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Role of Calcium and Protein Kinase C in Platelet Activating Factor-induced Activation of Peritoneal Macrophages*

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ABSTRACT

Particulate or soluble stimuli appear to stimulate phagocytic cell's response by the change of Ca^{2+} mobilization and by the activation of protein kinase C. In contrast, it is reported that activation of protein kinase C could attenuate agonist-stimulated elevation of Ca^{2+}i in neutrophils.

PAF elicited an increase of Ca^{2+}i in peritoneal macrophages in a dose dependent fashion and Ca^{2+} extrusion was accompanied. PAF-induced elevation of Ca^{2+}i was not affected by TMB-8, verapamil and TTX. TEA stimulated PAF-induced mobilization of Ca^{2+}i and delayed lowering of Ca^{2+}i. Five mM EGTA almost completely inhibited PAF-induced mobilization of Ca^{2+}i. After the addition of PAF, membrane permeability was markedly increased up to 5 min and then slowly increased. PAF-induced LDH release was slightly decreased by EGTA plus TMB-8. PAF-stimulated superoxide generation was inhibited by EGTA, TMB-8 and verapamil but not affected by TTX and TEA. PAF-induced elevation of Ca^{2+}i, increased membrane permeability and superoxide generation were inhibited by IQSP, chlorpromazine and propranolol. PAF-induced LDH release was significantly inhibited by chlorpromazine and minimally decreased by propranolol. After the pretreatment with PMA, the stimulatory effect of PAF on the elevation of Ca^{2+}i and LDH release in macrophages was significantly decreased.

These results suggest that PAF may exert the stimulatory action on peritoneal macrophages of mouse by the elevation of Ca^{2+}i and by the activation of protein kinase C. Preactivation of protein kinase C appears to attenuate the stimulatory action of PAF on macrophage response.

Key Words: Ca^{2+} mobilization, Degranulation, Superoxide generation, Peritoneal macrophage, Platelet activating factor, Human neutrophil

INTRODUCTION

Platelet activating factor (PAF) is 1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine (alkyl acetyl GPC). PAF is an extremely active biosynthetic product of cell phospholipids and has been implicated in platelet aggregation, vascular permeability changes, activation of neutrophils and macrophages and bronchospasm (Braquet *et al.*, 1987). It can be synthesized by many cells including platelets, mast cells, macrophages, basophils, eosinophils and neutrophils. PAF is formed by a two stage process in which 1-0-alkyl-2-acyl-sn-glycero-3-phosphocholine is degraded to lyso-PAF by phospholipase A_2 and then acetylated to

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PAF (Braquet *et al.*, 1987; Snyder, 1989). PAF receptor is a plasma membrane protein that is coupled to a guanine nucleotide binding proteins (Hwang *et al.*, 1986). When PAF binds to the receptor protein, it activates the G protein which in turn can activate either phospholipase C (MacIntyre and Pollock, 1983; Hallam *et al.*, 1984) or can inhibit adenylate cyclase (Haslam and Vanderwel, 1982; Hwang *et al.*, 1986). The G proteins, phospholipase C and adenylate cyclase appear to mediate the action of PAF in various cells and tissues (Shimizu *et al.*, 1992). PAF may promote inflammatory responses from the phagocytic cells, such as chemotaxis, superoxide generation, lysosomal enzyme release and aggregation (Ingraham *et al.*, 1982; Rouis *et al.*, 1988) by elevation of Ca^{2+}i and by activation of protein kinase C (Barzaghi *et al.*, 1989; Kadiri *et al.*, 1990).

The stimulation of macrophages by PAF leads to the activation of the phospholipase C cascade and subsequently to a biphasic increase of Ca^{2+}i (Conrad and Rink, 1986; Randriamampita and Trautmann, 1989). The rise in Ca^{2+}i is reported to due to both release of Ca^{2+} form intracellular stores and influx from the extracellular medium (Barzaghi *et al.*, 1989; Randriamampita and Trautmann, 1989). On the other hand, Ca^{2+} influx is known to accompanied by a simultaneously activated Ca^{2+} extrusion. Ca^{2+} extrusion may be stimulated either directly by the rise of Ca^{2+}i or indirectly via calmodulin (Niggli *et al.*, 1979; Lew and Stossel, 1980). Other second messenger systems may also be involved in Ca^{2+} extrusion (Caroni and Carafoli, 1981). In addition, previous studies suggest that activation of protein kinase C stimulates the plasma membrane Ca^{2+} ATPase of neutrophils and erythrocytes (Lagast *et al.*, 1984; Smallwood *et al.*, 1988). In contrast, activation of protein kinase C with phorbol 12-myristate 13-acetate (PMA) and diacylglycerol (DAG) appears to attenuate agonist-stimulated elevations of Ca^{2+}i in neutrophils (Naccache *et al.*, 1985). Some reports suggest that activation of protein kinase C may decrease agonist-stimulated elevations of Ca^{2+}i in neutrophils by the change of Ca^{2+} mobilization (Smallwood *et al.*, 1988) and by the feedback inhibition of phospholipase C, leading to decreased inositol 1, 4, 5-trisphosphate (Della Bianca *et al.*, 1986). Thus, effect of PAF on functional responses in PMA pretreated phagocytic cells is still not clarified. In addition, action of PAF on cell response may be partially mediated by arachidonic acid (Kadiri *et al.*, 1990).

However it has also been shown that the activation of the respiratory burst in neutrophils could be insensitive to inhibitors of protein kinase C (Rossi *et al.*, 1989) and do not correlate with the increase in Ca^{2+}i and with the activation of phosphoinositide turnover (Rossi *et al.*, 1986). On the other hand, PAF has been reported to show the different effect on the respiratory burst of macrophages according to the tissue and the species (Yasaka *et al.*, 1982; Maridonneau-Parini *et al.*, 1985).

In the present study, influences of Ca^{2+} chelators, ionic channel blockers, inhibitors of protein kinase C and chlorpromazine on PAF-induced changes of Ca^{2+}i, membrane permeability and LDH release in peritoneal macrophages of mouse and superoxide generation in human neutrophils were investigated. Effect of protein kinase C activation on PAF-induced macrophage response was examined.

MATERIALS AND METHODS

L-α-Phosphatidylcholine, β-acetyl-γ-o-hexadecyl (PAF), verapamil, tetrodotoxin (TTX), tetraethylammonium chloride (TEA), ethyleneglycol-bis (β-amino-ethyl-ether), N, N, N', N',-tetraacetic acid (EGTA), 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine dihydrochloride (IQSP), chlorpromazine, propranolol, phorbol 12-myristate 13-acetate (PMA), quin 2/AM, NAD, digitonin, ethidium bromide, ferricytochrome c, Hanks' balanced salt solution (HBSS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma Chemical Co., 8-(Diethylamino) ocytl, 3, 4, 5-trimethoxybenzoate hydrochloride (TMB-8) was obtained from Aldrich Chemical Co.; Murexide from J.T. Baker Chemical Co.; Thioglycollate medium from Difco Laboratories.; Heat inactivated fetal bovine serum from JRH Biosciences. Other chemicals were of analytical reagent grade.

Macrophage elicitation and cultivation

Macrophages were elicited by injection of 1 ml of 3% thioglycollate medium into the peritoneal

cavity of ICR female mouse weighing about 20 g. Cells were harvested 4 days after injection. The mouse was killed by cervical dislocation. Cells were removed after intraperitoneal injection of 10 ml of HBSS containing 10 U/ml sodium heparin and recovered by centrifugation at 400 g for 5 min at 4℃. RBC was lysed by the addition of a hypotonic solution (0.2% NaCl) and after 20 sec. 1.6% NaCl was added to make an isotonic solution. Cells were washed two times with HBSS does not contain heparin. Washed cells were suspended in DMEM supplemented with 10% heat-inactivated (56℃, 30 min) fetal bovine serum and plated on 35 mM plastic culture petri dishes. After incubation for 60 min at 37℃ in 5% CO_2-95% air, nonadherent cells were removed by aspirating with a sterile Pasteur pipet and adherent macrophages were washed two times with 5 ml of HBSS. Adherent macrophages were harvested with HBSS. Adherent macrophages were harvested with HBSS using a cell harvestor. For long-term cultures, the medium with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin was changed after 24 h and thereafter at 2 day intervals (Johnston *et al.*, 1978).

Preparation of neutrophils

Neutrophils were isolated from ACD treated venous blood of healthy donors by dextran (average molecular weight 465,000) sedimentation of erythrocytes and treatment with 0.85% ammonium chloride (Trush *et al.*, 1978). The purity of neutrophil suspensions averaged 90% as judged by Wright-Giemsa stain.

Measurement of cytosolic free calcium

Quin 2 loading and fluorescence measurement were performed by the modification of the method of Tsien *et al.* (1982). Macrophages (approximately 10^8/ml) were loaded with 5 μl of 20 mM quin 2/AM at 37℃ for 20 min in 1.0 ml of the reaction mixtures containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM dextrose. The suspension was then diluted to 10 fold with the above reaction mixture and further incubated at 37℃ for 40 min. After loading, the suspension was centrifuged at 1,500 g for 5 min and macrophages were resuspended in the above reaction mixture as approximately 10^7/100 μl.

Fluorescence measurement was done with a Turner Spectrofluorometer (Model/430). Preloaded macrophages (10^7) were suspended in the same reaction mixture in a final volume of 2.0 ml. After preincubation at 37℃ for 5 min, the response was initiated by the addition of PAF. The fluorescence change was read at an excitation wavelength of 339 nm and emission wavelength of 492 nm.

Measurement of calcium release

Calcium release was measured by the spectrophotometric method using an Aminco-Chance dual wavelength-split beam spectrophotometer. The reaction mixtures contained 4×10^6 cells/ml of macrophages, 50 μM murexide, HBSS buffer and 20 mM HEPES-tris, pH 7.4. After preincubation at 37℃ for 10 min, the response was initiated by the addition of various concentration of PAF with 1 mM calcium and a final volume was a 1.0 ml. The rate and extent of calcium release by macrophages was measured with the absorbance changes of calcium chelating dye, murexide, at 507-540 nm in a 1.0 ml cuvette (Malmström and Carafoli, 1979).

Measurement of plasma membrane permeability

Permeability of the plasma membrane was assessed by measuring the uptake of the DNA stain ethidium bromide, a fluorescent dye (Picello *et al.*, 1990). The reaction mixtures contained 2×10^6 macrophages, 20 μM ethidium bromide, other compounds and HBSS buffer in a total volume of 2 ml. After preincubation of 5 min with ethidium bromide, the response was initiated by the addition of PAF. Uptake of ethidium bromide was measured fluorometrically at the wavelength of excitation of 360 nm and emission, 580 nm.

Measurement of lactic dehydrogenase (LDH) release

Released amount of lactic dehydrogenase from activated macrophages was spectrophotometrically measured at 340 nm by reduction of NAD. The reaction mixtures contained 4×10^6 macrophages, 1 mM NAD, 54 mM sodium lactate, pH 7.0, 50 mM sodium phosphate buffer, pH 8.8 and other compounds in a total volume of 2 ml. After 5 min of preincubation at 37℃, the response

was initiated by the addition of PAF and NAD. Released lactic dehydrogenase is expressed as absorbance at 340 nm/2×10^6 cells (Wacker *et al.*, 1956).

Measurement of superoxide radical generation

The superoxide dependent reduction of ferricytochrome C was measured by the method of Markert *et al.* (1984). The reaction mixtures in plastic microfuge tubes contained 10^6 neutrophils, HBSS buffer, 75 μM ferricytochrome c, PAF, 20 mM HEPES-tris, pH 7.4 and other compounds in a total volume of 500 μl. The reactions were performed in a 37℃ shaking water bath for 10 min. The reaction was then stopped by placing the tubes in melting ice and the cells were rapidly pelleted by centrifuging at 1,500 g for 5 min at 4℃. The supernatants were taken and the amount of reduced cytochrome C was measured at 550 nm. The amount of reduced cytochrome C was calculated by using an extinction coefficient of $1.85\times10^4\ M^{-1}\ cm^{-1}$ at 550 nm.

RESULTS

PAF-induced calcium mobilization

Elevation of Ca^{2+}i is an early event in the response of phagocytic cells to many agonists, fMLP and PAF (Andersson *et al.*, 1986; Shimizu *et al.*, 1992). The Ca^{2+}i was measured with the increase of fluorescence due to the complex formation of an increased Ca^{2+} with quin 2. PAF elicited an increase of Ca^{2+}i in peritoneal macrophages in a dose dependent fashion (Fig. 1). The maximum mobilization occured within 5 sec post addition and decreased gradually to a lower level after 1 to 3 min. Ca^{2+} extrusion may be stimulated directly by the rise of Ca^{2+}i. PAF is suggested to activate Ca^{2+} efflux without causing a preceding increase in Ca^{2+}i (Randriamampita and Trautmann, 1990). As shown in Fig. 2, 10 μM PAF induced Ca^{2+} release from peritoneal macrophages. PAF-induced elevation of Ca^{2+} was not affected by 0.5 mM TMB-8, an intracellular Ca^{2+} chelator,

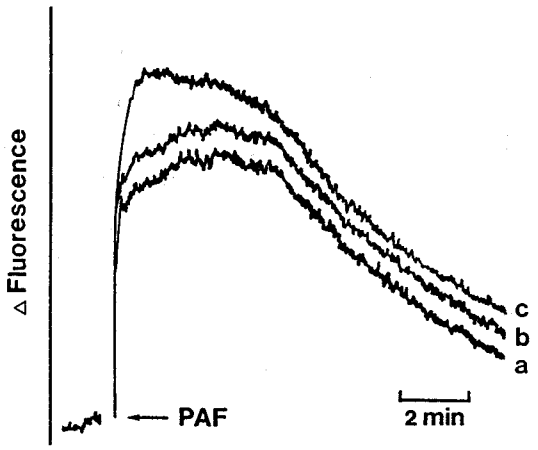

Fig. 1. PAF-induced elevation of Ca^{2+}i in peritoneal macrophages. Change of intracellular free Ca^{2+} level in PAF-stimulated macrophages (4×10^6 cells/ml) was measured as a fluorescence change of quin 2, a specific Ca^{2+} chelator. Experimental conditions were the same as described in Materials and Methods. The response was initiated by the addition of PAF. Fluorescence of quin 2-Ca^{2+} complex was read at the wavelength pair 390-492 nm. a, 5 μM PAF; b, 10 μM PAF; c, 20 μM PAF.

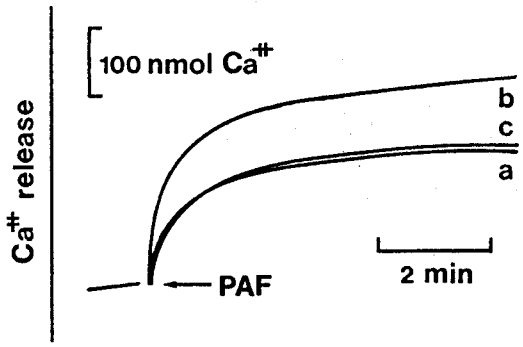

Fig. 2. PAF-induced Ca^{2+} release from peritoneal macrophages. The reaction mixtures contained 4×10^6/ml macrophages, 50 μM murexide, HBSS and 20 mM HEPES-tris, pH 7.4. After preincubation with or without 0.5 mM TMB-8, the release was initiated by the addition of PAF. Ca^{2+} release was measured spectrophotometrically at the wavelength pair 507-540 nm. a, 1 μM PAF; b, 10 μM PAF; c, 10 μM PAF +0.5 mM TMB-8.

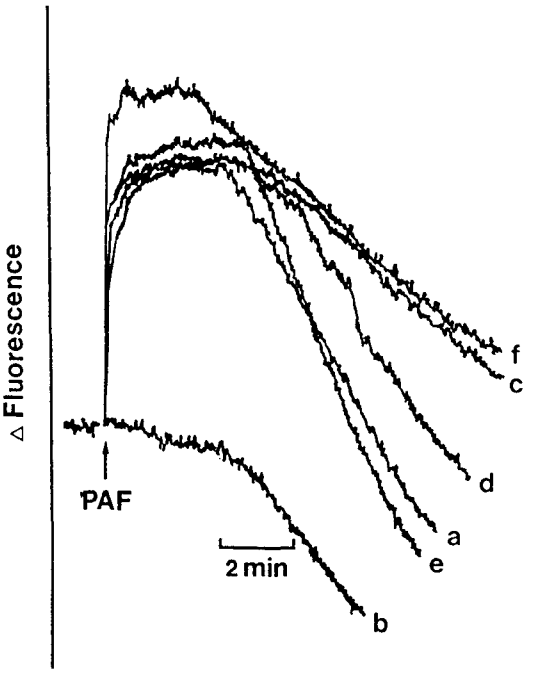

Fig. 3. Effects of Ca^{2+} chelators and ionic channel blockers on PAF-induced elevation of Ca^{2+}i. Macrophages (4×10^6 cells/ml) were preincubated with compounds for 5 min and then the response was initiated by the addition of PAF. a, no addition; b, 5 mM EGTA; c, 0.5 mM TMB-8; d, 0.5 mM verapamil; e, 15 μM TTX; f, 100 μM TEA in the presence of 10 μM PAF.

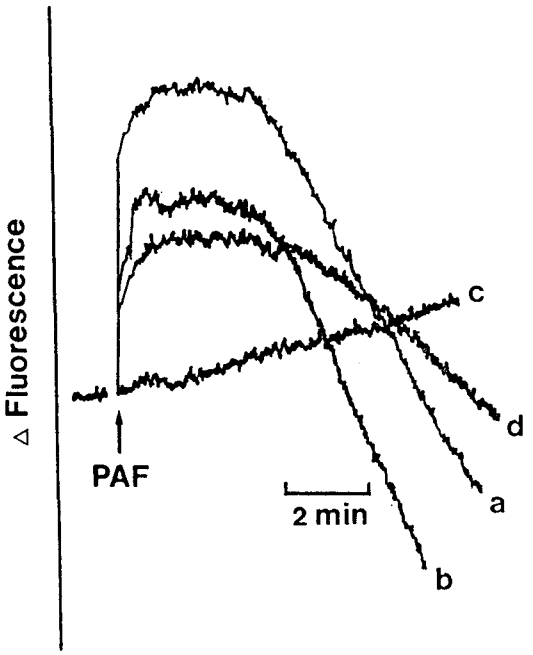

Fig. 4. Effects of IQSP, chlorpromazine and propranolol on PAF-induced elevation of Ca^{2+}i. Macrophages (4×10^6 cells/ml) were preincubated with compounds for 5 min and then the response was initiated by the addition of PAF. a, no addition; b, 50 μM IQSP; c, 100 μM chlorpromazine; d, 100 μM propranolol in the presence of 10 μM PAF.

0.5 mM verapamil, a Ca^{2+} channel blocker and 15 μM TTX, a Na^+ channel blocker. And in the presence of TMB-8, Ca^{2+} lowering was delayed (Fig. 3). On the other hand, 100 μM TEA, a K^+ channel blocker, stimulated PAF-induced mobilization of Ca^{2+}i and delayed lowering of Ca^{2+}. Five mM EGTA almost completely inhibited PAF-induced mobilization of Ca^{2+}i.

Changes of PAF-induced Ca^{2+}i mobilization by the inhibition and activation of protein kinase C

Activation of protein kinase C is reported to affect agonist-induced the influx and efflux of Ca^{2+} in neutrophils (McCarthy *et al.*, 1989). Fig. 4 showed that 50 μM IQSP, 100 μM chlorpromazine and 100 μM propranolol which are known to inhibit protein kinase C inhibited 10 μM PAF-induced mobilization of Ca^{2+}. In the presence of 100 μM chlorpromazine, action of PAF on Ca^{2+} mobilization in macrophage was almost completely inhibited. The phorbol ester PMA appears to decrease both agonist-and ionophore-induced elevation of Ca^{2+}i in neutrophils. As can be seen in Fig. 5, after the pretreatment of 10 ng/ml PMA for 2 min, the enhancing action of 10 μM PAF on Ca^{2+}i mobilization was significantly inhibited. In the presence of PMA, PAF induced a slight increase of Ca^{2+}i and 1 min later, Ca^{2+}i level was gradually decreased to a lower level. After 2 min, Ca^{2+}i level was lower than the resting level of Ca^{2+}i.

PAF-induced change of membrane permeability

Permeability of the plasma membrane was assayed by measuring the uptake of ethidium bro-

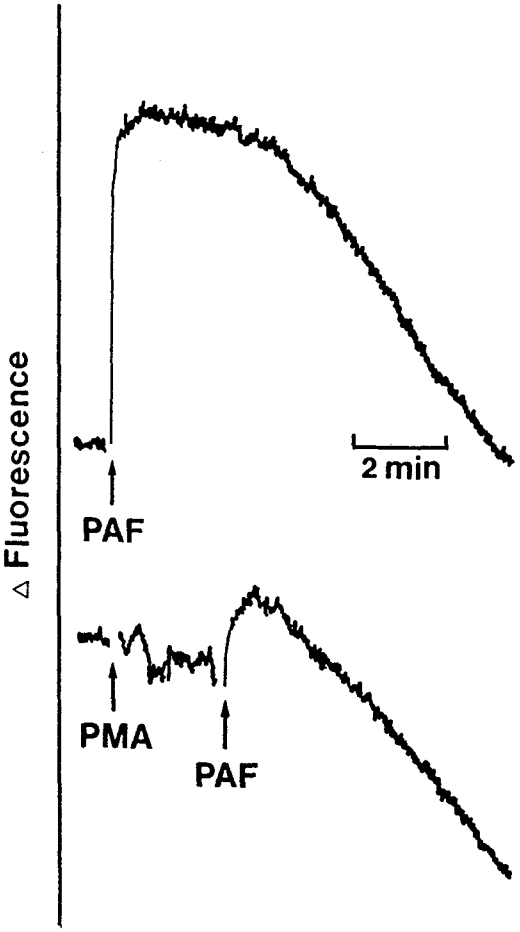

Fig. 5. Effect of PMA pretreatment on PAF-induced elevation of $Ca^{2+}i$ in macrophages. Macrophages (4×10^6 cells/ml) were incubated with 10 ng/ml PMA and then 10 μM PAF was added at the arrow point.

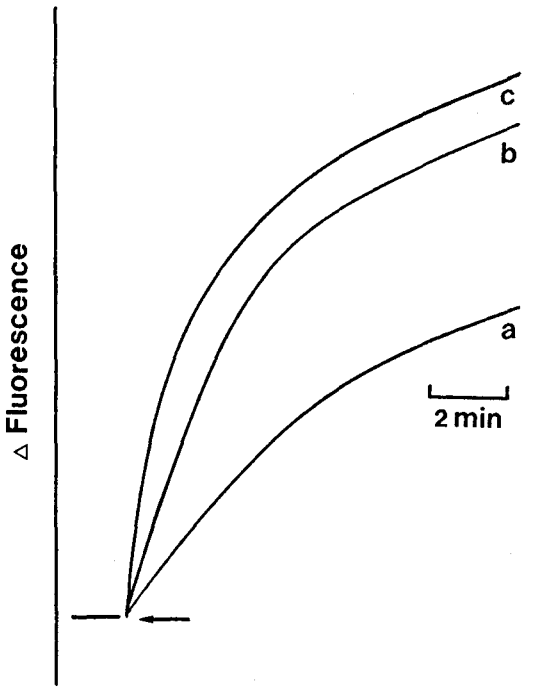

Fig. 6. PAF-induced increase of membrane permeability in macrophages. Change of membrane permeability in PAF-stimulated macrophages (10^6 cells/ml) was assayed by measuring the uptake of ethidium bromide, a cell penetrable fluorescent compound. The res-ponse was initiated by the addition of 10 μM PAF in the presence of 10 μM ethidium bromide. Fluorescence change was read at the wavelength pair 360-580 nm. a, 10 μM PAF; b, 30 μM PAF; c, 10 μM digitonin.

mide. The increase in plasma membrane permeability was selective for low molecular weight solutes and ethidium bromide which showes a fluorescence is able to access freely to the cytoplasm of macrophages (Picello *et al.*, 1990). Membrane permeability of macrophages which is responsible for digitonin was increased by PAF (Fig. 6). After the addition of PAF, membrane permeability was markedly increased up to 5 min and then slowly increased. To investigate role of protein kinase C on the regulation of membrane permeability, effects of protein kinase C inhibitors on PAF-induced change of membrane permeability was investigated. Fig. 7 showed that 10 μM PAF-induced increase of membrane permeability was inhibited by 50 μM IQSP and 100 μM chlorpromazine.

Changes of PAF-induced degranulation by the inhibition and activation of protein kinase C

After macrophages were preincubated for 5 min with various compounds, LDH release was initiated by the addition of 5 μM PAF. As shown in Fig. 8, 5 μM PAF-induced LDH release was not affect-

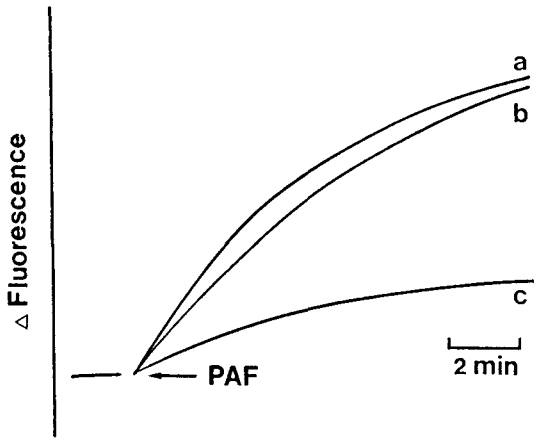

Fig. 7. Effects of IQSP and chlorpromazine of PAF-induced increase of membrane permeability. Macrophages (10^6 cells/ml) were preincubated with compounds for 5 min and then the response was initiated by the addition of PAF. a, no addition; b, 50 μM IQSP; c, 100 μM chlorpromazine in the presence of 10 μM PAF.

ed by 5 mM EGTA. In the presence of 5 mM EGTA and 0.5 mM TMB-8, PAF-induced LDH release was slightly decreased. PAF-induced LDH release was significantly inhibited by 100 μM chlorpromazine but not affected or minimally decreased by 50 μM IQSP and 100 μM propranolol (Fig. 9). In PMA pretreated macrophages, effect of PMA on LDH release was examined. Fig. 10 showed that 10 ng/ml PMA alone induced LDH release, but in the presence of PMA action of 5 μM PAF on LDH release was almost completely abolished.

PAF-stimulated superoxide generation in human neutrophils

PAF can activate neutrophils and monocytes via specific cell surface receptors. However, it is suggested that effect of PAF on the respiratory burst may be variable according to a function of the tissue and the species from which the macrophages are derived (Rouis *et al.*, 1988). In the pres-

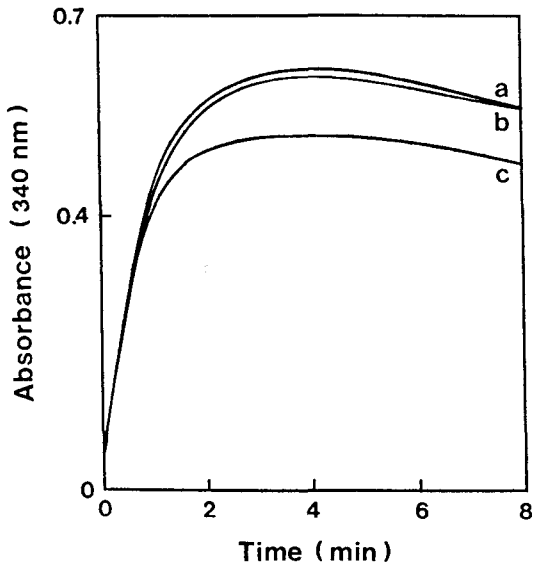

Fig. 8. Effects of EGTA and TMB-8 on PAF-induced LDH release from macrophages. Macrophages (2×10^6 cells/ml) were preincubated for 5 min at 37℃ with compounds. LDH release was initiated by the addition of PAF. Activity was expressed as $\triangle OD/2\times10^6$ cells. a, no addition; b, 5 mM EGTA; c, 5 mM EGTA+0.5 mM TMB-8 in the presence of 5 μM PAF.

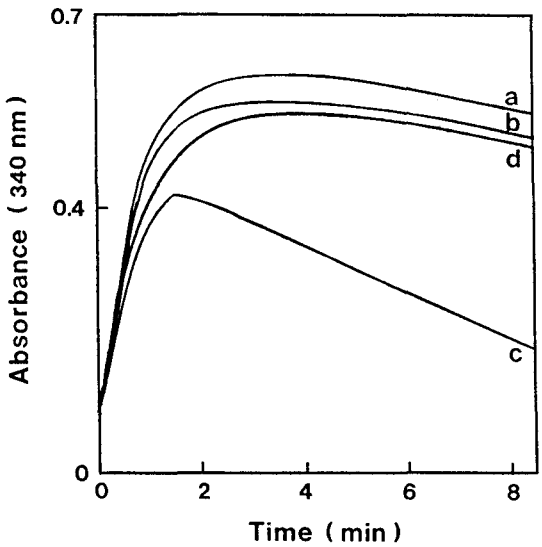

Fig. 9. Effects of IQSP, chlorpromazine and propranolol on PAF-induced LDH release. Macrophages (2×10^6 cells/ml) were preincubated with compounds and then LDH release was initiated by the addition of PAF. Activity was expressed as $\triangle OD/2\times10^6$ cells. a, no addition; b, 50 μM IQSP; c, 100 μM chlorpromazine; d, 100 μM propranolol in the presence of 5 μM PAF.

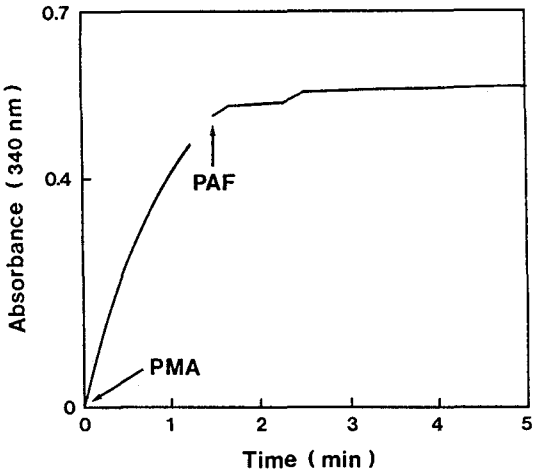

Fig. 10. Effect of PMA pretreatment on PAF-induced LDH release. Macrophages (2×10^6 cells/ml) were incubated with 10 ng/ml PMA and then 5 μM PAF was added at the arrow point. Activity was expressed as $\triangle OD/2\times10^6$ cells.

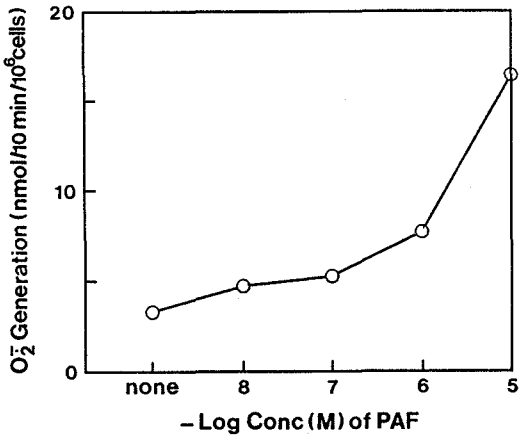

Fig. 11. PAF-stimulated superoxide generation in human neutrophils. Neutrophils (10^6 cells/0.5 ml) were incubated with varying concentration of PAF for 10 min at 37℃. Points are means of 7 experiments.

Table 1. Effects of Ca^{2+} chelators and ionic channel blockers on PAF-stimulated superoxide generation

Compounds		Superoxide nmol/ 10 min/10^6 cells
PAF 10 μM		16.79±1.50
+EGTA	10 mM	10.51±0.93
+TMB-8	0.5 mM	12.67±0.78
+Verapamil	0.1 mM	10.04±0.79
+TEA	0.1 mM	16.29±2.47
+TTX	10 μM	17.29±0.80

Neutrophils (10^6 cells/ 0.5 ml) were preincubated with compounds in HBSS for 5 min and then the response was initiated by the addition of PAF. Values are means±SD of 4~9 experiments.

Table 2. Effects of IQSP and chlorpromazine on PAF-stimulated superoxide generation

Compounds		Superoxide nmol/ 10 min/10^6 cells
PAF 10 μM		16.50±0.67
+IQSP	10 μM	13.03±0.89
+CPZ	10 μM	12.68±0.70

Neutrophils (10^6 cells/0.5 ml) were preincubated with compounds in HBSS for 5 min and then the response was initiated by the addition of PAF. Values are means±SD of 5 experiments.

ent study, PAF up to 30 μM did not show any significant effect on superoxide generation in peritoneal macrophages from mouse (data not shown). Thus, effect of PAF on superoxide generation was investigated in human neutrophils. Fig. 11 showed that PAF stimulated superoxide generation in intact neutrophils in a dose dependent fashion. Amount of superoxide generated in neutrophils activated by 10 μM PAF was 16.46 nmol/10 min/ 10^6 cells. PAF-stimulated superoxide generation was inhibited by 10 mM EGTA, 0.5 mM TMB-8 and 0.1 mM verapamil but not affected by 10 μM TTX and 0.1 mM TEA (Table 1). Role of protein kinase C in the respiratory burst was examined. As can be seen in Table 2, 10 μM PAF-stimulated superoxide generation was inhibited by 10 μM IQSP and 10 μM chlorpromazine.

DISCUSSION

Activated macrophages liberate reactive oxygen species, O_2^- and H_2O_2 (Johnston *et al.*, 1978), secrete lysosomal enzymes including collagenase, elastase-like enzyme, β-glucuronidase and lysozyme (Gordon *et al.*, 1974; Werb and Gordon, 1975a; Werb and Gordon, 1975b) and produce lipid inflammatory mediators, such as leukotrienes and platelet activating factor (Rankin, 1982; Albert and Synder, 1983). These secretory products are known to mediate various macrophage functions, immunoregulation and microbicidal and tumoricidal activity (Sadada and Johnston, 1980). When phagocytic cells are exposed to a variety of soluble or particulate substances, molecular and functional changes take place in the plasma membrane or intracellular components. These changes include the sodium influx (Showell and Becker, 1976), change of the membrane potential (Korchak and Weissmann, 1980), mobilization of calcium (Bareis *et al.*, 1982), phospholipid turnover (Takenawa *et al.*, 1985) and oxidation of surface and intracellular sulfhydryl groups (Shin *et al.*, 1989). These changes are followed by the alteration of functional responses of phagocytic cells which consists of chemotaxis, phagocytosis, release of lysosomal enzymes and superoxide generation (Fantone and Ward, 1982).

Platelet activating factor (PAF) is a potent lipid autacoid which mediates many types of anaphylactic and inflammatory responses (Braquet *et al.*, 1987). PAF promotes aggregation of platelet, neutrophil and monocyte. It stimulates neutrophils to release leukotrienes and lysosomal enzymes and to generate superoxide radical (Ingraham *et al.*, 1982; Lin *et al.*, 1982). PAF appears to exert its action by binding to G protein-linked, cell surface receptors at the plasma membrane (Hwang, 1988). Stimulation of these receptors causes activation of phospholipases C and A_2, promoting the formation of inositol 1, 4, 5-trisphosphate, 1, 2-diacylglycerol and arachidonate (Kawaguchi and Yasuda, 1986; Schwertschlag and Whorton, 1988). These mediators are responsible for the release of calcium from intracellular stores (Berridge and Irvine, 1984) and the activation of protein kinase C (Nishizuka, 1984). A rise in the cytosolic calcium concentration is considered to be an important factor in the stimulation of neutrophil responses including degranulation and superoxide generation according to the surface stimulation by external stimuli.

PAF elicited an increase of $Ca^{2+}i$ in peritoneal macrophages in a dose dependent fashion, which is attributed to the change of membrane permeability. PAF-induced elevation of $Ca^{2+}i$ was not affected by TMB-8, verapamil and TTX. These data suggest that PAF induces the release of Ca^{2+} from intracellular storage sites, which is similar with action of arachidonic acid (Wolf *et al.*, 1986; Sim *et al.*, 1992). In macrophages, it has been shown that PAF stimulates arachidonic acid production mainly as a result of phospholipase A_2 activation (Nakashima *et al.*, 1989). EGTA markedly inhibited PAF-induced elevation of $Ca^{2+}i$. In the presence of TMB-8, delayed decrease of elevated $Ca^{2+}i$ to lower level indicates that PAF-induced elevation of $Ca^{2+}i$ is attained partially by Ca^{2+} influx. Ca^{2+} influx appears to be counter-balanced by a simultaneously activated Ca^{2+} extrusion. Ca^{2+} extrusion may be accomplished by Ca^{2+}-ATPase and Na^+-Ca^{2+} exchanger (Niggli *et al.*, 1981; Carafoli, 1988). It is reported that arachidonic acid activates Ca^{2+} extrusion by its stimulatory effect on Ca^{2+}-ATPase (Randriamampita and Trautmann, 1990). Thus, PAF-induced Ca^{2+} extrusion, which is partly derived from the intracellular stores, may be due to the activation of Ca^{2+}-ATPase. In addition, blockade of potassium channel probably promotes the stimulatory effect of PAF on Ca^{2+} mobilization.

The functional responses of neutrophils, such as degranulation can be altered by the change of cytosolic nucleotide level as well as Ca^{2+} mobilization (Zurier *et al.*, 1974; Smolen *et al.*, 1981). The release of lysosomal enzymes is significantly inhibited by EGTA and the agents which elevate the cytosolic cAMP level. PAF-induced LDH release from peritoneal macrophages was inhibited by EGTA plus TMB-8 but not affected by EGTA alone. Thus, stimulatory effect of PAF on LDH release may be chiefly affected by cytosolic free calcium level. The potential role of PAF as an activator of the generation of reactive oxygen species by mononuclear phagocytes is suggested. PAF has been reported to stimulate the res-

piratory burst in peritoneal or alveolar macrophages of guinea pig, in murine macrophages derived from mouse bone marrow and in human monocyte-derived macrophages (Rouis *et al.*, 1988). In contrast, some reports found no effect of PAF on human monocytes and rat alveolar macrophages. In the present study, PAF up to 30 μM did not show any significant effect on superoxide generation in mouse peritoneal macrophages (data not shown). Thus, effect of PAF on superoxide generation was investigated in human neutrophils. Although PMA does not produce a measurable change of Ca^{2+}i, it can stimulate superoxide generation in neutrophils (Tauber, 1987). However, superoxide generation is partially dependent on the change of Ca^{2+}i. Ca^{2+} may be prerequisite for the activation of NADPH oxidase activity, because in Ca^{2+} free medium the stimulatory effect of arachidonic acid on NADPH oxidase which is obtained from resting neutrophils is not detected (Sim *et al.*, 1992). Previous study reported that arachidonic acid-stimulated superoxide generation in the intact neutrophils was inhibited by EGTA, TMB-8 and verapamil but not affected by TTX and TEA (Sim *et al.*, 1992). This finding is also investigated in PAF-stimulated superoxide generation in human neutrophils and two data shows a similar results. It is indicated that effect of PAF on superoxide generation may be mediated partially by arachidonic acid. The extra-and intracellular Ca^{2+} is considered to be required to the expression of functional responses, such as the respiratory burst in PAF-activated neutrophils.

In various cell types, fMLP and PAF stimulate phospholipase C which is linked with a pertussis toxin-sensitive G protein and promote the translocation of protein kinase C from the cytosol to the membrane (Horn and Karnnovsky, 1986). Activation of protein kinase C appears to stimulate neutrophil response, release of lysosomal enzyme and superoxide generation (Sha'afi *et al.*, 1983; Korchak *et al.*, 1984) and the plasma-membrane Ca^{2+}-ATPase of neutrophils and erythrocytes (Lagast *et al.*, 1984; Smallwood *et al.*, 1988). On the other hand, activation of protein kinase C with PMA and diacylglycerol is known to attenuate agonist-stimulated elevations of Ca^{2+}i in neutrophils (Naccache *et al.*, 1985). Several reports indicate that activation of protein kinase C may decrease agonist-stimulated elevations of Ca^{2+}i by the stimulated Ca^{2+} efflux and by the feedback inhibition of phospholipase C, leading to decreased inositol 1, 4, 5-trisphosphate (Della Bianca *et al.*, 1986; Smallwood *et al.*, 1988). In addition, it is reported that activation of protein kinase C can inhibit agonist-stimulated elevations of Ca^{2+}i in neutrophils by causing blockade of stimulated influx of bivalent cations from the extracellular medium and by partially inhibiting release of Ca^{2+} from intracellular storage sites (McCarthy *et al.*, 1989). Previous report suggests that activation of protein kinase C with PMA blocks Ca^{2+} influx into neutrophils via the closure of Ca^{2+} channels (Grigorian *et al.*, 1988). It has also been proposed that DAG produced as a result of phospholipase C activation may play a role in regulating phospholipase A_2 activity (Dawson *et al.*, 1983; Kramer *et al.*, 1987). The incorporation of DAG into the phospholipid substrate stimulates phospholipase A_2 activity.

PAF-induced elevation of Ca^{2+}i, increase of membrane potential and release of LDH was effectively inhibited by IQSP, propranolol and chlorpromazine. These results support that activation of protein kinase C may be implicated in the initiation and expression of phagocytic cell response. However, after macrophages were pretreated with PMA, the stimulatory effect of PAF on responses, Ca^{2+} mobilization and LDH release was almost completely abolished. This finding is coincided with the previous report and activation protein kinase C may attenuate PAF-induced functional changes of mouse peritoneal macrophages. It is reported that after a PMA treatment, agonist-induced arachidonic acid mobilization in mouse marcophages is noticeably enhanced, whereas agonist-induced inositol phosphates production is markedly inhibited (Portilla *et al.*, 1988; Silvka and Insel, 1988). This finding indicates that PMA treatment dissociates phospholipase A_2 from phsopholipase C. However, this suggestion does not elucidate clearly, because PAF-induced change of response may be partially mediated by arachidonic acid. In addition, activator of protein kinase C may induce down-regulation of PAF receptors. It is reported that activators of protein kinase C down regulate LTB_4 high affinity receptors and thereby reduce neutrophil responses (O'Flaherty *et al.*, 1990).

From these results, PAF may exert the stimula-

tory action on peritoneal macrophages of mouse by the activation of protein kinase as well as Ca^{2+} mobilization. Preactivation of protein kinase C appears to cause the inhibiton of Ca^{2+} mobilization and the desensitization of PAF receptors.

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=국문초록=

Platelet Activating Factor에 의한 대식세포의 활성화에 있어서 칼슘과 Protein Kinase C의 역할

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입자 또는 용해성 자극 물질들은 칼슘 이동의 변화와 protein kinase C의 활성화를 초래하여 식 세포의 반응을 자극하는 것으로 추정하고 있다. 이에 비해서 protein kinase C가 활성화되면 호중구에서 agonist에 의한 세포 칼슘 농도의 증가가 억제된다고 보고하고 있다.

PAF는 peritoneal macrophage에서 세포내 칼슘 농도를 용량에 따라 증가시켰으며 칼슘의 유출이 동반되었다. PAF에 의한 세포내 칼슘 농도의 증가는 TMB-8, verapamil과 TTX의 영향을 받지 않았다. TEA는 PAF에 의한 세포내 칼슘 이동을 자극하였으며 세포내 칼슘 농도의 감소를 지연시켰다. 5 mM EGTA는 거의 완전히 PAF에 의한 세포내 칼슘 이동을 억제하였다. PAF의 첨가 후에 세포막 투과성은 반응 5분까지 현저하게 증가하였으며 이후 느리게 증가하였다. PAF에 의한 LDH 유리는 EGTA와 TMB-8에 의하여 약간 감소하였다. PAF에 의하여 자극된 superoxide 생성은 EGTA, TMB-8과 verapamil에 의하여 억제되었으나 TTX와 TEA의 영향은 받지 않았다. PAF에 의한 세포내 칼슘 농도의 증가, 세포막 투과성의 증가와 superoxide 생성은 IQSP, chlorpromazine과 propranolol에 의하여 억제되었다. PAF에 의한 LDH 유리는 chlorpromazine에 의하여 유의하게 그리고 propranolol에 의하여 다소 적게 억제되었다. PMA 전처리 후에 macrophage에서 세포내 칼슘 농도의 상승과 LDH 유리에 대한 PAF의 자극 효과는 유의하게 감소되었다.

이상의 결과로 부터 PAF는 세포내 칼슘 농도를 증가시키고 protein kinase C를 활성화시킴에 의하여 마우스 peritoneal macrophage에 자극 작용을 나타낼 것으로 시사된다. Protein kinase C를 미리 활성화시키면 macrophage 반응에 대한 PAF의 자극 작용은 억제될 것으로 추정된다.