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P48-Triggered transmembrane signaling transduction of human monocytes: mobilization of calcium ion and activation of protein kinase C (PKC)[1]

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ABSTRACT

P48 is a cytokine which induces monocyte differentiation and the induction of cytotoxic activity. In this study, the signal transduction events involved in the stimulation of monocytes with the membrane form of P48 (mP48) were investigated. Monocyte stimulation with mP48 was found to involve the mobilization of intracellular calcium (Ca^{2+}) and the activation and translocation of PKC from the cytosol to the membrane. Membane P48 induced a rapid rise of intracellular Ca^{2+} in a dose dependent maner. Similarly, the stimulation of monocytes with P48 was found to involve the activation and translocation of PKC. The translocation of PKC was rapid (within 0-5 min) yet transient with PKC activity returning to control levels by 8 min. The functional role of protein kineses in P48 induced TNF secretion was studied using various kinese inhibitors. The PKC inhibitors, H-7 and sphingosine, were found to inhibit P48 induced TNF secretion with 50% inhibition at 5 μ M. HA1004, which inhibts cyclic nucleotide-dependent kinase (PKA, Ki 1.2 μM), did not inhibit TNF secretion. H-8 (PKA inhibitor) was found to be an effective inhibitor of TNF secretion only at high concentrations(30 μM). The Calmodulin-dependent kinase inhibitor, W7 (Ki 12 μM) was found to be effective at concentration above 5 μM.

1. Dedicated to Professor Zhen YAO's 80th Birthday.

These findings suggest that P48-triggered TNF secretion, involves transmembrane Ca^{2+} signaling and the subsequent activation of at least two protein kineses, PKC and CaMK.

Key words: *P48 monocyte differentiation inducing factor, Signal transduction, Ca^{+} mobilization, PKC activation, TNF secretion.*

INTRODUCTION

The differentiation of cells along the monocyte-macrophage pathway and the signals involved in these cells acquiring the ability to kill tumor cells are not fully understood. We have been studing a molecule which appears to be an important member of the cytokine network involved in the regulation monocyte activation. This cytokine termed P48 was isolated from the human null cell leukemia cell line Reh. It has been purified to homogeneity and found to be distinct from interferon gamma, colony stimulating factors (CSFs) and TNF alpha and beta[l, 2]. Functionally, this molecule was identified by this ability to induce human leukemia cells (HL-60) to differentiate along the monocyte-macrophage pathway and to develop cytotoxic activity[l, 2]. Using human monocytes, P48 was found to possess potent stimulatory activity inducing the secretion of TNF and IL-1[3]. Recently, we have shown TNF inducing activity in fixed Reh cells and plasma membrane from Reh cells but not K562 or P815 cells[4]. Furthermore, P48 was extracted with Triton X-114, identified by immunopreciptation, and the TNF inducing activity was removed from Reh membrane preperations using an anti-P48 affinity column. Thus, we have identified P48 as an integral membrane protein, which like the secreted moleculae is a potent stimulator of TNF secretion[4]. Monocytes and its TNF secretion play an important role in immume and in flammatory responses[5-9]. However, the mechnisms by which P48 induces cytokine release from monocyte are not known.

The stimulation of Ca^{2+} influx and the activation of protein kinase C following ligand binding with a receptor is a common signal transduction mechanism in a variety of cells[10]. These mediators have been found to play a major important role in the cell growth, differentiation, gene expression and secretion[1, 2, 10, 11]. The interaction of ligand with receptor results in a rapid, transient hydrolysis of cell membrane phosphoinositides that are cleaved by phospholipases C to generate 1, 2-diacylglycerol (DAG) and 1, 4, 5-trisphosphate (IP3). In turn, these mediators induce Ca^{2+}. mobilization and stimulate the activation and translocation of PKC. In this study, we have found that mP48 mediates a rapid elevation in intracellular Ca^{2+} which appears to be coupled to the activation of two independent protein kinases. Information regarding the transmembrane signal transduction pathways of

human monocytes may aid the understanding of the biochemical mechanisms which regulate TNF secretion.

MATERIALS AND METHODS

Media and reagents

Culture medium consisted of RPMI 1640 (Cellgro, Mediatech, Washington, DC) supplemented with 2 mM glutamine (GIBCO, Grand Island, NY), 10% (V/V) of fetal bovine serum (GIBCO), and the antibiotics: penicillin (100 U/ml) and streptomycin (100 U/ml) (GIBCO). Except where otherwise noted, all chemicals were purchansed from Sigma Chemical Co. (St. Louis, MO). Protein kinase inhibitors (H-7, H-8, HA1004, W-7) were purchased from CALBIOCHEM (La Jolla, CA).

Preparation of monocytes

Peripheral blood mononucler cells (PBMC) were isolated from healthy donors by centrifugation over Ficoll-Hypaque (Pharmacia, Piscataway, N J) at 600 g for 20 min. PBMC were washed 3 times, placed in 10 cm petri dishes (3×10^7/dish) in complete medium and incubated for 60 min at 37℃ in an atmosphere of 5% CO_2 in air. Nonadherent cells were removed by washing (3 times) with HBSS. The adherent cells were incubated in cold HBSS (Ca^{2+}, Mg^{2+} free, 0.2% EDTA) 20 min and the cells were recovered by gentle scrapping with a rubber policeman. The cells were washed with HBSS, and ultillized in the assays. The cells were greater than 90% monocytes as assessed by morphology and phagocytosis of latex beads (Dow Diagnostics, IN), with a viability greater than 90%.

Preparation of membrane P48

The isolation of plasma membrane containing mP48 was previously described in detail[12]. In brief, Reh cells were hypotonically lysed and the membranes were centrifuged at 175,000 gfor 1 h and washed twice with 10 mM HEPES, pH 7.0. The pellet was layered onto a 35% sucrose cushion and cetrifuged at 250,000 g for 2 h. Plasma membrane banding at the interface was removed, diluted with 10 mM HEPES (pH 7.0) and pelleted at 175,000 g for 1h. The palsma membrane pellet was resuspended at 1 mg/ml (total memranre proteins) in 10 mM HEPES (pH 7.0) stored at -70 ℃ prior to use in the monocyte stimulation. Plasma membranes from the human chronic mylegenous leukemia cell line, K562, were similarly prepared as control membrane preperation.

PKC assay

Quantitative analysis of PKC was carried using a modification of the method of Melton and Kiley[13-14]. Monocytes (3 x 10^6/ ml)were stimulated for various period of time at 37 ℃ with P48 or PMA. The cells were suspended in 50/μl of ice-cold hypotonic lysing buffer (20 mM Tris-HCL, 2 mM EDTA, 1 m*M* DTT, 10 μg/ml leupeptin and 1 m*M* PMSF, pH 7.5) and lysed by repeated, rapid aspiration through a 50μ1 Hamilton syringe. Following lysis, the preparation was diluted in 1.0 ml of ice-cold lysing buffer containing 0.33 M sucrose and the cytosolic and membrane fractions were separated by centrifugation at 100,000 g for 1 h at 4 ℃. The membrane pellets were solubilized by sonication for 15 sec. in cold sample buffer containing 1% Triton ×-100 and stored overnight at 4 ℃. To partially purify PKC, the samples were centrifuged for 8 min at 12,000 rpm and the supernatant from the solubilized membranes as well as the cytosol fractions were applied to 0.6 ml DE-52 cellulose (Whatman BioSystem Ltd., England) columns equilibrated with hypotonic buffer. Before locading the sample, each column was washed once with 2 ml of sample buffer. Partially purified PKC was eluted from the column with 1 ml of 100 m*M* NaCI in hypotonic buffer.

PKC activity was determined by measuring the incorporation of (γ-^{32}p) ATP into histone type-III. The reaction was carried out in assay buffer (20 mM HEPES, 10 mM MgC12, 0.5 mM Ca^{2+}, 5 mM DTT, 60μg/ml phosphatidylserine (PS), 6 μg/ml 1,2-diacylglycerol (DAG), and 10 mg /ml

lysine-rich histone III, pH 7.5). Basal kinase activity was measured in the presence of 2 mM EGTA instead of PS DAG, and Ca^{2+}. Reactions were initiated by the addtion of 0.25 μCi of (γ -^{32}p) ATP (Specific activity, 6000 Ci/mM, NEN, Boston, MA) and carried out for 10 min at 30 ℃ The ^{32}p-labeled histone III was isolated by pipetting 20 μl of the reaction mixture onto Whatman P-81 phosphocellulose paper (1.5 × 1.5 cm). The papers were washed with 10 % trichloroacetic acid (TCA), dried, and enumerated by liquid scintillation counting (LS2800, Beckman). Protein was quantitated using the BCA protein assay (Pierce, Rockford, IL). Results are given as mean ± S.D. of triplicated samples and expressed as pM of ^{32}p incorprated/10 min/mg protein. Specific PKC activity was caculated as the difference in kinase activity in the presence and absence of Ca^{2+}, DAG, and PS.

Intracellular Ca^{2+} measurements

Fura2/AM (Calbiochem, La Jolla, CA) was prepared as a 10 m*M* stock solution in DMSO. Monocytes (5 x 10^6/ml) were incubated with 5 μM Fura 2/AM for 20 min at 37 ℃ in complete medium. The cells were washed 2 times and resuspended at 5 x 10^5 /ml in assay buffer (145 m*M* NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 m*M* $CaCl_2$, 0.5 m*M* $MgSO_4$, 5 m*M* glucose, 10 m*M* HEPES, pH 7.4)[15]. The cells were equilibrated to 37 ℃ and transferred to a 1-cm quartz cuvette equipped with a stirring bar in a LS-5B Luminescence Spectrometer (Perkin-Elmer). The excitation (340 and 380 nM) and emission wave lenghts (510 n*M*) were set respectively. Baseline signal was determined by the addition of buffer (control) for 60 s before stimulating the cells, with P48 and A23187.

The Ca^{2+} concentriation was calculated with the software program Fura 2 (Perkin-Elmer) using a modification of the Grynkiewicz equation[16-17]:

$$[Ca^{2+}] = (R- Rmin)/(Rmax-R) \times KD \times SFB$$

where Rmin is minimum ratio (340/380) and Rmax is maximum (both derived from the calibration data). KD is the dissociation constant of Fura2 (220). R is the fluorescence ratio at unknown [Ca^{2+}] and SFB is the fluorescence ratio at the baseline wavelengh (380 nM) in EGTA (40 m*M* and 0.2% Triton X-100.

TNF assay

Tumor necrosis factor (TNF) activity in culture supernatants from monocytes was measured as previously reported[9]. The murine L929 fibroblasts (CCL 1. American Type Culture Collection, Rockville, MD) were grown to confluency in RPMI 1640 medium. Cells were gently removed using Pancreatin (GIBCO) and washed medium. Cells genetly removed using Pancreatin (GIBCO) and washed with fresh medium. Cells (2.5 × 10^4/well) were seeded in 96 well flat bottom plastes (Corning, Corning, NY) in complete medium. Following incubation for 18 h, the medium was aspirated and replaced with 100 μl of medium containing 1 μg/ml of actinomycin D sulfate. The monocyte supernatants to be tested were serially diluted through 8 cells in triplicate. Human TNF-alpha (40 units/ml, interim standard, National Biological Standard Board, NCL), and medium alone were included as positive and negative controls. The plates were incubated for 18 h at 37 ℃ and 5% CO_2, the cells were rinsed with HBSS, and stained in 50 μl of crystal violet (0.5% in 20% of methanol) for 10 min. The stained and fixed cells were washed in water and lysed with 33% acetic acid. The uptake of crystal violet was quantitated by absorbance at 592 nM on a microtiter plate reader (Titertk, Flow Laboratories). TNF activity in the supernatants was determined by comparisons to the TNF standard using the PARLIN program for the statistical analysis of parallel line bioassays.

RESULTS

P48-induced intracellular calcium mobilization

Calcium mobilization by mP48 was investigated using Fura 2/AM technique.

When monocytes were loaded with Fura 2 and stimulated with mP48, a rapid rise in intracellular Ca^{2+} was observed. The rise in Ca^{2+} concentration occurred immediatly upon addtion of P48 and reached a maximum level within 1 to 2 min and was followed by a gradual decline. The increase of intracellular calcium was not observed with the control membrane preparation isolated from K562 cells (Fig 1). As a positive control, the ionophore A23187 (50 ng/ml) was found to increase Ca^{2+} to a greater extent than P48 did. The calcium antagonist, EGTA (40 mM) used to chelate media Ca^{2+} totally abrogated P48 and A23187-induced Ca2+ influx in our assay system.

The mP48 induced increase in intracellular Ca^{2+} was dose-dependent in the range of 0.1-10 μg/ml of plasma membrane protein (Tab 1). Because of a drift in the baseline flurescence over the course of the experiment, the data is presented as net increase in calcium concentration. The data show a net increase from 4-124 n*M* of intracellular calcium. These data suggest a role for calcium as an initial event in P48 induced intracelular signalling in monocytes.

Tab 1. Membrane P48 stimulates Ca^{2+} influx in human monocytes

Protein concentration (μg/ml)	Net [Ca^{2+}] (μM) increase by	
1	**K562**	**P48**
0.1	**2**	**4**
0.4	**3**	**29**
1.25	**2**	**53**
2.5	**2**	**76**
5.0	**2**	**103**
10.0	**3**	**124**

P48-induced PKC activity

Next, we sought to determine whether the increase in calcium results in the activation of PKC. Initially, we measured the subcellular distribution of PKC activity in unstimulated human monocytes and in cells stimulated with phorbol, 12-myristate, 13-acetate ester (PMA). In unstimulated monocytes, 70 $\pm$ 13% of the PKC activity was found in the cytosol while 30 $\pm$ 11% was found in the membrane fraction. After stimulation with PMA (100 n*M*), a significant translocation of PKC from cytosol to the membrane was observed (Fig 2). The stimulation of monocytes with PMA (100 nM) resulted in a 3.4 fold increase of PKC activity in the membrane (1534 $\pm$ 11 to 5219 $\pm$ 177 p mol/10 min/mg protein) by 2 min. Concomitant with the increase of PKC activity in membrane, there was a decrease of PKC activity in cytosol (802 $\pm$ 19 to 313 $\pm$ 15 p mol/min/mg protein) by 2 min. This translocation was sustained through out the 8 min time course of the experiment.

Next, monocytes were stimulated with P48 and the translocation of PKC was investigated. Fig 3 shows the P48 induced PKC redistribution in monocytes. The stimulation of monocytes with 10 μg/ml of mP48 induced a decrease cytosolic PKC

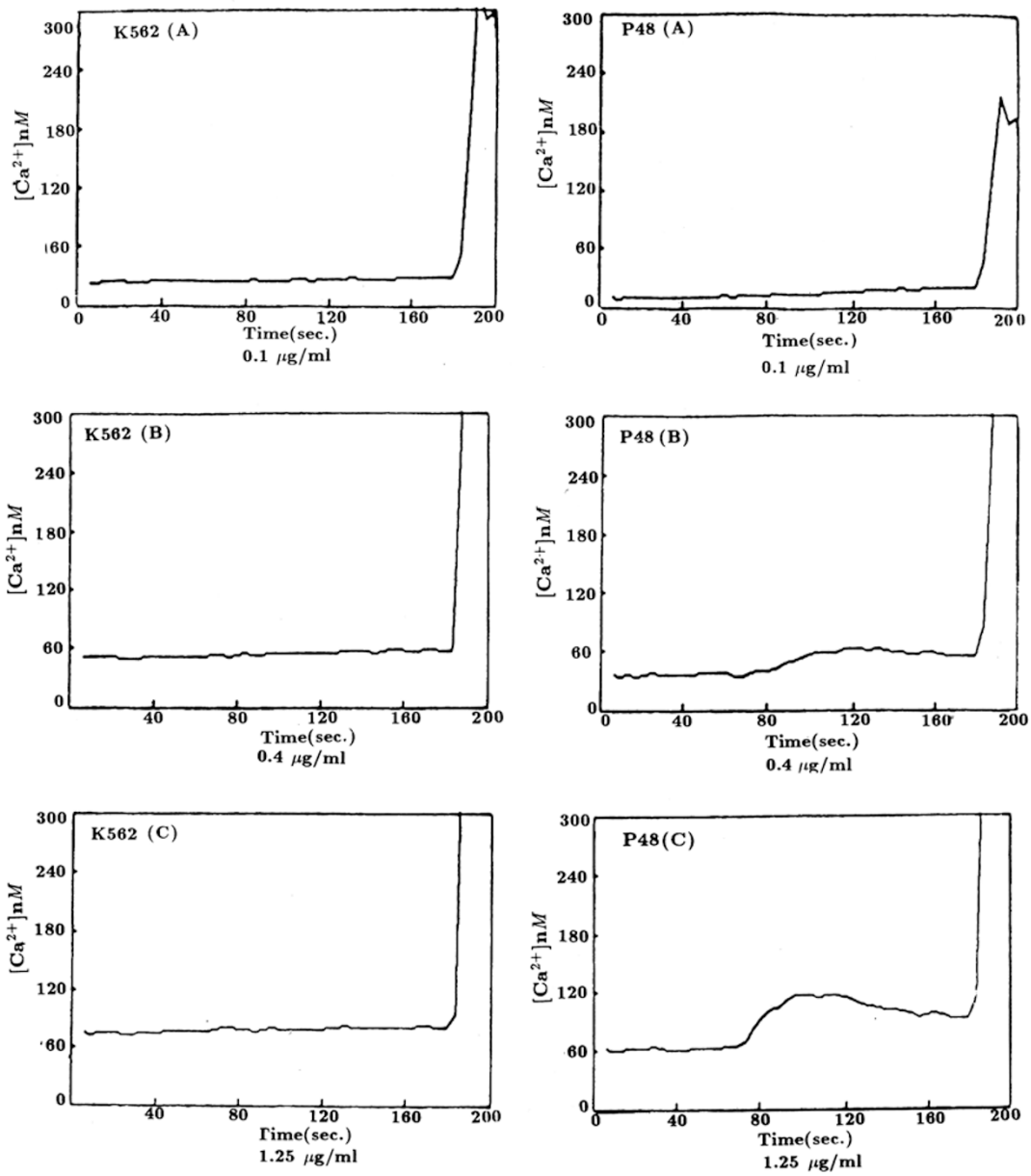
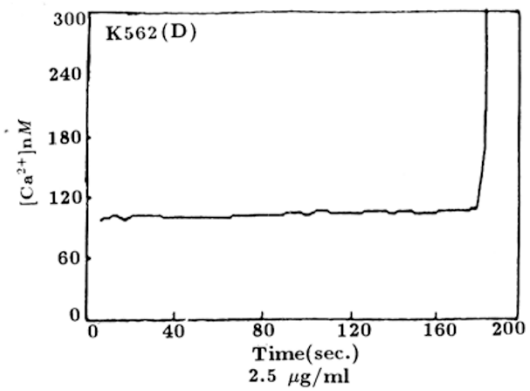
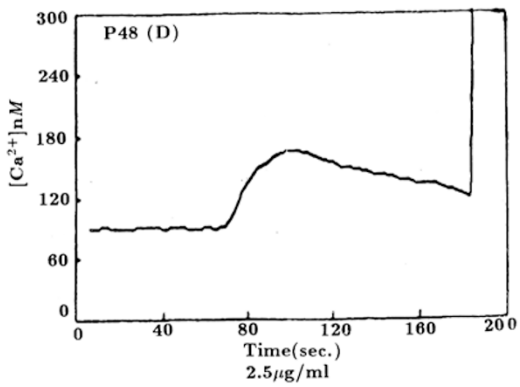
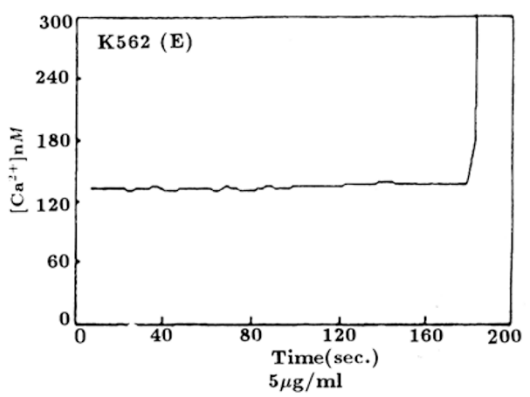
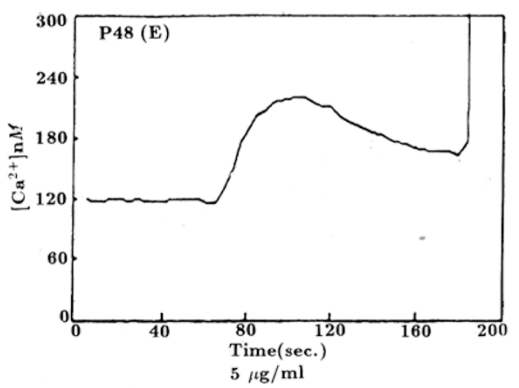


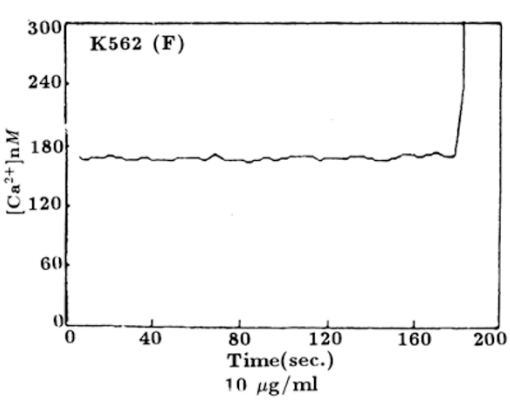
Fig 1. P48 induces an increase in intracellular calcium in human monocytes.
Monocytes were loaded with Fura-2 AM and resuspended in HBSS as described in the Materials and Methods. In traces A to F, Fura-2 loaded monocytes were treated with P48. The following concenttrations of K562 (membrane control) and P48 were added: A, 0.1 μg/ml; B, 0.4 μg/ml; C, 1.25μg/ ml; D, 2.5 μg/ml; E, 5.0 μg/ml; F, 10μg/ml. Triton X-100, 0.3% was added at 180 second (Fmax); and EGTA 20 m*M* was added at 200-220 second at (Fmin). The experiments lasted two hours from A to F. Results shown are representative of at least five separate experiments.

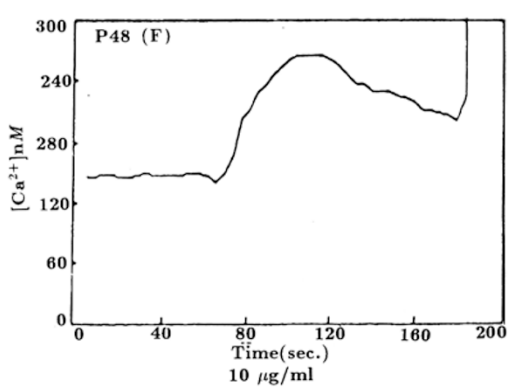

K562(D)
[Ca2+]nM
300
240
180
120
60
0
0
40
80
120
160
200
Time(sec.)
2.5 μg/ml


P48 (D)
[Ca2+]nM
300
240
180
120
60
0
0
40
80
120
160
200
Time(sec.)
2.5μg/ml


K562 (E)
[Ca2+]nM
300
240
180
120
60
0
0
40
80
120
160
200
Time(sec.)
5μg/ml


P48 (E)
[Ca2+]nM
300
240
180
120
60
0
0
40
80
120
160
200
Time(sec.)
5 μg/ml


K562 (F)
[Ca2+]nM
300
240
180
120
60
0
0
40
80
120
160
200
Time(sec.)
10 μg/ml


P48 (F)
[Ca2+]nM
300
240
280
120
60
0
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40
80
120
160
200
Time(sec.)
10 μg/ml

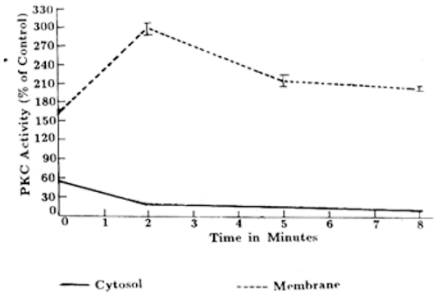

Fig 2. Kinetics of PMA-induced PKC translocation. The time course of PMA-induced PKC translocation from human monocytes was assaied. The monocytes were treated with PMA (100 a *M*) as indicated times. The radioactivities associated with net PKC activity (p mol. ^{32}P-incorporation /10 min/mg protein) extracted from cytosol and membrane of monocytes at time 0 were 1481 ±63 and 951 ± 31, respectively. Whereas, those from PMA-treated monocytes were 802 ± 19 and 1534 ±11 respectively. At 0 to 8 min, the membrane PKC activity was significant increase in comparison to unstimulated control ($P < 0.001$). On the contrary, the PKC activity in the cytosol was dramaticly decreases ($P < 0.01$). Data points represent the mean ± S.D of triplicate determinations. The results are representative of three similar experiments.

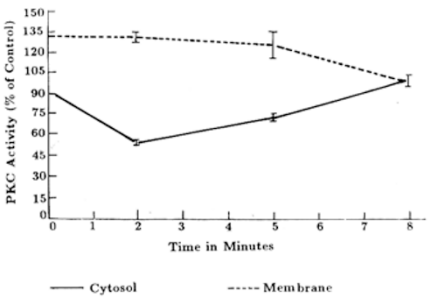

Fig 3. Kinetics of P48-induced PKC translocation, the time course of P48-induced PKC translocation of human monocytes was done. At the end of each incubated times as indicated. PKC was isolated from cytosol and membrane and the net PKC activities were measured as described in Materials and Methods. The radioactivities associated with net PKC activity (p mol. ^{32}p-incorporation /10 min/mg protein) extracted from cytosol and membrane of monocytes at time 0 were 2165 -± 88 and 886 ± 33 respectively. Whereas, those from P48-treated monocytes were 1961 ± 36 and 1178 ± 34. Data points represent the mean ± S.D. of triplicate determinations. At 0, 2, and 5 min, the membran PKC was significantly increased in comparison to unstimulated monocytes control ($P<0.01$). Whease, the cytosol PKC activity was significantly descreased at 2 and 5 min ($P< 0.01$) in comparison to that unstimulated monocytes. The results are representative of three similar experiments.

activity within 2 min, whereas the PKC activity of membrane was significantly increased over the control within the first 5 min. Interestingly, P48 did not alter the absolute levels of PKC as noted following PMA stimulation. The PKC activity in P48 stimulated monocytes returned to unstimulated levels by 8 min.

Effect of inhibitors of PKC on P48-induced TNF section.

To examine the functional role of PKC in the monocytes response to P48, the effect of protein kinase inhibitors on P48 induced TNF secretion was determined. As shown in Fig 4, H-7 significantly inhibited mP48 induced TNF secretion in a dose-dependent fashion, compared to medium or HA1004 as a control for the isoquinoline sulfonamide derivative (Fig 4). HA1004 had no effect on P48-induced TNF secretion event at concentration as high as 15 μM. The effect of another potent PKC inhibitor, sphingosine, on P48 induced TNF secretion was examined. Sphingosine was found to inhibit TNF secretion at similar concentrations as H-7 (Fig 5). In monocytes treated with various concentrations of H-8, a potent PKA inhibitor (Fig 6), H-8 reduced P48-induced TNF secretion only at higher concentrations (50% inhibition at 30 μM).

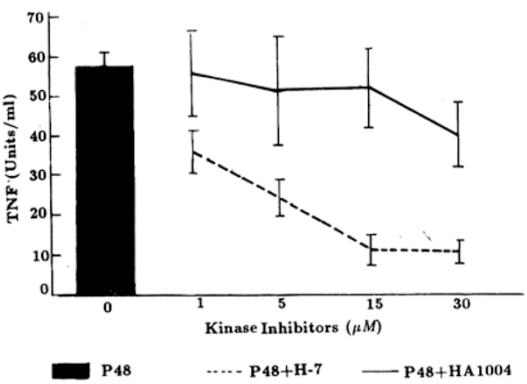

Fig 4. Effect of H-7 and HA1004 on P48-induced TNF secretion. Monocyte monolayers were pretreated with various concentrations of H-7 and HA1004 for 15 min, and were then incubated with 5μg/ml of mP48 for an additional 6 h. Supernatants were harvested from monocyte monolayers for TNF measurements as described in Materials and Methods. Each point represents the mean $\pm$ S.D. of triplicate assays. The results represent of four similar experiments.

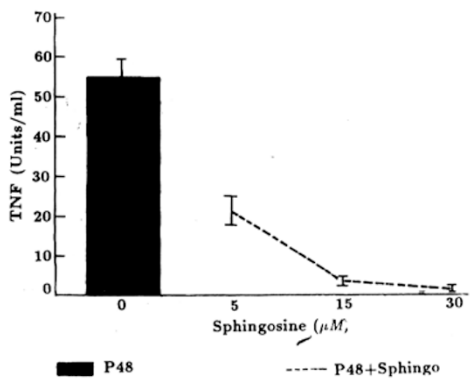

Fig 5. Effect of sphingosine on P48-induced TNF secretion. Adherent monocytes were incubated with various concentrations of sphingosine for 15 min, and then were stimulated with 5 μg/ml of P48 for an additional 6 h. Supernatants were removed from the cultured monocytes for assayed for TNF activity. Each point represents the mean $\pm$ S.D. of triplicate assays. The results are representative of four similar experiments.

Our data clearly suggests the involvement of Ca^{2+} mobilization and Ca^{2+}-phosphlipid-dependent PKC activation in P48-induced TNF secretion by monocytes. In order to investigate the possibility that other calcium dependent enzymes are involved, we utilized W-7, N-(6-aminohexyl)-5-chloro-l-naphthalene-sulfonamide hydrochloride, an inhibitor of calmodulin-dependent kinase (CaMk). When monocytes were

treated with of W-7 (1-30 μM), P48-induced TNF production was significantly reduced in a W7 dose-dependent fashion. The 50% inhibition of P48 induced TNF secretion by W7 was observed at 5 μM, with complete inhibition at 30 μM (Fig 7). These results suggest that both PKC and CaMk may be involved in the P48 induced signal transduction.

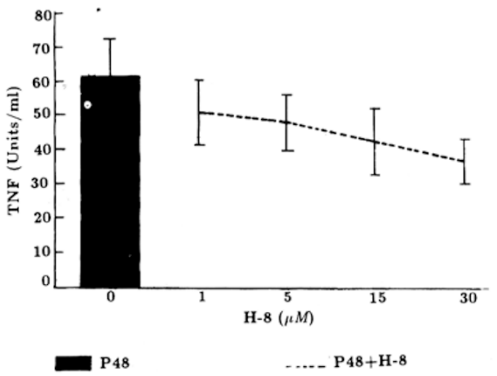

Fig 6. Effect of H-8 on P48-induced TNF secretion. Human monocytes were treated with various doses of H-8 for 15 min, and then incubated with 5 μg/ml of mP48 for 6 h at 37 ℃. Supernata were harversted from the adherent monocytes and assayed TNF activity. Results are expressed as mean ± S.D. of triplicated determination. Data are representative of four similar experiments.

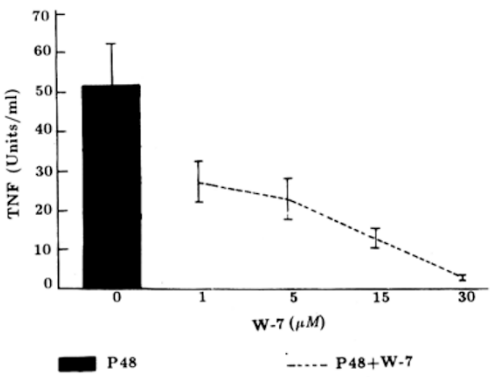

Fig 7. Effect of W-7 on P48-induced TNF secretion. Adherent monocytes were treated with various concentrations of W-7 for 15 min, and stimulated with 5 μg/ml of P48 for an additional 6 h. Supernantans were harvested from the adherenet monocytes and assayed for TNF activity. Results are expressed as mean ± S.D. of triplicated determinations. Data are representative of four similar experiments.

DISCUSSION

We have previously identified a novel cytokine involed in monocyte differentiation and the induction of cytotoxic activity[1-4]. We have identified P48 in the supernatant and membrane of the null cell line Reh. Both the secreted protein and the membrane moleculae have been shown to be potent stimulators of TNF secretion by human monocytes[3, 4]. In the present study, we have examined the signal transduction pathways involved in P48 induced TNF secretion using the membrane form of P48. We have found that mP48 is a potent stimulator of Ca^{2+} mobilization and the subsequent activation and translocation of PKC activity.

The mobilization of intracellular Ca^{2+} and the stimulation of a Ca^{2+}-phospholipid dependent PKC has been implicated in a common signal transduction pathway involving hydrolysis of phosphotidylionstitol. Stimulation of this pathway by receptor ligand bining results in the rapid hydrolysis of phosphatidylinositol 4, 5-bisphosphate

(PIP2) and subsequent generation of DAG and IP3. These second mediators have been implicated in Ca^{2+} fluxes (IP3), as well as the activation of PKC. The subcellular redistribution of PKC from the cytosol or a loosely associated membrane compartment to a more integral membrane protein has been demonstrated. This translocation event is thought to be necessary for PKC activation by Ca^{2+} and DAG in vivo[18].

The translocation of PKC induced by P48 was found to be transient, within 0-5 min and returned to control levels by 8 min. This was found to be substantially different from the response to PMA which was prolonged and did not return to control levels. The difference between PMA and P48 may be explained by the fact that PMK is metabolized very slowly and prolonged exposure to PMA results in the degradation of cellular PKC[19-20]. Thus it is would be expected to induce a protracted stimulation of PKC[21]. However, in repeated experiments using monocytes stimulated with P48 or PMA, we found that TNF secretion was induced only by P48 and not by PMA (data not shown). Numerous investigators have reported that PMA stimulates the induction of TNF messenger RNA, however, few reports demonstrate the secretion of TNF activity after PMA treatment. Thus, it appeares that although PMA stimulates PKC activation and TNF messenger RNA production, it dose not represent an functional signal for secretion of the protein.

In order to further investigate the functional role of PKC in P48-induced TNF secretion, we have used various kinase inhibitors. A number of synthetic isoquinoline sulfonamide derivative are available which differentially inhibit protein kinase due to different binding affinities for the various enzymes. H-7 is the most potent inhibitor of PKC (Ki 6 μM) while, H-8 and HA1004 are much less potent than H-7[22-23]. Sphingosine is a widely used, naturally occuring inhibitor of PKC activation[24-26]. The treatment of human monocytes with the PKC inhibitors, H-7 and sphingosine, blocked P48 induced TNF secretion. The PKA inhibitor, H-8 (Ki=1.2 μM) was found to inhibit P48 induced TNF secretion only at higher concentrations (15-30 μM). Since the Ki of H-8 for PKC is in this range[27], it suggests that the inhibition we observed with H-8 is due to inhibition of PKC rather than PKA. Additionally, HA1004 had no inhibitory activity on P48 induced TNF secretion further suggesting that PKC and not PKA is involed in P48 induced TNF secretion.

An interesting observation in our studies was that W-7, an inhibitor of calmodulin-dependent kinase, significantly blocked P48 induced TNF secretion. In six experiments, we found that W-7 inhibited P48-induced TNF production in a dose dependent manner. W-7 inhibits CaMK with a Ki=12 μM, while it inhibits PKC with a Ki=110 μM[28]. Therefore, it appears that the inhibitory activity of W-7 is due to the inhibition of CaMK and not PKC. It has been reported that LPS induced TNF-alpha mRNA in mouse macrophages can be blocked by a PKC inhibitor, H-7, but not W-7[29]. One possible explanation for thr difference in our data is that they used a high dose (10 μg/ ml) of LPS to treat the macrophages. In our hands, W-7 can block TNF secretion in monocytes induced with optimal concentrations of

LPS (100 ng /ml) but not in monocytes stimulated with 10 μg/ml of LPS (data not shown). Thus, the inhibition of TNF secretion by W-7 was dependent on the concentration of LPS used for inducing of TNF production, these results suggest that the biochemical pathways involved in P48 induced TNF secretion involve multiple kinase activies.

Recently, it has been suggested that transmembrane signals involved in the stimulation of cytokine secretion by monocytes may involve different pathways. Taniguchi et al., have found that H-7 and W-7 inhibit pertussis toxin PT induced IL-1 production in human monocytes is involved the different pathways: both calmodulin- and PKC-dependent processed are necessary[30]. On the other hand, CaMK has been shown its functional new aspects, such as regulates a number of secretory responses[31], since it phosphorylates myosin light chain (MLC-8), may also phosphorylate other substrates that alter the activation of c-fos, c-myb, c-myc and lymphokine genes as well as their receptor genes[32]. The future determination of the role of PKC and CaMK for P48-induced late events, such as the activation of the regulatory DNA-binding protein, gene expression and TNF secretion, should lead to an elucidation of P48-triggered multiple transmembrane signal transduction pathways.

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