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Evidence for the involvement of protein kinase C inhibition by norathyriol in the reduction of phorbol ester-induced neutrophil superoxide anion generation and aggregation

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Abstract

Norathyriol, a xanthone aglycone, inhibited superoxide anion ($O_2^{\cdot -}$) generation and O_2 consumption in phorbol 12-myristate 13-acetate (PMA)-activated rat neutrophils in a concentration-dependent manner. In addition, norathyriol inhibited PMA- but enhanced formylmethionyl–leucyl–phenylalanine (fMLP)-induced neutrophil aggregation. Norathyriol suppressed neutrophil cytosolic protein kinase C as well as rat brain protein kinase C over the same range of concentrations at which it inhibited the respiratory burst. Norathyriol did not affect [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDB) binding to neutrophil cytosolic protein kinase C, but effectively attenuated trypsin-treated rat brain protein kinase C activity. Moreover, norathyriol was found to be a noncompetitive inhibitor with respect to ATP and peptide substrate (N-terminal acetylated, amino acid sequence 4–14 of the myelin basic protein, Ac-MBP-(4–14)). Unlike staurosporine, norathyriol did not affect porcine heart protein kinase A activity. On the immunoblot analysis of protein kinase C subcellular distribution, the PMA-induced translocation of protein kinase C-β from the cytosol to the membrane was not affected by norathyriol. These results show that the inhibition by a plant product, norathyriol, of PMA-induced respiratory burst and aggregation is, at least partly, attributed to the direct suppression of protein kinase C activity through blockade of the catalytic region, but is not due to interference with the membrane translocation of protein kinase C during PMA-induced cell activation. © 1997 Elsevier Science B.V.

Keywords: Norathyriol; Neutrophil, rat; Respiratory burst; Aggregation; Protein kinase C; Phorbol 12-myristate 13-acetate; Translocation

1. Introduction

The neutrophil is a prominent inflammatory cell. The primary physiological function of neutrophils is to protect the host from infections. When neutrophils are exposed to soluble stimuli or ingest solid particles, their O_2 uptake from the surrounding medium is increased and large amounts of superoxide anion ($O_2^{\cdot -}$) are generated, which subsequently leads to the formation of other toxic O_2 metabolites (Badwey and Karnovsky, 1980). These reactive O_2 species produced during the respiratory burst process are believed to serve as bactericidal agents (Baehner et al., 1975). A functional defect of this process is an important contributory factor to an increased susceptibility to infections. However, the generation of excess reactive O_2 species by uncontrolled neutrophils may deleteriously affect adjacent cells or structural matrix components of tissue. This is probably involved in the pathogenesis of many diseases (Halliwell and Gutteridge, 1990). Neutrophil aggregation contributes to the recruitment of inflammatory cells and to the pathophysiological obstruction of vessels. Therefore, one approach for the treatment of inflammation is to develop agents that prevent the activation of neutrophils.

It has been proposed that the signal transduction mechanisms of receptor-mediated neutrophil activation involve the breakdown of phosphatidylinositol 4,5-bisphosphate through the activation of phospholipase C to give inositol trisphosphate (Di Virgilio et al., 1985), which increases intracellular Ca^{2+}, and diacylglycerol (Rossi, 1986), which activates protein kinase C, whereas phorbol 12-myristate 13-acetate (PMA) bypasses the membrane receptor and directly activates protein kinase C (Castagna et al., 1982). Protein kinase C has been postulated to play a role in the phosphorylation of $p47^{phox}$ (Kramer et al., 1988), which in turn enhances the assembly of cytosolic factors of NADPH

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oxidase (mainly $p47^{phox}$ and $p67^{phox}$) to membrane flavocytochrome b_{558}, leading to activation of NADPH oxidase and the production of $O_2^{\cdot-}$ through a univalent reduction of O_2 (Segal and Abo, 1993). The mechanism for the activation of neutrophil aggregation has not been fully elucidated; however, β_2-integrin CD11b/CD18 and L-selectin are essential for aggregation (Simon et al., 1993). Activation of protein kinase C results in a prolonged neutrophil aggregation that is associated with intense phosphorylation of the CD18 β-chain (Merrill et al., 1990). Therefore, protein kinase C has a critical role in the neutrophil respiratory burst and aggregation.

Several inhibitors of protein kinase C have been reported. These include the microbial products, staurosporine and calphostin C (Tamaoki et al., 1986; Kobayashi et al., 1989), synthetic staurosporine-related compounds (Meyer et al., 1989; Martiny-Baron et al., 1993) and many other synthetic compounds such as trifluoperazine (Schatzman et al., 1981), H-7 (Kawamoto and Hidaka, 1984), aminoacridines (Hannun and Bell, 1988), tamoxifen (Issandou et al., 1990), mefloquine (El Benna et al., 1992) and GF 109203X (Heikkila et al., 1993). However, only a few natural plant products such as chelerythrine, isolated from *Macleaya cordata* and *microcarpa* (Herbert et al., 1990) and flavonoids (Ferriola et al., 1989) have been reported to exert an inhibitory effect on protein kinase C activity.

Norathyriol, an aglycone of a xanthone *C*-glycoside mangiferin, which was isolated from the aerial parts of *Tripterospermum lanceolatum* (**Hayata**) **Hara ex Stake** (*Gentianaceae*) (Lin et al., 1982), has been found to inhibit rabbit platelet aggregation (Teng et al., 1989), prolong tail bleeding time in mice (Teng et al., 1991) and to relax the rat thoracic aorta (Ko et al., 1991). Recently, norathyriol was shown to inhibit the release of chemical mediators from rat peritoneal mast cells in vitro and to suppress cutaneous plasma extravasation in mice caused by inflammatory mediators in vivo (Wang et al., 1994). In the present study, we found that the natural plant product norathyriol inhibited the PMA-induced neutrophil respiratory burst and aggregation, and that these effects can be attributed to the suppression of protein kinase C activity through actions on the catalytic region.

2. Materials and methods

2.1. Materials

Norathyriol was obtained from the reaction of mangiferin, isolated from *Tripterospermum lanceolatum* (**Hayata**) **Hara ex Stake** (*Gentianaceae*), with hydriodic acid as previously described (Lin et al., 1982). All chemicals were purchased from Sigma (St. Louis, MO, USA) except for the following: dextran T-500 (Pharmacia Biotechnology, Uppsala, Sweden); KT 5720 (Biomol Research Laboratories, Plymouth Meeting, PA, USA); rat brain protein kinase C and protein kinase C substrate (N-terminal acetylated, amino acid sequence 4–14 of myelin basic protein, Ac-MBP-(4–14)) (Boehringer-Mannheim, Mannheim, Germany); DE-52 cellulose and Whatman GF/C filter (Whatman, Singapore); Hanks' balanced salt solution and protein kinase A assay kit (Gibco BRL, Gaithersburg, MD, USA); [^{3}H]phorbol 12,13-dibutyrate (DuPont NEN, Boston, MA, USA); [γ-^{32}P]ATP, protein kinase C assay kit and enhanced chemiluminescence kit (Amersham International, Amersham, UK); Immobilon-P (Millipore, Bedford, MA, USA); anti-protein kinase C-β monoclonal antibody (Transduction, Lexington, KY, USA). Dimethylsulfoxide (DMSO) was the solvent used for the inhibitors.

2.2. Isolation of neutrophils

Rat blood was collected from the abdominal aorta and neutrophils were purified by dextran sedimentation, hypotonic lysis of erythrocytes and centrifugation through Ficoll-Hypaque (Wang et al., 1995). Purified neutrophils with $>95\%$ viability were resuspended in Hanks' balanced salt solution containing 4 mM $NaHCO_3$ and 10 mM HEPES, pH 7.4 (HBSS) and kept in an ice bath before use.

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

Whole cell $O_2^{\cdot-}$ generation was determined spectrophotometrically by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* as described previously (Wang et al., 1995). For continuous measurement, a double-beam spectrophotometer (Hitachi, U-3210) was used. O_2 consumption by neutrophils was measured with a Clark-type oxygen electrode using a YSI oxygen monitor (Model 5300), and was determined in continuous assay as described (Ingraham et al., 1982). Assays were conducted at 37°C with 2×10^6 cells (for $O_2^{\cdot-}$ generation), or with 6×10^6 cells (for O_2 consumption), which were preincubated for 5 min with stirring to permit temperature equilibration. The reaction was started by the addition of PMA to reaction mixture.

2.4. Measurement of neutrophil aggregation

Neutrophil aggregation was measured as previously reported (Buyon et al., 1988) with some modifications. A total of 0.5 ml of suspension containing 1×10^6 cells was preincubated with test drugs at 37°C for 3 min in a siliconized cuvette and placed in a whole-blood aggregometer (Chrono-Log), and then PMA or formylmethionyl–leucyl–phenylalanine (fMLP) was added to the cell suspensions. The changes in light transmission were recorded.

2.5. Measurement of protein kinase C activity

For the preparation of cytosolic protein kinase C, neutrophils (6×10^7 cells/ml) were disrupted in buffer A (50

mM Tris–HCl, pH 7.5, 0.25 M sucrose, 50 mM 2-mercaptoethanol, 2 mM phenylmethylsulphonyl fluoride, 5 mM EDTA, 10 mM EGTA, 0.01% leupeptin and 10 mM benzamidine) by sonication and then centrifuged at $100\,000 \times g$. The supernatant was applied to a DE-52 cellulose column. Protein kinase C was eluted with buffer B (50 mM Tris–HCl, pH 7.5, 50 mM 2-mercaptoethanol, 2 mM phenylmethylsulphonyl fluoride, 1 mM EDTA, 1 mM EGTA, 0.01% leupeptin and 10 mM benzamidine) containing 0.4 M NaCl. The method has been described in detail elsewhere (Wang et al., 1995). Neutrophil cytosolic protein kinase C and rat brain protein kinase C activities were assayed by measuring the incorporation of ^{32}P into peptide substrates using a protein kinase C assay kit, based on the mixed micelle method previously described (Hannun et al., 1986). Briefly, the reaction mixture contained 50 mM Tris–HCl buffer, pH 7.5, 1 mM $CaCl_2$, 15 mM magnesium acetate, 2.5 mM dithiothreitol, 6 mM phosphatidylserine, 2 μg/ml of PMA, 50 μM ATP (0.2 μCi [γ-^{32}P]ATP per tube), 75 μM protein kinase C substrate and protein kinase C sample. After addition of stop reagent, an aliquot of the mixture was spot onto a phosphocellulose disc. Phosphorylated substrate, which bound to the filter paper, was washed and counted. In some experiments, brain protein kinase C was partially digested with trypsin to generate the catalytic region (Inoue et al., 1977). Trypsin-treated protein kinase C activity was determined as described above except that $CaCl_2$, phosphatidylserine and PMA were left out of the reaction mixture. For the determination of inhibition of protein kinase C by norathyriol with respect to ATP and peptide substrate, assays were performed in the presence of 75 μM protein kinase C substrate with various concentrations of ATP, or in the presence of 50 μM ATP with various concentrations of protein kinase C substrate, Ac-MBP-(4–14).

2.6. Measurement of protein kinase A activity

Porcine heart protein kinase A activity was assayed by measuring the incorporation of ^{32}P into kemptide in the presence of cAMP, using a protein kinase A assay kit, based on the method previously described (Roskoski, 1983). The assay tubes contained 50 mM Tris–HCl buffer, pH 7.5, 10 mM $MgCl_2$, 100 μM ATP (0.3 μCi [γ-^{32}P]ATP per tube), 50 μM kemptide, protein kinase A sample and 10 μM cAMP for 5 min at 30°C. Phosphorylated substrate, which bound to the filter paper, was washed and counted. In some experiments, protein kinase A activity was determined in the absence of cAMP.

2.7. [^{3}H]PDB binding assay

The reaction mixture contained 20 mM Tris–HCl, pH 7.2, 100 mM KCl, 50 μg/ml of phosphatidylserine, 0.5 mM $CaCl_2$, 30 nM [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDB) and neutrophil cytosolic protein kinase C. For the determination of non-specific binding, 30 μM PDB was present in the reaction mixture. After addition of ice-cold 0.5% DMSO solution to terminate the reaction, the mixture was poured onto a Whatman GF/C filter. The filter was then washed and counted (d.p.m.) as described in detail elsewhere (Tanaka et al., 1986).

2.8. Immunoblot analysis of protein kinase C-β

Neutrophils (4×10^7 cells/ml) were stimulated with 0.2 μM PMA for 5 min at 37°C. The reaction was stopped by the addition of 4 volumes of ice-cold HBSS, and the cells were resuspended in disruption solution (0.34 M sucrose, 10 mM Tris–HCl, pH 7.0, 1 mM phenylmethylsulphonyl fluoride, 1 mM EGTA, 10 mM benzamidine, 10 μg/ml of leupeptin and antipain). After sonication, the lysate was centrifuged at $800 \times g$ at 4°C for 5 min to remove the unbroken cells, and then further centrifuged at $100\,000 \times g$ at 4°C for 30 min. The pellet (as membrane fraction) and supernatant (as cytosol fraction) were boiled in Laemmli sample buffer, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and then transferred to an Immobilon-P membrane. These membranes were incubated with 5% non-fat milk in TST buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20) and with a monoclonal antibody against protein kinase C-β (1:500 dilution in TST buffer with 0.5% non-fat milk). Detection was with the enhanced chemiluminescence kit.

2.9. Statistical analysis

Statistical analyses were performed using the Bonferroni t test method after analysis of variance. A P value less than 0.05 was considered significant for all tests.

3. Results

3.1. Neutrophil respiratory burst and aggregation

When 3 nM PMA was added to the rat neutrophil suspension, $O_2^{\cdot-}$ generation was observed by detecting the absorbance changes in the superoxide dismutase-inhibitable reduction of ferricytochrome *c*. Addition of 10 nM PMA to the neutrophil suspension in the presence of 1 mM NaN_3 evoked non-mitochondrial O_2 consumption. Norathyriol concentration dependently inhibited the PMA-induced $O_2^{\cdot-}$ generation and O_2 consumption (Fig. 1A,B), and significant inhibitions were observed at concentrations of norathyriol ≥ 10 μM and ≥ 30 μM, respectively. More than 95% of cells treated with 100 μM norathyriol for 5 min were viable.

PMA and fMLP induced homotypic neutrophil aggregation, with PMA being more potent than fMLP. Preincubation with 0.3 μM staurosporine, a protein kinase inhibitor,

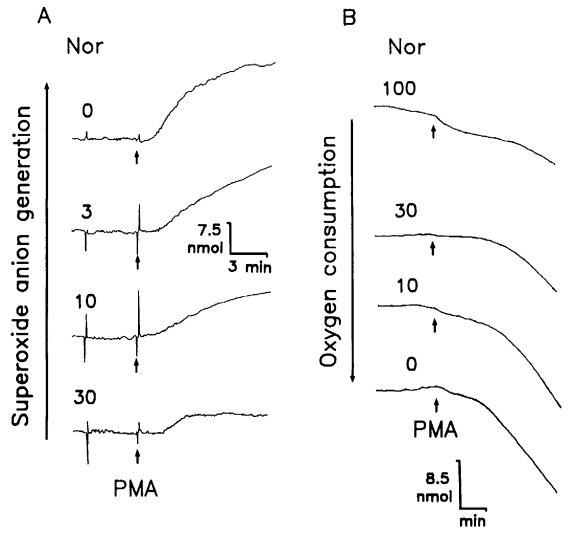

Fig. 1. Effect of norathyriol on PMA-induced $O_2^{\cdot-}$ generation and O_2 consumption in rat neutrophils. Neutrophils were preincubated with various concentrations of norathyriol (Nor, 3–100 μM) for 3 min at 37°C before the addition (arrow) of (A) 3 nM PMA to induce $O_2^{\cdot-}$ generation, or (B) 10 nM PMA to evoke O_2 consumption. $O_2^{\cdot-}$ generation and O_2 consumption were continuously measured as described in Section 2. The results shown are representative for 5 separate experiments.

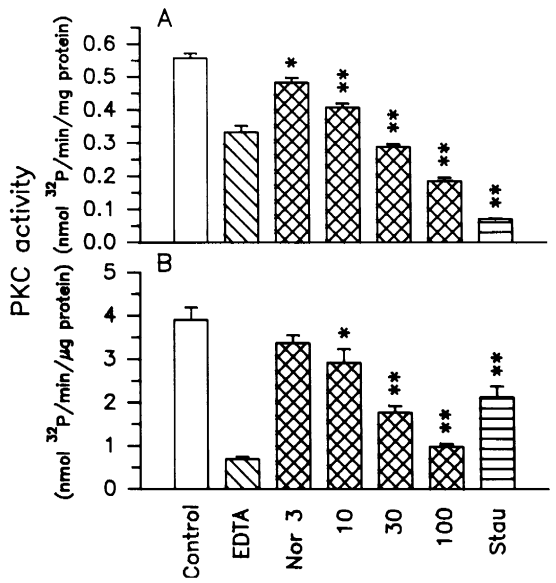

Fig. 2. Effect of norathyriol on protein kinase C activity. (A) Neutrophil cytosolic protein kinase C or (B) rat brain protein kinase C was preincubated with DMSO (as control, open column) and various concentrations of norathyriol (Nor, 3–100 μM) or 3 nM staurosporine (Stau) at 25°C for 3 min in the presence of Ca^{2+}/phosphatidylserine, [γ-^{32}P]ATP and protein kinase C substrate before addition of PMA to start the reaction. In some experiments, 3 mM EDTA replaced $CaCl_2$ in the reaction mixture. After termination of the reaction, phosphorylated protein was harvested on a filter, and the radioactivity on the filter was counted as described in Section 2. Data are expressed as the means ± S.E.M. of 4–6 independent experiments. * $P < 0.05$, ** $P < 0.01$ compared to the corresponding control values.

significantly reduced the PMA-induced response but enhanced the fMLP-induced response. Similar reactive profiles were also observed with 100 μM norathyriol-treated cells (Table 1).

3.2. Protein kinase C activity

In the presence of $CaCl_2$, phosphatidylserine and PMA, the incorporation of ^{32}P from [γ-^{32}P]ATP into peptide substrate was demonstrated in preparations of neutrophil cytosolic protein kinase C and rat brain protein kinase C. Like staurosporine, norathyriol effectively attenuated the protein kinase C activity of both preparations (Fig. 2A, B) in a concentration-dependent manner. In the presence of EDTA, marked Ca^{2+}-independent protein kinase C activity was demonstrated (accounting for 60% of the total protein kinase C activity) in cytosolic protein kinase C preparations, whilst only minor Ca^{2+}-independent protein kinase C activity (account for ≤ 20% of the total protein kinase C activity) was observed in rat brain protein kinase C preparations.

Table 1
Effect of norathyriol on neutrophil aggregation

Drugs [a]	(μM)	Aggregation (%) [b]
PMA	2	39.1 ± 1.9
+ Norathyriol	100	20.8 ± 1.7 [c]
+ Staurosporine	0.3	17.9 ± 4.7 [d]
fMLP	10	15.0 ± 1.0
+ Norathyriol	100	24.6 ± 1.6 [c]
+ Staurosporine	0.3	34.3 ± 4.1 [d]

[a] Cells were preincubated with drugs at 37°C for 3 min before the addition of PMA or fMLP to start the reaction.
[b] Neutrophil aggregation was measured by detecting changes in light transmission as described in Section 2. Data are expressed as means ± S.E.M. of 4–5 separate experiments.
[c] $P < 0.05$, compared to the corresponding control values.
[d] $P < 0.01$, compared to the corresponding control values.

3.3. Binding of [^{3}H]phorbol 12,13-dibutyrate to protein kinase C

The binding of [^{3}H]PDB to neutrophil cytosolic protein kinase C was determined by the rapid filtration assay. Non-specific binding was less than 15% of the total binding. Unlike 1 nM PMA and 10 μM 1-oleoyl-2-acetyl-*sn*-glycerol, which produced about 75% and 70% inhibition, respectively, of [^{3}H]PDB binding to neutrophil cytosolic protein kinase C (Fig. 3), norathyriol, even in concentrations up to 100 μM, which caused about 70% inhibition of protein kinase C activity, had negligible effects on [^{3}H]PDB binding to protein kinase C.

3.4. Trypsin-treated protein kinase C activity

After rat brain protein kinase C was partially digested with trypsin to remove the regulatory region, protein ki-

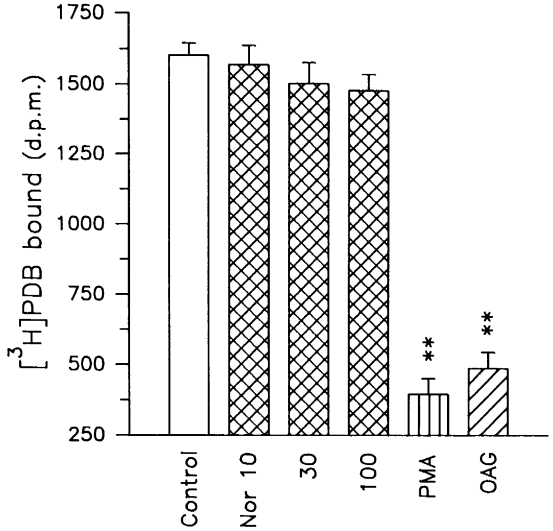

Fig. 3. Effect of norathyriol on [^{3}H]PDB binding to protein kinase C. Neutrophil cytosolic protein kinase C was preincubated with DMSO (as control, open column) and various concentrations of norathyriol (Nor, 10–100 μM), 1 nM PMA, or 10 μM 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) at 30°C for 3 min before the addition of [^{3}H]PDB to the reaction mixture. After termination of the reaction, protein was harvested on a filter, and the radioactivity on the filter was counted as described in Section 2. Data are expressed as the means ± S.E.M. of 4–5 independent experiments. ** $P < 0.01$ compared to the control value.

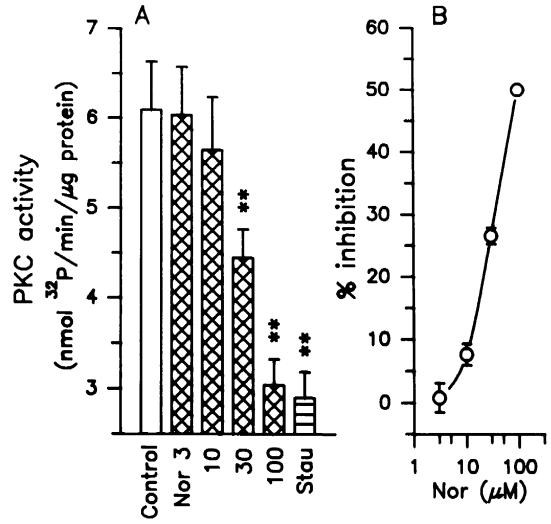

Fig. 4. Effect of norathyriol on trypsin-treated protein kinase C activity. (A) Trypsin-treated rat brain protein kinase C was incubated with DMSO (as control, open column) and various concentrations of norathyriol (Nor, 3–100 μM) or 3 nM staurosporine (Stau) for 3 min at 25°C before the addition of [γ-^{32}P]ATP and protein kinase C substrate to start the reaction. After termination of the reaction, phosphorylated protein was harvested on a filter, and the radioactivity on the filter was counted as described in Section 2. (B) Inhibition of protein kinase C by norathyriol was calculated as a percent inhibition of control. Data are expressed as the means ± S.E.M. of 4 independent experiments. ** $P < 0.01$ compared to the control value.

nase C activity was measured by detecting the incorporation of ^{32}P from [γ-^{32}P]ATP into peptide substrate in the absence of $CaCl_2$, phosphatidylserine and PMA. Norathyriol as well as staurosporine effectively attenuated the trypsin-treated protein kinase C activity (Fig. 4A, B); the inhibition elicited by norathyriol was concentration dependent. For the determination of inhibition by norathyriol of protein kinase C with respect to ATP and to peptide substrate, assays were performed at a fixed concentration of protein kinase C substrate (Ac-MBP-(4–14), 75 μM) with various concentrations of ATP (Fig. 5A), or at a fixed concentration of ATP (50 μM) with various concentrations of protein kinase C substrate (Fig. 5B). Norathyriol was found to be a noncompetitive inhibitor of protein kinase C with respect to ATP and to protein kinase C peptide substrate.

3.5. Protein kinase A activity

Porcine heart protein kinase A phosphorylated kemptide in the presence of ATP and activator cAMP. This response was significantly attenuated by 3 μM KT 5720, a protein kinase A inhibitor (Kase et al., 1987), and by 30 nM staurosporine. In contrast, norathyriol (up to 100 μM) failed to affect the protein kinase A activity (Fig. 6). In addition, norathyriol (up to 100 μM) alone did not affect the protein kinase A activity in the absence of cAMP (data not shown).

3.6. Redistribution of protein kinase C-β

In order to determine the membrane translocation of protein kinase C-β, immunoblot analysis was carried out by using specific anti-protein kinase C-β antibody. In the

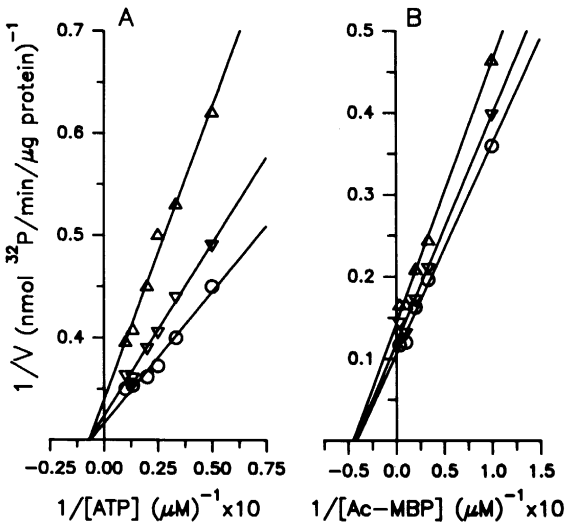

Fig. 5. Double reciprocal plot for trypsin-treated protein kinase C in the presence of norathyriol. Inhibition of trypsin-treated protein kinase C by norathyriol with respect to (A) ATP (20–100 μM) and (B) peptide substrate, Ac-MBP-(4–14) (Ac-MBP, 10–300 μM), was determined as described in Section 2. (○) DMSO, (▽) 30 and (△) 100 μM norathyriol were present in the assay mixture.

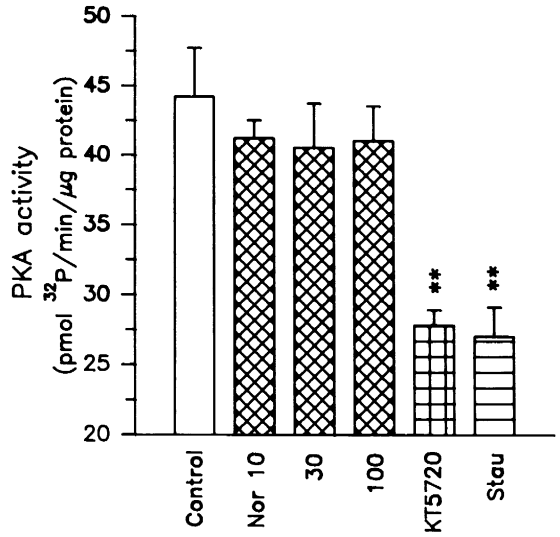

Fig. 6. Effect of norathyriol on protein kinase A activity. Porcine heart protein kinase A was incubated with DMSO (as control, open column) and various concentrations of norathyriol (Nor, 10–100 μM), 30 μM KT5720, or 30 nM staurosporine (Stau) for 3 min at 30°C in the presence of Mg^{2+}, [γ-^{32}P]ATP, cAMP, and kemptide. After termination of the reaction, phosphorylated protein was harvested on a filter, and the radioactivity on the filter was counted as described in Section 2. Data are expressed as the means ± S.E.M. of 3–4 independent experiments. ** $P < 0.01$ compared to the control value.

resting cells, protein kinase C-β was enriched in the cytosol fraction. Upon 0.2 μM PMA treatment, protein kinase C-β was translocated from the cytosol to the membrane (Fig. 7). This PMA-induced response was unaffected by norathyriol (10–100 μM). In addition, norathyriol (100 μM) alone did not affect the distribution of protein kinase C between the membrane and cytosol.

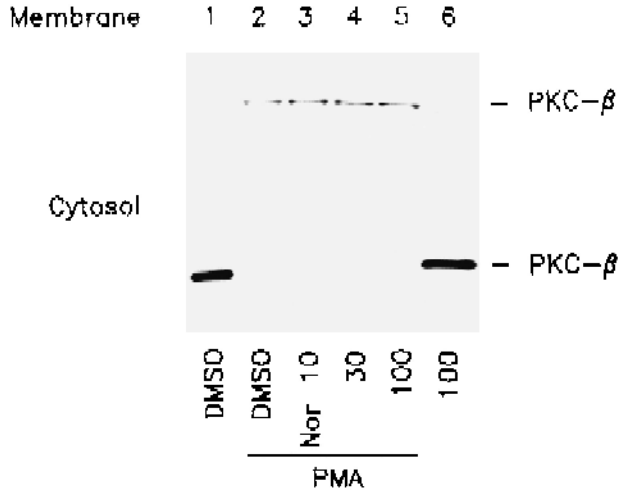

Fig. 7. Effect of norathyriol on the membrane translocation of protein kinase C-β. Neutrophils (4×10^7 cells) were preincubated with (lane 2) DMSO and (lanes 3–5) norathyriol (Nor, 10–100 μM) for 5 min at 37°C and then stimulated with 0.2 μM PMA for another 5 min. Cells were also incubated with (lane 1) DMSO or (lane 6) 100 μM norathyriol alone for 10 min. After termination of the reaction, cells were disrupted by sonication, and then centrifuged. Membrane and cytosol proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Analysis was performed by immunoblotting with a monoclonal antibody to protein kinase C-β as described in Section 2. The results shown are representative of 3 separate experiments.

4. Discussion

In the present study, we demonstrated that a xanthine aglycone, norathyriol, is able to inhibit the respiratory burst in PMA-activated neutrophils. It has been reported that PMA bypasses neutrophil membranes and directly activates protein kinase C and induces $O_2^{\cdot-}$ generation (Castagna et al., 1982; Smallwood and Malawista, 1992). The phosphorylation of CD18 β-chain by protein kinase C is responsible for arming the CD11b/CD18 leukocyte integrin with the ability to mediate homotypic neutrophil aggregation, and both PMA-induced aggregation and phosphorylation are abolished by staurosporine (Merrill et al., 1990). Conversely, fMLP appears to activate CD11b/CD18 by a distinct mechanism, and fMLP-induced aggregation is enhanced by protein kinase C inhibitor (Merrill et al., 1990). The observation that norathyriol and staurosporine exerted comparable effects on the PMA- and fMLP-induced responses suggests the inhibition of protein kinase C by norathyriol. Therefore the effect of norathyriol on protein kinase C activity was investigated.

The mammalian protein kinase C family consists of at least 12 different isoforms (Dekker and Parker, 1994), of which α, β and ζ have been identified in neutrophils (Pontremoli et al., 1990; Stasia et al., 1990). Protein kinase C participates in the activation of NADPH oxidase, probably through the phosphorylation of $p47^{phox}$ (Kramer et al., 1988). In the presence of Ca^{2+} and phosphatidylserine, PMA activated the cytosolic protein kinase C of resting neutrophils. In the presence of EDTA, Ca^{2+}-independent protein kinase C activity was also observed in the cytosolic protein kinase C preparations. Since the rat brain protein kinase C preparation contains mainly Ca^{2+}-dependent isoforms (α, β and γ isoforms) (Go et al., 1987), only minor protein kinase C activity was observed in the presence of EDTA. Norathyriol inhibited cytosolic protein kinase C as well as rat brain protein kinase C over the same range of concentrations at which it inhibited the PMA-induced respiratory burst. These results indicate that norathyriol, like the protein kinase inhibitor staurosporine, may directly inhibit protein kinase C, and that the inhibition of protein kinase C contributes to the attenuation of the respiratory burst.

Protein kinase C comprises both regulatory and catalytic regions (Nishizuka, 1986). In order to determine which one of the regions is the site of action of norathyriol, experiments investigating PDB binding to protein kinase C and trypsin-treated protein kinase C activity were performed. Phorbol esters are believed to bind to the C1 (diacylglycerol/phorbol ester binding site) domain of

the protein kinase C regulatory region (Newton, 1995). In the assay of PDB binding, norathyriol, unlike PMA and 1-oleoyl-2-acetyl-*sn*-glycerol, did not inhibit the binding of [^{3}H]PDB to neutrophil cytosolic protein kinase C, which suggests that the inhibition of protein kinase C by norathyriol may not involve blockade of the regulatory region of protein kinase C.

The regulatory and catalytic regions are separated by a hinge region which is sensitive to protease. After partial digestion of protein kinase C with trypsin, the regulatory region is removed from the native protein kinase C (Lee and Bell, 1986). The remaining catalytic region of protein kinase C has enzyme activity independent of Ca^{2+}, phosphatidylserine and diacylglycerol (Inoue et al., 1977). Staurosporine is a competitive inhibitor with respect to ATP (Tamaoki et al., 1986) and thus inhibited native as well as trypsin-treated protein kinase C. Like staurosporine, norathyriol inhibited trypsin-treated protein kinase C activity. In addition, staurosporine had little effect on the binding of PDB to protein kinase C (Tamaoki et al., 1986). These results lead us to suggest that the site of action of norathyriol is probably the catalytic region. Determination of the inhibition by norathyriol of trypsin-treated protein kinase C with respect to ATP and to peptide substrate indicated that norathyriol is a noncompetitive inhibitor. Staurosporine inhibits a variety of kinases including protein kinase C, protein kinase A, protein kinase G and myosin-light chain kinase (Tamaoki et al., 1986; Matsumoto and Sasaki, 1989). Unlike staurosporine, norathyriol (up to 100 μM) had no effect on protein kinase A activity.

Protein kinase C activity is primarily cytosolic in unstimulated neutrophils (Wolfson et al., 1985), but becomes firmly associated with the membrane fraction after PMA treatment. The translocation of protein kinase C to the membrane is mediated by diacylglycerol or phorbol ester binding to the C1 domain and phosphatidylserine/Ca^{2+} binding to the C2 domain of the protein kinase C regulatory region (Newton, 1995). Isoform β is the major Ca^{2+}-dependent protein kinase C isoform and is translocated from the cytosol to the membrane in response to treatment with phorbol ester (Majumdar et al., 1991). In the assay of the subcellular distribution of protein kinase C-β, norathyriol had no effect on the PMA-induced translocation of protein kinase C-β from the cytosol to the membrane. These results indicate that the inhibition of protein kinase C by norathyriol can not be attributed to an influence of the redistribution of protein kinase C-β during cell activation.

In conclusion, we found that a plant product, norathyriol, inhibited the respiratory burst and homotypic aggregation in PMA-activated neutrophils. This inhibition is probably attributable to the direct suppression of protein kinase C activity by norathyriol through blockade of the catalytic region and is not due to the interference with the subcellular redistributions of protein kinase C.

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