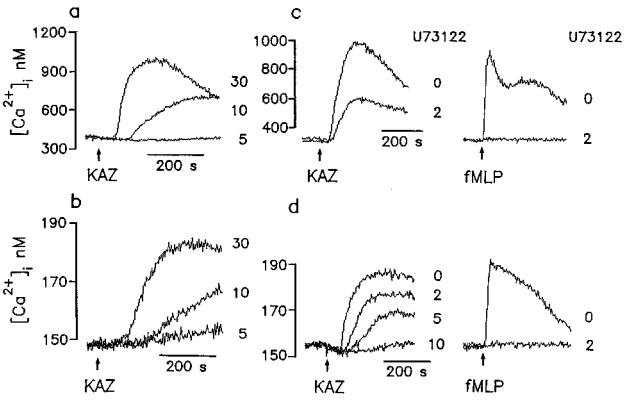

Figure 4 Effect of kazinol B (KAZ) on $[Ca^{2+}]_i$ of rat neutrophils. Fura 2-loaded neutrophils were stimulated (arrow) with various concentrations of kazinol B (5–30 μM) in the (a) presence or (b) absence of 1 mM extracellular Ca^{2+} at 37°C. In some experiments, cells were preincubated with U73122 (2–10 μM) for 3 min before stimulation (arrow) with 0.3 μM fMLP or 30 μM kazinol B in the (c) presence or (d) absence of 1 mM extracellular Ca^{2+}. $[Ca^{2+}]_i$ change was monitored as the fluorescence change. The traces shown are representative of 4–6 separate experiments.

by fMLP whether the extracellular Ca^{2+} was present or not. Kazinol B (30 μM)-induced Ca^{2+} response was either greatly attenuated (54.9$\pm$5.1% inhibition, $P<0.01$) by 2 μM U73122 in the presence of extracellular Ca^{2+} (Figure 4c) or concentration-dependently inhibited by U73122 (IC_{50} 4.7$\pm$0.6 μM) in the absence of Ca^{2+} in the medium (Figure 4d). Pretreatment of cells with 1 μg ml^{-1} of pertussis toxin for 2 h at 37°C abolished the fMLP-induced response, whereas, the kazinol B-induced response remained relatively unchanged under these conditions (data not shown).

Addition of 0.3 μM fMLP to *myo*-[^{3}H]inositol-loaded neutrophils in the presence of 10 mM LiCl at 37°C for 10 s, evoked a significant generation of IP_2 and IP_3. Cells treated with 30 μM kazinol B for 10 s (data not shown) or 0.5 min did not stimulate the IP_2 and IP_3 generation. However, a significant increase in cellular IP_2 and IP_3 levels were observed following the addition of 30 μM kazinol B for 1 and 3 min (Figure 5a). Under the same conditions, 10 μM kazinol B, the concentration which maximally induced $O_2^{\cdot-}$ generation, did not significantly affect the cellular IP_2 and IP_3 levels (data not shown). In a Ca^{2+}-free medium, addition of fMLP to fura 2-loaded neutrophil suspension greatly attenuated the $[Ca^{2+}]_i$ changes caused by a subsequent stimulation of cells with fMLP (data not shown). The kazinol B (30 μM)-induced $[Ca^{2+}]_i$ changes were also significantly inhibited (45.7$\pm$4.5% inhibition, $P<0.01$) in cells pretreated with 0.3 μM fMLP. Moreover, pretreatment of cells with 30 μM kazinol B abolished the $[Ca^{2+}]_i$ changes following the stimulation with 0.3 μM fMLP (Figure 5b,c).

Kazinol B stimulated PKC activation

In order to determine the subcellular distribution of PKC, immunoblot analysis was carried out by using specific mouse monoclonal antibodies against PKC isoforms. In the resting cells, PKC isoforms were enriched in the cytosol fraction. Upon exposure to 0.2 μM PMA, PKC-α and θ were translocated from cytosol to particulate fraction. In addition, the cellular PKC-θ (and -δ, -ε, -μ, data not shown) but not PKC-α (and -β, -γ, data not shown) became associated with the particulate fraction of cells in response to the stimulation with

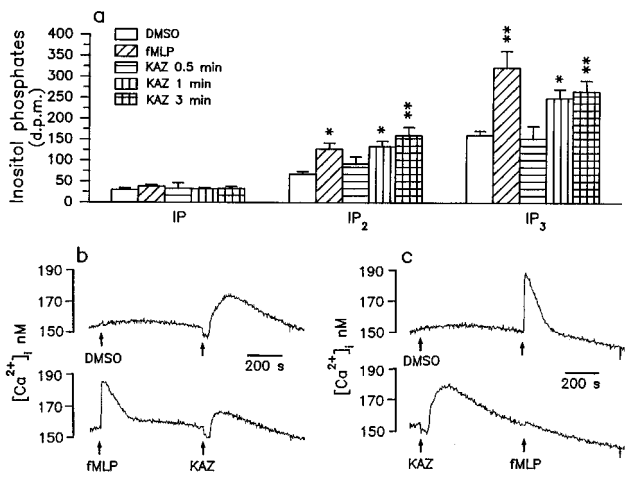

Figure 5 Kazinol B (KAZ) stimulates inositol phosphates formation and empties the Ca^{2+} pool used by formylmethionyl-leucyl-phenylalanine (fMLP) in rat neutrophils. (a) *myo*-[^{3}H]Inositol-loaded cells were treated with DMSO (vehicle control), 0.3 μM fMLP for 10 s, or 30 μM kazinol B for 0.5, 1 and 3 min at 37°C in the presence of 10 mM LiCl. After extraction and separation, the levels of IP, IP_2, and IP_3 were counted. Values are expressed as means ± s.e.mean of 4 separate experiments. $^*P<0.05$, $^{**}P<0.01$ compared to the corresponding control values. In the absence of 1 mM extracellular Ca^{2+}, fura 2-loaded cells were treated with (b) DMSO or 0.3 μM fMLP and then 30 μM kazinol B, or with (c) DMSO or 30 μM kazinol B followed by 0.3 μM fMLP. $[Ca^{2+}]_i$ change was monitored as the fluorescence change. The traces shown are representative of 4 separate experiments.

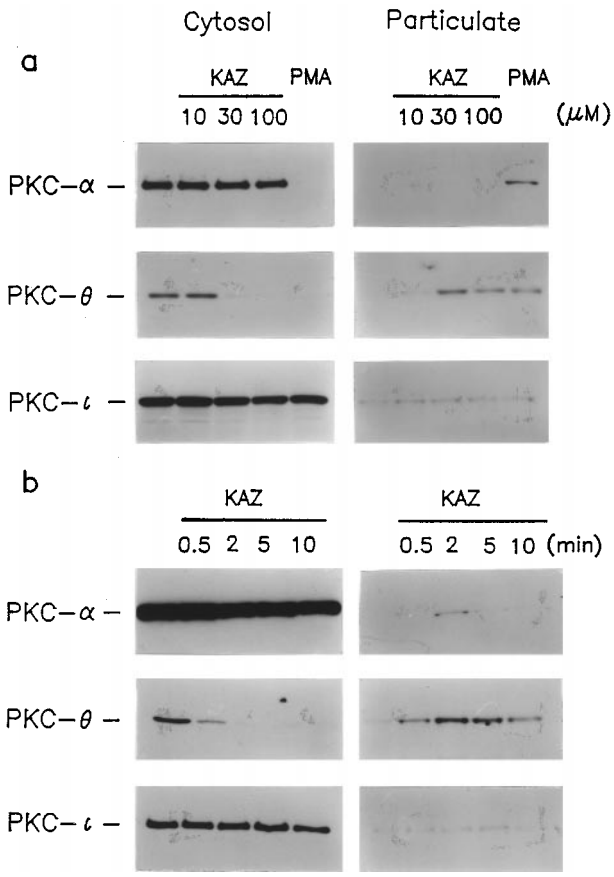

Figure 6 Effect of kazinol B (KAZ) on PKC redistribution. Neutrophils (4×10^7 cells) were treated with (a) DMSO (lane 1, vehicle control), 10–100 μM kazinol B for 10 min or 0.2 μM PMA for 5 min, or with (b) DMSO (lane 1, vehicle control) or 30 μM kazinol B for 0.5–10 min at 37°C. Cells were then disrupted and centrifuged. Particulate and cytosol proteins were subjected to SDS–PAGE, immunoblotted with a specific monoclonal antibody to PKC-α, PKC-θ or PKC-ι. The results shown are representative of 4 separate experiments.

kazinol B ($\geqslant$30 μM) for 10 min. The subcellular distribution of PKC-ι was affected by neither PMA nor kazinol B (Figure 6a). For the kinetic analysis, the labelled intensity of PKC-α (and -β, -γ, data not shown) associated with the particulate fraction was increased at 2 min after exposure of cells to 30 μM kazinol B and then decreased. Whereas, the accumulation of PKC-θ (and -δ, -ε, -μ, data not shown) in the particulate fraction was apparent at the earliest time analysed, 0.5 min, attained maximal level at 2 min and then progressively declines (Figure 6b).

The binding of [^{3}H]PDB to neutrophil cytosolic PKC was determined by means of the rapid filtration assay. Non-specific bound [^{3}H]PDB was less than 15% of the total bound (270.0 ± 17.1 vs 1873.0 ± 52.3 d.p.m.). Pretreatment of cytosolic PKC for 3 min with 10 μM kazinol B or 10 μM 1-oleoyl-2-acetylglycerol (OAG) showed significant inhibition of the [^{3}H]PDB binding (30.6 ± 3.4% inhibition for kazinol B, $P<0.05$; 70.1 ± 4.0% inhibition for OAG, $P<0.01$).

Effect of kazinol B on protein tyrosine phosphorylation and PLD activity

Pretreatment of cells with a tyrosine kinase inhibitor genistein (Akiyama *et al*., 1987) 100 μM for 30 min greatly reduced the $O_2^{\cdot-}$ generation caused by 0.3 μM fMLP but left alone the PMA (1 nM)- and kazinol B (10 μM)-induced responses (Figure 7a). In addition, immunoblotting of lysates of rat neutrophils with specific monoclonal anti-phosphotyrosine antibody revealed that several proteins were labelled to variable extent in the resting cells. Stimulation of cells with 0.1 μM fMLP for 1 min induced a substantial phosphotyrosine accumulation on a protein of 62 kDa. Whereas, incubation of cells with 10 μM kazinol B for 1, 5, and 10 min had no effect on the labelled intensity (Figure 7b).

We next determined whether the PLD activity of neutrophils was activated by kazinol B. PLD catalyzes the hydrolysis primarily of phosphatidylcholine to produce PA. In the presence of ethanol, PA *via* a transphosphatidylation reaction yields phosphatidylethanol (PEt). Cells pretreated with 0.5% (v/v) ethanol greatly reduced the $O_2^{\cdot-}$ generation caused by 0.3 μM fMLP but had no effect on the PMA (3 nM)- and kazinol B (10 μM)-induced responses (Figure 7c). Moreover, fMLP (1 μM) stimulated the PA and PEt generation in 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine-loaded neutrophils in the presence of 0.5% (v/v) ethanol. Whereas, cells treated with 10 (data not shown) or 30 μM kazinol B for 0.5, 1 or 3 min failed to alter the mass of PA and PEt (Figure 7d).

Discussion

Stimulation of neutrophils leads to the generation of $O_2^{\cdot-}$ that is believed to participate in the microbicidal function of these cells. Activation of NADPH oxidase likely occurs through more than one signal transduction pathway, depending on the type of stimuli. In the present study we show that a natural isoprenylated flavan kazinol B stimulates the respiratory burst in rat neutrophils. The neutrophil $O_2^{\cdot-}$ generation caused by kazinol B was found to be transient with a lag time. This result

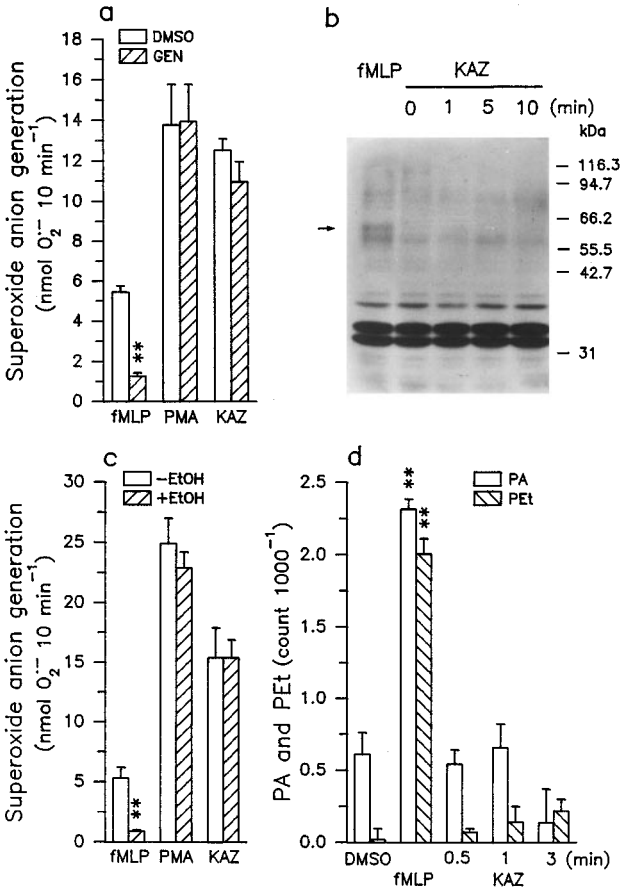

Figure 7 The role of protein tyrosine phosphorylation and PLD activity on kazinol B (KAZ)-stimulated $O_2^{\cdot-}$ generation in rat neutrophils. Neutrophils (1×10^6 cells ml^{-1}, at 37°C) were preincubated with (a) DMSO (vehicle control) or 100 μM genistein (GEN) for 30 min before addition of 0.3 μM fMLP plus 5 μg ml^{-1} of CB, 1 nM PMA or 10 μM kazinol B, or (c) with or without 0.5% (v/v) ethanol (EtOH) for 3 min before addition of 0.3 μM fMLP plus 5 μg ml^{-1} of CB, 3 nM PMA or 10 μM kazinol B for $O_2^{\cdot-}$ generation. Values are expressed as means±s.e.mean of 4–5 separate experiments. $^{**}P<0.01$ compared to the corresponding control values. (b) Neutrophils were stimulated with 0.1 μM fMLP plus 5 μg ml^{-1} of CB for 1 min or 10 μM kazinol B for 0–10 min at 37°C, and then sedimented, boiled in Laemmli sample buffer, and subjected to SDS–PAGE, immunoblotted with a specific monoclonal antibody to phosphotyrosine. The arrow points to the proteins of 62 kDa. The results shown are representative of 3 separate experiments. (d) 1-*O*-[^{3}H]Octadecyl-*sn*-glycerol-3-phosphocholine-loaded cells were treated with DMSO (vehicle control), 1 μM fMLP plus 5 μg ml^{-1} of CB for 0.5 min, or 30 μM kazinol B for 0.5–3 min at 37°C in the presence of 0.5% (v/v) ethanol. The lipids were extracted and separated, the PA and PEt were counted. Values are expressed as means±s.e.mean of 3 separate experiments. $^{**}P<0.01$ compared to the corresponding control values.

contrasts with the rapid and transient or the slow and long lasting responses induced by fMLP and PMA, respectively. Unlike AA, which could mimic the effect of p47^{phox} phosphorylation (Fuchs *et al*., 1995) and produce active NADPH oxidase, kazinol B did not evoke $O_2^{\cdot-}$ generation in a cell-free system, suggesting that kazinol B interacted with certain signal transduction steps rather than directly activated NADPH oxidase. Since the profiles of kazinol B-induced response differ from those of fMLP and PMA, one can speculate that they may use different signal transduction mechanisms to activate NADPH oxidase.

It is generally found that prior exposure of neutrophils to one stimulus could potentiate the respiratory burst to the subsequent addition of a second heterologous stimulus (Della Bianca *et al*., 1986). The mechanism of priming has yet to be defined. In contrast, exposure of cells to sequential concentrations of homologous stimuli desensitized the $O_2^{\cdot-}$ generation has been observed (McPhail *et al*., 1984). fMLP activates neutrophils by binding to its receptor and activating the pertussis toxin-sensitive G protein-linked receptor on the membrane (Polakis *et al*., 1988). In contrast, PMA bypasses membrane receptors and directly activates PKC (Castagna *et al*., 1982). Pretreatment of neutrophils with fMLP or PMA significantly enhanced the stimulation of $O_2^{\cdot-}$ generation by kazinol B. The evidence points to the activation of respiratory burst by kazinol B *via* different signalling pathways. Whether kazinol B acts through its own cell-surface receptor, or a known cell-surface receptor or directly activates intracellular signalling pathways needs further investigation.

There is a general consensus that tyrosine phosphorylation and PLD activation play regulatory roles in the signal transduction pathway leading to the respiratory burst (Bonser *et al*., 1989; Torres *et al*., 1993), although the identities of many of the tyrosine-phosphorylated substrates present in stimulated neutrophils remain unknown. In this study, the most prominent phosphotyrosine-containing protein was 62 kDa after stimulation with fMLP. Kazinol B did not affect the tyrosine phosphorylation on 62 kDa, nor on other proteins. PLD catalyzes the hydrolysis primarily of phosphatidylcholine to produce PA. PA could act on the respiratory burst through the activation of PKC or NADPH oxidase (Bellavite *et al*., 1988). Cells pretreated with ethanol, to reduce the generated PA, or with a tyrosine kinase inhibitor genistein (Akiyama *et al*., 1987) attenuated the respiratory burst caused by fMLP but not by PMA and is consistent with previous reports (Rossi *et al*., 1990; Yasui *et al*., 1994). The results that ethanol and genistein had no effect on the kazinol B-induced response preclude the involvement of these two signalling pathways in kazinol B-induced respiratory burst.

The lipid kinase PI3K has been reported to play an important role in regulating neutrophil $O_2^{\cdot-}$ generation (Arcaro & Wymann, 1993). Recently, evidence pointing to the existence of another signalling pathway, p38 MAPK, which participates in the neutrophil $O_2^{\cdot-}$ generation (Nick *et al*., 1997), although p38 MAPK activation alone is probably not sufficient to induce this response. Utilizing the p38 MAPK inhibitor SB203580 (Cuenda *et al*., 1995) and the PI3K inhibitor wortmannin (Arcaro & Wymann, 1993), we can investigate the potential role of these two signalling pathways in kazinol B-induced response. The inhibition of p38 MAPK and PI3K activation affected fMLP- but not PMA-induced respiratory burst reconciling these earlier reports (Arcaro & Wymann, 1993; Nick *et al*., 1997). The finding that the kazinol B-induced response was not modified by the both inhibitors, obviates the requirement for these two signalling pathways in kazinol B-induced $O_2^{\cdot-}$ generation.

fMLP- but not PMA-induced $O_2^{\cdot-}$ generation is a Ca^{2+}-dependent process (Lehmeyer *et al*., 1979). Kazinol B-induced $O_2^{\cdot-}$ generation was attenuated in the presence of EGTA, suggesting the $[Ca^{2+}]_i$ elevation signalling pathway is involved in the activation of oxidase. The finding that kazinol B stimulated $[Ca^{2+}]_i$ elevation in neutrophils confirms this hypothesis. However, kazinol B differs from fMLP in the profiles of $[Ca^{2+}]_i$ changes and the resistance to pertussis toxin inhibition suggest that an additional mechanism of action must contribute to the kazinol B-induced response. The rise in $[Ca^{2+}]_i$ caused by fMLP results from both Ca^{2+} release from IP_3-sensitive internal stores and Ca^{2+} influx from the extracellular environment (Meldolesi *et al*., 1991). fMLP- as

well as kazinol B-induced $[Ca^{2+}]_i$ changes were inhibited by a PLC inhibitor U73122 (Smith *et al*., 1990) providing pharmacological evidence for the role of IP_3 in kazinol B-induced response.

Additional studies show that kazinol B stimulated IP_2 and IP_3 formation with a slow onset. The lag of IP_3 formation appears parallel to the lag of $[Ca^{2+}]_i$ elevation strengthening the role of IP_3 in initiating the $[Ca^{2+}]_i$ changes. The results that pretreatment with fMLP or kazinol B greatly reduced the $[Ca^{2+}]_i$ changes caused by a subsequent addition of kazinol B or fMLP, respectively, reinforced the proposal that kazinol B removed Ca^{2+} from the IP_3-sensitive Ca^{2+} store. The ability of these two stimulants act through different mechanisms of action is suggested by the following observations. First, a slow onset profile of kazinol B-induced IP_3 formation is different from that of fMLP (max. at 10 s) (Smith *et al*., 1990). Second, the kazinol B-induced response is more resistant than that of fMLP to the inhibition by U73122. The slow increase in $[Ca^{2+}]_i$ was previously reported in a Ca^{2+}-ATPase inhibitor cyclopiazonic acid (CPA)-induced response (Wang, 1996), which is also less sensitive to be suppressed by U73122. Since CPA evokes $[Ca^{2+}]_i$ changes without increasing cellular IP_3 levels (Demaurex *et al*., 1992), it is unlikely that kazinol B and CPA act *via* the same mechanism of action. It has been reported that neutrophil contains PLC-β and -γ (Dusi *et al*., 1994; Jiang *et al*., 1997), which are regulated by G protein and tyrosine phosphorylation, respectively. Since genistein did not affect the kazinol B-induced $O_2^{\cdot-}$ generation, precluding the role of PLC-γ activation in kazinol B-induced response. It is uncertain whether the kazinol B-induced cellular IP_3 formation occurs through a pertussis toxin-insensitive G protein-linked surface receptor or acts directly on PLC. The precise mechanism by which kazinol B induced IP_3 formation remain to be determined.

PKC has been proposed to play a role in the activation of $O_2^{\cdot-}$ generation (Cox *et al*., 1986). PKC participates in the activation of NADPH oxidase probably through the phosphorylation of $p47^{phox}$ (Kramer *et al*., 1988). It is generally accepted that the fMLP-induced activation of oxidase occurs *via* mainly PKC-independent signal transduction pathways, whereas the PMA-induced activation occurs largely *via* PKC pathway. Therefore, the protein kinase inhibitor staurosporine (Tamaoki *et al*., 1986) is more potent in the inhibition of PMA- than fMLP-induced respiratory burst (Kessels *et al*., 1993). The observations that staurosporine inhibited the kazinol B-induced response, suggesting the respiratory burst induced by kazinol B is PKC-dependent. However, the inhibition by staurosporine appears less potent in kazinol B- than in PMA-induced responses indicates that the PKC-dependent signalling pathway play a more important role in PMA- than in kazinol B-stimulated neutrophil $O_2^{\cdot-}$ generation.

In resting cells PKC is found in the cytosol, and PKC becomes tightly associated with the particulate fraction upon activation (Wolfson *et al*., 1985). This enzyme translocation can be used as an index of the enzyme activation. Recently we found that rat neutrophil contains PKC isoforms of four categories: conventional PKCs (α, β and γ), novel PKCs (δ, ε and θ), atypical PKCs (ι, λ and ζ), and PKC-μ, although PKC-λ and -ζ are barely detected (Tsao & Wang, 1997). However, the isoform specific regulation and function remain unclear yet. Like PMA, kazinol B activated the neutrophil PKC. The atypical PKC isoforms are affected neither by phorbol esters (Blobe *et al*., 1996) nor by kazinol B as assessed by the detection of immunoreactive PKC isoforms in the particulate fraction suggest that the conventional and novel PKC isoforms might contribute to the PKC activation upon exposure of cells to kazinol B. Moreover, the novel PKC isoforms may play the major role since these are more sensitive and more rapidly activated by kazinol B than conventional isoforms. Phorbol esters compete with DAG for the same binding site of PKC regulatory region. Since kazinol B only partially inhibited the [^{3}H]PDB binding to PKC, we speculate that the action on DAG binding site may not contribute greatly to the activation of PKC by kazinol B. It has been reported that PKC can be activated by other second messengers besides DAG (Blobe *et al*., 1996). These include the products of PI3K (phosphatidylinositol bis- and trisphosphates); product of phospholipase A_2 (AA); and product of PLD (PA). The observations that kazinol B-induced $O_2^{\cdot-}$ generation is not mediated by PI3K and PLD rule out the possibility that the products of these two signalling pathways play roles in kazinol B-induced PKC activation. However, further study will be required to clarify the possibility of AA plays a role in kazinol B-induced response since the $[Ca^{2+}]_i$ elevation caused by kazinol B may probably activate PLA_2.

In summary, we have shown that plant product kazinol B is capable of stimulating $O_2^{\cdot-}$ generation in rat neutrophils. The underlying signal transduction mechanism was also assessed. The stimulation of respiratory burst by kazinol B is probably not through the activation of p38 MAPK, PI3K, PLD and protein tyrosine phosphorylation but *via* the elevation of $[Ca^{2+}]_i$, through the IP_3 generation, and the activation of PKC. Although a little higher concentration range of kazinol B (10–30 μM) was required to activate PKC and elevate $[Ca^{2+}]_i$ than was active in the $O_2^{\cdot-}$ generation, the previous studies using the combination of calcium ionophore, which increases $[Ca^{2+}]_i$ but is not a sufficient signal for $O_2^{\cdot-}$ generation (Korchak *et al*., 1988), with phorbol esters have demonstrated the synergistic effect in stimulating $O_2^{\cdot-}$ generation in neutrophils (Penfield & Dale, 1984; Robinson *et al*., 1984). Therefore, it is plausible to speculate that the kazinol B-induced $O_2^{\cdot-}$ generation is attributable to the synergism between the elevation of $[Ca^{2+}]_i$ and the activation of PKC. Since neutrophil adhesion, phagocytosis and degranulation processes are probably mediated by PKC activation and elevation of $[Ca^{2+}]_i$ (O'Flaherty *et al*., 1991; Bengtsson *et al*., 1993; Sullivan *et al*., 1994), kazinol B may also exert stimulatory effect on these responses. This needs further investigation.

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