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Possible Involvement of Protein Kinase C Inhibition in The Reduction of Phorbol Ester-induced Neutrophil Aggregation by Magnolol in the Rat

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Abstract

The influence of the plant product magnolol on neutrophil aggregation has been investigated in the rat.

Magnolol inhibited phorbol 12-myristate 13-acetate (PMA)-activated rat neutrophil aggregation in a concentration-dependent manner with an IC50 (concentration resulting in 50% inhibition) of 24·2 ± 1·7 μM. Magnolol suppressed the enzyme activity of neutrophil cytosolic and rat brain protein kinase C (PKC) over the same range of concentrations at which it inhibited the aggregation. Magnolol did not affect PMA-induced cytosolic PKC-α and -δ membrane translocation or trypsin-treated rat-brain PKC activity, but attenuated [^{3}H]phorbol 12,13-dibutyrate binding to neutrophil cytosolic PKC.

These results suggest that the inhibition of PMA-induced rat neutrophil aggregation by magnolol is probably attributable, at least in part, to the direct suppression of PKC activity through blockade of the regulatory region of PKC.

The primary physiological function of neutrophils is to protect the host from infection. For this purpose they are equipped for chemotaxis, phagocytosis, degranulation, and generation of toxic oxygen metabolites (Borregaard 1988). Because neutrophil aggregation contributes to the recruitment of inflammatory cells, any functional defect of this process is an important contributory factor to increased susceptibility to infection. Neutrophil aggregation might contribute to the pathophysiological obstruction of vessels and to tissue injury, and because a pathogenic role for neutrophil aggregation has been postulated in haemodialysis neutropenia, adult respiratory distress and ischaemic retinopathy (Hammerschmidt et al 1979; Hosea et al 1980), neutrophil aggregation is also an important pathogenic mechanism.

Although signal-transduction mechanisms in neutrophil activation have been well documented, the link between the signalling pathways and aggregation is poorly elucidated. Neutrophil aggregation mediated by direct receptor activation might be regulated by phosphatidylinositol 3-kinase and mitogen-activated protein kinase (Pillinger et al 1996; Wise 1996). Diacylglycerols activated protein kinase C (PKC) in a similar manner, with potencies that paralleled their respective abilities to stimulate neutrophil aggregation (Nishihira & O'Flaherty 1985). Although the mechanism of activation of neutrophil aggregation has not been fully elucidated, β_2-integrin (CD11b/CD18) and L-selectin are essential for aggregation (Simon et al 1993). PKC has a critical role in neutrophil aggregation because activation of the compound results in prolonged neutrophil aggregation associated with intense phosphorylation of CD18 (Merrill et al 1990).

In this study we have found that suppression of PKC activity by the action of magnolol on the regulatory region is probably involved in the inhibition of phorbol 12-myristate 13-acetate (PMA)-induced homotypic neutrophil aggregation.

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Materials and Methods

Materials

Magnolol was obtained and purified from the cortex of *Magnolia officinalis* (Magnoliaceae) as

described elsewhere (Fujita et al 1972). The purity of magnolol (> 99%) was determined by high-performance liquid chromatography (HPLC) with 3-D photodiode-array detection and by nuclear magnetic resonance (NMR) spectroscopy (the spectrum was free from impurity signals). Other materials were purchased from Sigma (St Louis, MO) except for dextran T-500 (Pharmacia, Uppsala, Sweden), Hanks, balanced salt solution (Gibco, Gaithersburg, MD), rat brain PKC (Boehringer Mannheim, Germany), DE-52 cellulose and Whatman GF/C filters (Whatman, Singapore), [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDB; DuPont NEN, Boston, MA), [γ-^{32}P]ATP, PKC assay kit and enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK), Immobilon-P membrane (Millipore, Bedford, MA) and mouse monoclonal anti-PKC isoforms antibodies (Transduction, Lexington, KY). Dimethylsulphoxide (DMSO) was used as solvent for the inhibitors.

Isolation of neutrophils

Rat blood was collected from the abdominal aorta of pentobarbital-anaesthetized rats, and neutrophils were purified by dextran sedimentation, hypotonic lysis of erythrocytes, and centrifugation through Ficoll-Hypaque (Wang et al 1995). Purified neutrophils (> 95% pure and > 95% viable as assessed by differential counting and trypan-blue exclusion, respectively) were normally resuspended in Hanks, balanced salt solution (HBSS) containing 4 mM $NaHCO_3$ and 10 mM HEPES, pH 7·4 and kept in an ice-bath before use.

Measurement of neutrophil aggregation

Neutrophil aggregation was measured as reported elsewhere (Buyon et al 1988) with some modifications. Suspension (0·5 mL containing 1×10^6 cells) was pre-incubated with test drugs at 37°C for 3 min in a siliconized cuvette and placed in a whole-blood aggregometer (Chrono-log, Havertown, PA; Model 560); PMA (2 μM) was then added to the cell suspensions and changes in light transmission were recorded.

Measurement of protein kinase C activity

For preparation of cytosolic PKC, neutrophils (6×10^7 cells mL^{-1}) were disrupted in buffer A (50 mM Tris-HCl, pH 7·5, 0·25 M sucrose, 50 mM 2-mercaptoethanol, 5 mM EDTA, 10 mM EGTA, 2 mM phenylmethylsulphonyl fluoride, 0·01% (w/v) leupeptin and 10 mM benzamidine) by sonication and then centrifuged at 100 000 *g*. The supernatant was applied to a DE-52 cellulose column. PKC 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% (w/v) leupeptin and 10 mM benzamidine) containing 0·4 M NaCl. The method has been described in detail elsewhere (Wang et al 1995). Neutrophil cytosolic PKC and rat brain PKC activity were assayed by measuring the incorporation of ^{32}P into peptide substrate using a PKC assay kit, based on the mixed-micelle method described elsewhere (Hannun & Bell 1988). 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 (PS), 2 μg mL^{-1} PMA, 50 μM ATP (0·2 μCi [γ-^{32}P]ATP per tube), 75 μM PKC substrate and PKC sample. After addition of stop reagent, a sample of the mixture was spotted on to a phosphocellulose disc. Phosphorylated substrate, which became bound to the filter paper, was washed and counted. In some experiments brain PKC was partially digested with trypsin to generate the catalytic region (Inoue et al 1977). Trypsin-treated PKC activity was determined as described above except that $CaCl_2$, PS and PMA were left out of the reaction mixture.

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

The reaction mixture contained 20 mM Tris-HCl, pH 7·2, 100 mM KCl, 50 μg mL^{-1} PS, 0·5 mM $CaCl_2$, 30 nM [^{3}H]PDB and neutrophil cytosolic PKC. For determination of non-specific binding 30 μM PDB was present in the reaction mixture. After addition of ice-cold 0·5% (v/v) DMSO solution to terminate the reaction, the mixture was poured on to a Whatman GF/C filter. The filter was then washed and counted in disintegrations min^{-1} as described in detail elsewhere (Tanaka et al 1986).

Immunoblot analysis of subcellular distribution of PKC

Neutrophils (4×10^7 cells mL^{-1}) were stimulated with 0·2 μM PMA at 37°C, and then disrupted in Tris buffer (0·34 M sucrose, 10 mM Tris-HCl, pH 7·0, 1 mM phenylmethylsulphonyl fluoride, 1 mM EGTA, 10 mM benzamidine, 10 μg mL^{-1} leupeptin and antipain) by sonication, and fractionated as described elsewhere (Wang et al 1997). The cytosol and plasma membrane fractions were stored at −70°C until use. The membrane and cytosol fractions were boiled in Laemmli sample buffer, subjected to sodium dodecylsulphate-polyacrylamide-gel electrophoresis (SDS-PAGE), and then transferred to an Immobilon-P membrane, blocked with non-fat milk and probed with specific monoclonal antibodies against PKC isoforms (1 : 500 to 1 : 2000 dilution in 10 mM Tris-HCl, pH 8·0, 150 mM NaCl, 0·05% (v/v) Tween-20, and 0·5% (w/v) non-fat

milk). Detection was by means of the enhanced chemiluminescence kit.

Statistical analysis

Statistical analysis was performed by use of the Bonferroni *t*-test after analysis of variance. In all tests P values < 0.05 were considered indicative of significance. Analysis of the regression-line test was used to calculate IC50 values (concentrations resulting in 50% inhibition).

Results

Effect of magnolol on neutrophil aggregation

Addition of 2 μM PMA to the neutrophil suspension induced prolonged homotypic neutrophil aggregation. Cells pre-incubated with magnolol inhibited the PMA-induced response in a concentration-dependent manner with a IC50 value of 24.2 ± 1.7 μM (Table 1). Inhibition of neutrophil aggregation by the protein kinase inhibitor staurosporine (0·3 μM) is consistent with a previous report (Merrill et al 1990).

Effect of magnolol on PKC activity

In the presence of $CaCl_2$, PS and PMA, the incorporation of ^{32}P from [γ-^{32}P]ATP into peptide substrate was demonstrated in preparations of neutrophil cytosolic PKC and rat brain PKC. Like staurosporine, magnolol concentration-dependently attenuated neutrophil cytosolic PKC activity (35.6 ± 1.6% inhibition at 100 μM) (Figure 1) and rat brain PKC activity (37.3 ± 5.0% inhibition at 100 μM) (Figure 2). In the presence of EDTA marked Ca^{2+}-independent PKC activity (accounting for 60% of total PKC activity) was demonstrated in cytosolic PKC preparations, whereas only minor Ca^{2+}-independent PKC activity (accounting for ≤ 20% of total PKC activity) was observed in rat brain PKC preparations.

Table 1. Effect of magnolol on phorbol 12-myristate 13-acetate-induced rat neutrophil aggregation.

Treatment	Dose (μM)	Aggregation (%)
Control	–	26.7 ± 2.1
Magnolol	10	25.4 ± 1.9
	20	18.0 ± 2.2*
	30	12.8 ± 1.2†
Staurosporine	0·3	7.8 ± 1.4†

Values are means ± s.e.m. of results from four independent experiments. *$P < 0.05$, †$P < 0.01$, significantly different from control value.

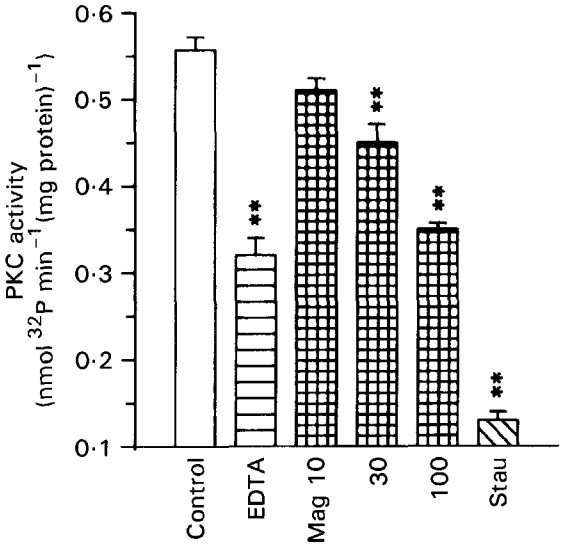

Figure 1. Effect of magnolol on neutrophil cytosolic protein kinase C activity. Neutrophil cytosolic protein kinase C was pre-incubated with dimethylsulphoxide (as control), magnolol (Mag, 10–100 μM) or staurosporine (Stau, 3 nM) at 25°C for 3 min in the presence of Ca^{2+}-phosphatidylserine, [γ-^{32}P]ATP and protein kinase C substrate and then phorbol 12-myristate 13-acetate was added 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. Values are means ± s.e.m. of results from six independent experiments. ** $P < 0.01$, significantly different from control value.

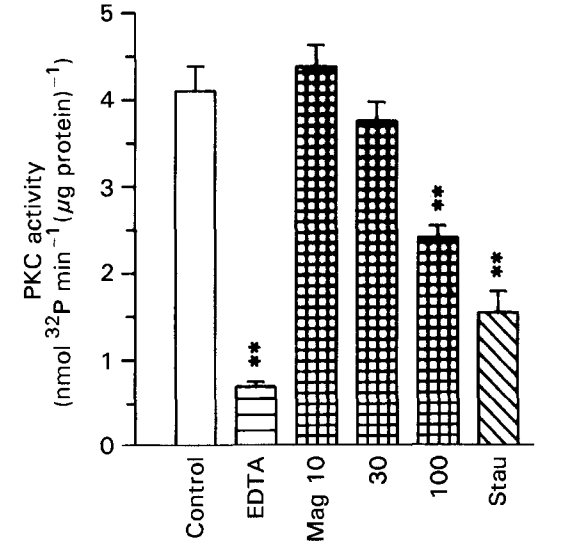

Figure 2. Effect of magnolol on rat brain protein kinase C activity. Rat brain protein kinase C was pre-incubated with dimethylsulphoxide (as control), magnolol (Mag, 10–100 μM) or staurosporine (Stau, 10 nM) at 25°C for 3 min in the presence of Ca^{2+}-phosphatidylserine, [γ-^{32}P]ATP and protein kinase C substrate and then phorbol 12-myristate 13-acetate was added 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. Values are means ± s.e.m. of results from four or five independent experiments. ** $P < 0.01$, significantly different from control value.

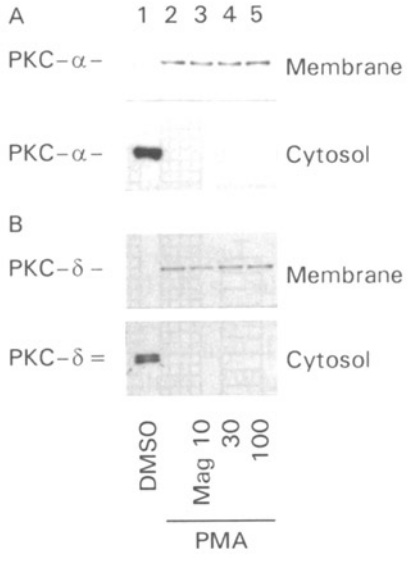

Figure 3. Effect of magnolol on protein kinase C membrane translocation. Neutrophils were treated with dimethylsulphoxide (lane 2) or magnolol (Mag, 10–100 μM, lanes 3–5) for 5 min at 37°C and then stimulated with 0·2 μM phorbol 12-myristate 13-acetate for another 5 min. Cells were also reacted with dimethylsulphoxide (lane 1) alone for total 10-min reaction time. After termination of the reaction cells were disrupted by sonication, and then centrifuged. Membrane and cytosol proteins were subjected to sodium dodecylsulphate-polyacrylamide-gel electrophoresis. Analysis was performed by immunoblotting with monoclonal antibodies to (A) protein kinase C-α and (B) protein kinase C-δ. The results shown are representative of three separate experiments.

Effect of magnolol on PKC membrane translocation

To determine the membrane translocation of PKC, immunoblot analysis was performed using specific anti-PKC isoforms (α, δ, and ι). In the resting cells PKC was enriched in the cytosol fraction. Upon treatment with 0·2 μM PMA, PKC-α and δ, but not PKC-ι (data not shown), were translocated from the cytosol to the membrane (Figure 3A, B). Magnolol (10–100 μM) had no effect on the PMA-induced membrane translocation of PKC.

Effect of magnolol on the binding of [^{3}H]PDB to PKC

The binding of [^{3}H]PDB to neutrophil cytosolic PKC was determined by rapid filtration assay. Non-specific binding was less than 15% of total binding. Concentration-dependent inhibition of [^{3}H]PDB binding to PKC was observed in reaction mixtures treated with 1-oleoyl-2-acetyl-*sn*-glycerol and with magnolol (Figure 4), in which 1-oleoyl-2-acetyl-*sn*-glycerol was more potent than magnolol.

Effect of magnolol on trypsin-treated PKC activity

After partial digestion of rat brain PKC with trypsin to remove the regulatory region, the trypsin-treated PKC was treated with magnolol (10–100 μM) or staurosporine (3 nM) for 3 min at 25°C before addition of [γ-^{32}P]ATP to measure the incorporation of ^{32}P into peptide substrate in the absence of $CaCl_2$, PS and PMA. Staurosporine effectively attenuated the trypsin-treated PKC activity whereas magnolol, at concentrations which significantly inhibited neutrophil aggregation and PKC activity, had no effect (Table 2).

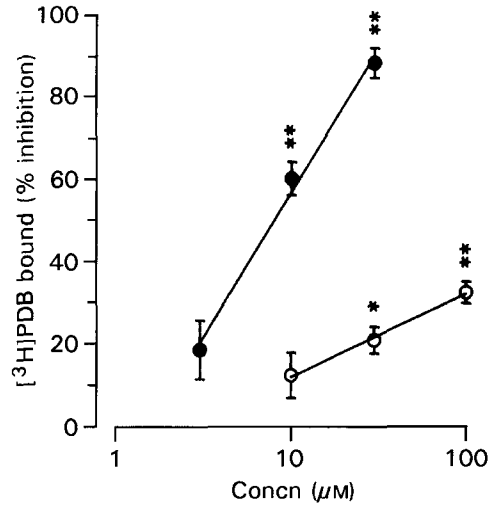

Figure 4. Effect of magnolol on [^{3}H]phorbol 12,13-dibutyrate binding to protein kinase C. Neutrophil cytosolic protein kinase C was pre-incubated with dimethylsulphoxide (as control), 10–100 μM magnolol (○) or 3–30 μM 1-oleoyl-2-acetyl-*sn*-glycerol (●) at 30°C for 3 min before addition of [^{3}H]phorbol 12,13-dibutyrate to the reaction mixture. After termination of the reaction protein was harvested on a filter, and the radioactivity on the filter was counted. Inhibition of [^{3}H]phorbol 12,13-dibutyrate binding (%) is expressed as means ± s.e.m. of results from four or five independent experiments. * $P < 0.05$, ** $P < 0.01$, significantly different from the control value (1873·0 ± 52·3 disintegrations min^{-1}).

Discussion

Activation of PKC induces several neutrophil functions including the respiratory burst, degranulation, surface adhesion and aggregation. PMA, a

Table 2. Effect of magnolol on trypsin-treated protein kinase C activity.

Treatment	Dose (μM)	Trypsin-treated PKC activity (nmol ^{32}P min^{-1} (μg protein)$^{-1}$)
Control	–	6·09 ± 0·54
Magnolol	10	6·03 ± 0·54
	30	6·03 ± 0·51
	100	6·09 ± 0·45
Staurosporine	0·003	2·90 ± 0·28*

Values are means ± s.e.m. of results from four or five independent experiments. * $P < 0.01$, significantly different from control value.

poorly hydrolysable diacylglycerol analogue, bypasses neutrophil membranes, directly activates PKC and induces more profound and prolonged effects (Castagna et al 1982). In the current study we have demonstrated that a natural product, magnolol, inhibits neutrophil aggregation in response to PMA. It has been reported that two types of molecule, L-selectin and β_2-integrin (CD11b/CD18), are involved in homotypic neutrophil aggregation (Simon et al 1993). β_2-Integrin has limited expression on the surface membrane of resting cells. PMA induces an increase in the surface expression of β_2-integrin from presynthesized intracytoplasmic stores. This adhesiveness is not simply the result of modulating the prevalence of the surface molecules but results from modification of β_2-integrin from a non-adhesive to an adhesive state (Buyon et al 1988). In response to PMA prolonged cellular aggregation occurs that is associated with de-novo phosphorylation of the CD18 cytoplasmic tail. Both aggregation and phosphorylation induced by PMA are completely abolished by staurosporine in a parallel concentration-dependent manner, which implies that PKC has a critical role in this pathway (Merrill et al 1990). Because it is plausible that inhibition of PMA-induced neutrophil aggregation by magnolol is a result of inhibition of PKC, the effect of magnolol on PKC activity was investigated.

The mammalian PKC family consists of at least 12 different isoforms (Dekker & Parker 1994); these isoenzymes' specific regulation and function remain unclear. We recently found that rat neutrophils contain PKCs of three isoform families (α, β, γ, δ, ε, θ, μ, ι, λ and ζ), although PKC λ and ζ are barely detected (Tsao & Wang 1997). In the presence of Ca^{2+} and PS, PMA activated the cytosolic PKC of resting rat neutrophils. In the presence of EDTA, Ca^{2+}-independent PKC activity was observed in the cytosolic PKC preparations. Because rat brain PKC preparation contains mainly Ca^{2+}-dependent isoforms (α, β, and γ isoforms; Go et al (1987)), only minor PKC activity was observed in the presence of EDTA. Magnolol inhibited cytosolic PKC over the same range of concentrations at which it inhibited the rat brain PKC, which implies that magnolol can directly inhibit PKC, or at least that of the Ca^{2+}-dependent PKC isoforms. These results suggest that inhibition of PKC by magnolol probably contributes to the attenuation of neutrophil aggregation. Although only approximately 35% of the total enzyme activity of cytosolic PKC was inhibited by 100 μM magnolol, an effect much less potent than the inhibitory effect of magnolol on neutrophil aggregation, more than 85% reduction of PKC activity was observed when the magnolol concentration was 100 μM (IC50 45·7 $\pm$ 6·5 μM) if only the Ca^{2+}-dependent PKC component was taken into account. Further study will be required to clarify which of the PKC isoforms was inhibited by magnolol.

PKC has both regulatory and catalytic regions (Nishizuka 1986) and to determine which of these is the site of action of magnolol experiments were performed on PDB binding to PKC and on trypsin-treated PKC activity. Phorbol esters are believed to bind to the C1 domain of the PKC regulatory region (Newton 1995). In the assay of PDB binding, magnolol and 1-oleoyl-2-acetyl-*sn*-glycerol inhibited the binding of [^{3}H]PDB to neutrophil cytosolic PKC. The parallel inhibition of PKC activity and PDB binding by magnolol implies that the blockade of regulatory region of PKC has a critical role.

The regulatory and catalytic regions are separated by a hinge region which is sensitive to protease. After partial digestion of PKC with trypsin the regulatory region is removed from the native PKC (Lee & Bell 1986). The remaining catalytic region of PKC has enzyme activity independent of Ca^{2+}, PS and diacylglycerol (Inoue et al 1977). Staurosporine, a competitive inhibitor of ATP (Tamaoki et al 1986), inhibited native and trypsin-treated PKC. Unlike staurosporine, magnolol did not affect the trypsin-treated PKC activity, suggesting that the site of action of magnolol is not at the catalytic region.

PKC activity is primarily cytosolic in unstimulated neutrophils (Wolfson et al 1985). Although conventional and novel PKC isoenzymes contribute to the increase in membrane-associated PKC activity in cells after PMA treatment, phorbol esters do not affect the atypical PKC isoenzymes (Blobe et al 1996; Tsao & Wang 1997). Membrane translocation is mediated by diacylglycerol or phorbol ester binding to the C1 domain and PS/Ca^{2+} binding to the C2 domain of the PKC regulatory region (Newton 1995). Magnolol did not affect the PMA-induced PKC -α and -δ associated with membrane, implying that magnolol probably interacted with the C1 domain only of the regulatory region.

In conclusion, we found that a plant product, magnolol, inhibited homotypic aggregation in PMA-activated neutrophils. This inhibition is probably attributable to the direct suppression of PKC activity by magnolol through blockade of the regulatory region.

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