Synthesis and Function of Platelet-activating Factor in the Central Nervous System

中枢神経系における血小板活性化因子の産生と機能

相原　一

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Abbreviation

PAF	platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine)
CNS	central nervous system
PAFR	PAF receptor
PAF-AH	PAF acetylhydrolase
NMDA	*N*-methyl-*D*-aspartate
MAPK	mitogen-activated protein kinase
t-ACPD	*trans*-1-amino-1.3-cyclopentanedicarboxylic acid
DMEM	Dulbecco's modified Eagles's medium "Nissui"2
MAP2	microtuble-associated protein 2
GFAP	glial fibrillous acidic protein
FITC	fluorescein isothiocyanate
BSA	bovine serum albumin
PBS	phosphate buffered saline
CHO	Chinese hamster ovary
cPLA2	cytoplasmic phospholipase A2
AA	arachidonic acid
PLL	poly-L-lysin
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
TRITC	tetramethylrhodamine isothiocyanate
BAPTA-AM	*O-O'*-bis(2-aminophenyl) ethylene glycol-*N,N,N',N'*-tetra-acetic acid, tetra-acetoxymethyl ester
RT-PCR	reverse transcription-polymerase chain reaction

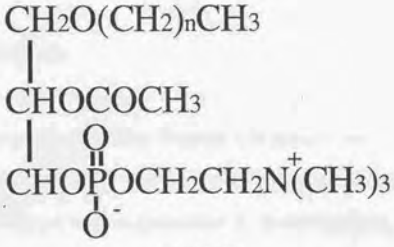
Introduction

Platelet-activating factor (PAF) (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) (**Figure 1**) is a potent activator of platelets and is later shown to have diverse biological effects on various cells and tissues (Hanahan, 1986; Braquet et al., 1987; Snyder, 1989; Prescott et al., 1990; Izumi and Shimizu, 1995). In the central nervous system (CNS), PAF is involved in various events (Feuerstein et al., 1990; Frerichs et al., 1990; Doucet and Bazan, 1992; Bazan, 1994). PAF induces long-term potentiation (Wieraszko et al., 1993; Kato et al., 1994), enhances excitatory postsynaptic transmission (Clark et al., 1992) and increases memory task performance (Izquierdo et al., 1995). PAF receptor (PAFR) antagonists are reported to suppress postischemic neuronal death (Panetta et al., 1989; Gilboe et al., 1991; Prehn and Krieglstein, 1993) and PAF is related to human immunodeficiency virus-associated neuronal cell death (Genis et al., 1992; Epstein and Gendelman, 1993; Gelbard et al., 1994; Lipton, 1994; Lipton et al., 1994). Additionally, PAF has been shown to be relevant to the development of the brain because a subunit of PAF inactivating enzyme, PAF acetylhydrolase (PAF-AH), has a 99 % homology with the gene coding for Miller-Dieker lissencephaly with the lack of gyri and sulci in the cerebral cortex (Reiner et al., 1993; Hattori et al., 1994). These events suggest that PAF plays a crucial role in the CNS.

Previous reports demonstrated the synthesis of PAF by application of acetylcholine (Sogos et al., 1990) and dopamine (Bussolino et al., 1986), during convulsant electrical stimuli (Kumar et al., 1988), ischemia (Baker and Chang, 1993; Baker and Chang, 1994; Kunievsky and Yavin, 1994; Francescangeli et al., 1996) or viral infection (Gelbard et al., 1994; Nishida et al., 1996). However, the cells in the brain responsible for the synthesis remain to be elucidated.

In point of the expression of PAFR, our previous study demonstrated that PAFR is expressed ubiquitously in the CNS of rat brain (Bito et al., 1992). Other studies showed that cultured astroglia (Petroni et al., 1994) and microglia (Rhigi et al., 1995) respond to PAF.

The present study was undertaken to clarify following points; which cells in the brain produce PAF, which cells respond to PAF and how targeted cells react to PAF. Here we found that (1) PAF was predominantly synthesized in neuronal cells by the stimulation of glutamic



1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine

Fig. 1 Structure of platelet activating factor

acid, especially through NMDA receptor activation, (2) PAFR mRNA was expressed in microglia and hippocampal neuron by *in situ* hybridization and immunohistochemisty, (3) PAFR expressed in microglia functionally elicited the intracellular calcium rise, (4) microglia showed chemotactic response to PAF, possibly through the activated mitogen-activated protein kinase (MAPK) cascade, and (5) PAF-activated microglia released arachidonic acid and glutamate. These findings suggest that PAF plays a critical role of neuron-microglial interaction in various events like inflammation, ischemia, neuronal cell death and apoptosis.

A. Synthesis of PAF in the rat brain

A-1 Experimental procedures

A-1-1 Materials

PAF (C-16) was purchased from Cayman Chemicals, Ann Arbor, MI. Glutamic acid, NMDA, kainate, t-ACPD and MK-801 were purchased from Research Biochemicals Inc., Natick, MA. A23187 calcium ionophore was purchased from Calbiochem, La Jolla, CA. PTX from Funakoshi, Tokyo, and MEK1 inhibitor PD98059 from New England Biolabs, Beverly, MA. WEB2086 was a geneous gift of Boeringer Ingerheim, Germany.

A-1-2 Primary cell culture

Primary cultures of hippocampal neurons were prepared from 15-19 days Wistar rat embryos by the method of Banker and Cowan (Banker and Cowan, 1977) with modifications. Bliefly, hippocampal tissues were removed and treated with 1 mg/ml trypsin (Gibco, Gaithersburg, MD) and 0.5 mg/ml DNase I (Boehringer Mannheim, Germany) in 120 mM NaCl, 5 mM KCl, 25 mM D-glucose, 20 mM PIPES, pH 7.0, for 5 min at 37 °C. The tissue suspension was centrifuged at 150 × g for 20 sec, and the cell pellet was resuspended in 0.3 mg/ml trypsin inhibitor (Gibco) and 40 μg/ml DNase I in the above buffer. After gentle trituration with a Pasteur pepette, the cells were centrifuged at 150 × g for 3 min. The cell pellet was resuspended in glutamate-free Dulbecco's modified Eagles's medium "Nissui"2 (DMEM) (Nissui, Tokyo)/10 % horse serum supplemented with 0.1 mg/ml transferrin (Sigma), 16 μg/ml putrescine (Sigma),

and 5 μg/ml insulin (Wako Chemicals, Osaka, Japan), 20 nM progesterone (Sigma), and 30 nM $NaSe_2O_4$ (Sigma) (Bottenstein, 1985). Cells were seeded at a density of 10^6 cells per well in the same medium with 10 % fetal calf serum (Upstate Biotechnology, NY). Cells were incubated at 37 °C in a humidified atomosphere of 5 % CO_2 in air. After incubation for 2 or 3 days, serum was deprived to prevent increase of astroglia or other fibroblastic cells. The purity of neuronal cells was estimated to be >99 % (data not shown) by staining with two monoclonal antibodies against neurofilament and microtuble-associated protein 2 (MAP2) (Sigma). The PAF synthesis was measured at 10 days after preparation.

Astroglia was prepared from the brain of rat pups (P3) by the previously reported method (Mori et al., 1990), with modifications. Briefly, after incubation of brain mixed culture for 10 day, non-astroglial cells were removed by shaking (MaCarthy and de Vellis, 1980) and subculturing twice. Isolated astroglia were cultured in DMEM supplemented by 10 % fetal calf serum. The final purity of astrocytes was estimated to be >99.5 % (data not shown) by staining with anti-GFAP antibody (Sigma). Subconfluent cells were used for determination of PAF synthesis.

Microglia was prepared from neonatal rat cerebral tissues, as described previously (Nakajima et al., 1989; Nakajima et al., 1992). Briefly, trypsin-treated cerebral cells were cultured in DMEM/10 % FCS for about two weeks and microglia was purified by shaking. Isolated microglia was cultured in DMEM with 0.1 % BSA (PENTEX, Miles Inc., Kankakee, IL) and used within 24 hr. The purity of microglia was estimated to be >99.5 % (data not shown) by staining with fluorescein isothiocyanate (FITC)-labelled-isolectin B4 (Sigma), a microglial surface marker.

A-1-3 Extraction of PAF

Before ligand stimulation, all cells were cultured at least over 12 hr in a serum-free medium containing 0.1 % BSA. Each ligand was prepared in the same medium. After ligand stimulation, the culture medium was collected. Then, cells were scraped and retrieved with 0.1 % phosphate buffered saline (PBS). From these 2 samples per well, lipids were extracted by the Bligh and Dyer method (Bligh and Dyer, 1959). The extracted lipids in chloroform were loaded onto a Sep-Pak silica column (Waters, Tokyo) prewashed with chloroform to purify PAF. The

column was sequentially washed by 100 % chloroform, acetone : methanol (1 : 1, v/v) and chloroform : methanol (7 : 3). PAF was eluted with chloroform : methanol : water (1 : 2 : 0.8). PAF was further extracted by the Bligh and Dyer method into chloroform solution. After evaporation with a rotatory evaporator, the dried precipitates containing PAF were dissolved into 20 mM HEPES/NaOH pH 7.4, 10 mM $MgCl_2$ and 0.1 % BSA buffer and vigourously sonicated in a bath sonicator for 15 min.

A-1-4 PAF radioreceptor binding assay

To measure PAF, we employed a radioreceptor binding assay which was established in our laboratory (Aoki et al., 1995). Aliquots of extracted samples were incubated with 40 nM [^{3}H]WEB2086 (Du Pont NEN, Tokyo) and 100 μg membrane from Chinese hamster ovary (CHO) cells carrying PAFR in the binding buffer; 20 mM HEPES, 10 mM $MgCl_2$ and 0.1 % BSA. After incubation for 60 min at 25 °C, the membrane was absorbed to GF-C glass filters (Packard, Meriden, CT). The filters were washed 5 times each 1 ml of with 20 mM HEPES, 10 mM $MgCl_2$ and 0.1 % BSA, and the radioactivity of the filters was measured with a Top Count radiodetector (Packard). The amount of PAF was quantified with a standard curve using various amounts of PAF.

A-2 Results

A-2-1 Synthesis of PAF in rat neuron

To clarify the synthesis of PAF and its producing cell types in the brain, we determined PAF using radioreceptor binding assay in rat primary culture systems. Previous *in-vivo* studies have shown the presence of PAF in the CNS by convulsant electrical stimuli (Kumar et al., 1988) and ischemia (Baker et al., 1993; Baker et al., 1994; Kunievsky et al., 1994; Francescangeli et al., 1996). Thus, we selected glutamic acid, NMDA and calcium ionophore as ligands to stimulate cultured cells. PAF was synthesized in neuron by 50 μM NMDA + 1 μM glycine stimulation **(Figure 2a)** , but not in astroglia or microglia **(Figure 4)**. PAF was synthesized with the peak of 1 min, and degraded rapidly **(Figure 2a)**. Similar results were obtained by the stimulation with

10 μM glutamic acid (**Figure 2b**). The synthesis of PAF was dependent on the concentration of NMDA (**Figure 3**). We also examined its synthesis in rat primary astroglia and microglia (**Figure 4**). Astroglia was stimulated by 10 μM glutamic acid, 50 mM KCl and 100 μM A23187 for 1 min and microglia was stimulated by 100 μM A23187 for 1 min. Though glutamic acid and A23187 at these concentrations were enough to cause a full elevation of intracellular calcium ion (data not shown), PAF was not detected in these cells or their culture media.

A-2-2 Requirement of NMDA receptor activation for PAF synthesis

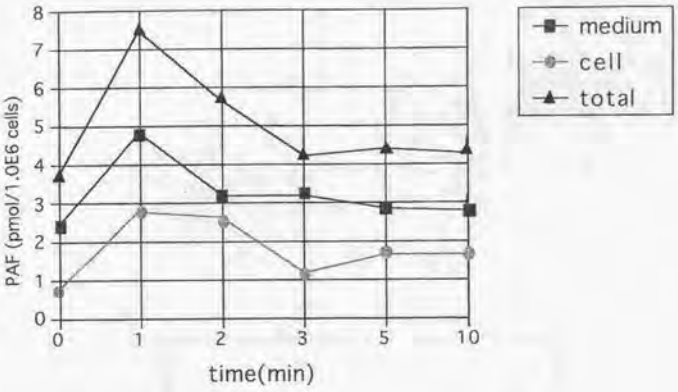
To investigate the signal to initiate the PAF synthesis, various ligands were applied in neuronal cell culture (**Figure 5**). As a positive control, 100 μM glutamic acid was used. To determine the glutamate receptor subtypes related to PAF synthesis, NMDA, kainate, t-ACPD and MK801 (an NMDA receptor blocker) were used. NMDA and A23187 synthesized PAF, and tACPD and KCl showed a production of PAF to smaller extent. AMPA/kinate receptor stimulation did not produce PAF. The effect of NMDA was inhibited by MK801 or extracellular calcium depletion. Taken together, the NMDA receptor activation and intracellular calcium elevation were required to trigger the synthesis of PAF.

A-3 Discussion

A-3-1 PAF synthesis and pathological events in CNS

Many previous studies indicate that PAF is present in the CNS and plays a crucial role in neuronal events pertinent to diseases such as ischemia, convulsion, trauma, virus infection and neuronal development (Panetta et al., 1989; Gilboe et al., 1991; Genis et al., 1992; Epstein et al., 1993; Prehn et al., 1993; Reiner et al., 1993; Wieraszko et al., 1993; Gelbard et al., 1994; Hattori et al., 1994; Kato et al., 1994; Lipton, 1994; Lipton et al., 1994). However, they have only shown the presence of PAF in particular and long-lasting conditions. The synthetic cells and mechanisms in the brain remain to be elucidated. Our data have shown first that even in a normal glutamic acid stimulation, PAF is synthesized in neuron mainly by NMDA receptor activation and is decomposed very rapidly (**Figure 2**). The presence of PAF as a cell-associated

a.



b.

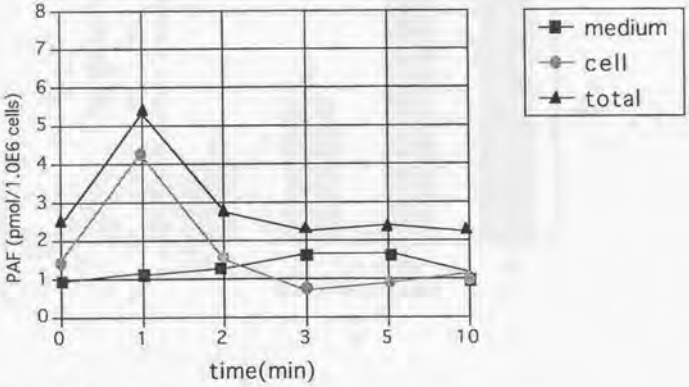


Fig. 2 PAF synthesis in rat hippocampal neuron
a. Time course of PAF synthesis by 50 μM NMDA stimulation.
b. Time course of PAF synthesis by 10 μM glutamic acid stimulation.
Rat hippocampal neuronal cells were stimulated by each ligand. At indicated times, cells and culture media were collected and PAF was extracted by Bligh-Dyer method. The determination of PAF was performed by radio-receptor binding assay using PAFR-expressed CHO cell membrane as described in Experimental Procedures. PAF was synthesized and degraded immediately after stimulation.

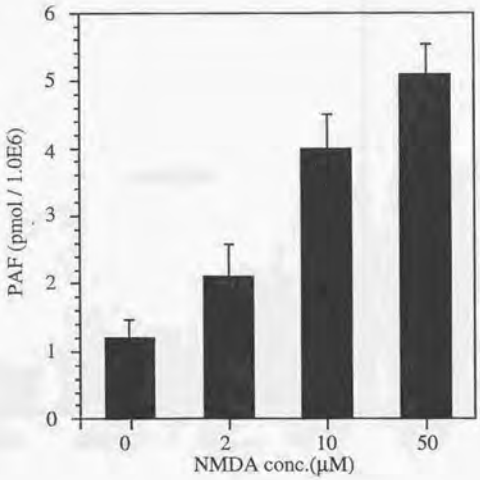


Fig. 3 Dose dependent synthesis of PAF in rat hippocampal neuron
Varying concentration of NMDA were applied to primary culture of rat hippocampal neuron. 1 min later, cells were collected and PAF was determined by radioreceptor assay. NMDA dose-dependently stimulated the synthesis of PAF in neuron. Columns and vertical bars denote the mean and SD, respectively.

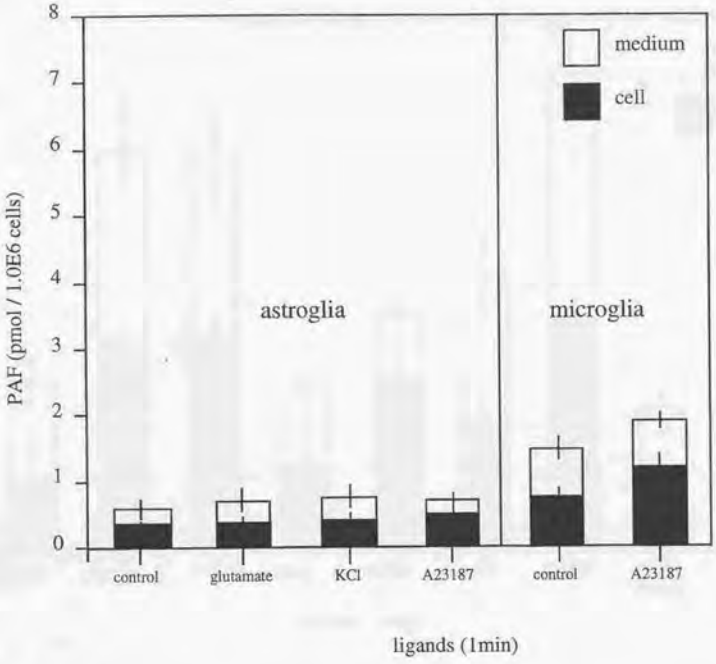


Fig.4 PAF synthesis in rat brain astroglia and microglia
Rat primary culture of astroglia and microglia were stimulated by various ligands; 10 μM glutamic acid, 50 mM KCl and 1 μM A23187 for 1 min. No significant production of PAF was detected in stimulated cells or culture media. Columns and vertical bars denote the mean and SD, respectively.

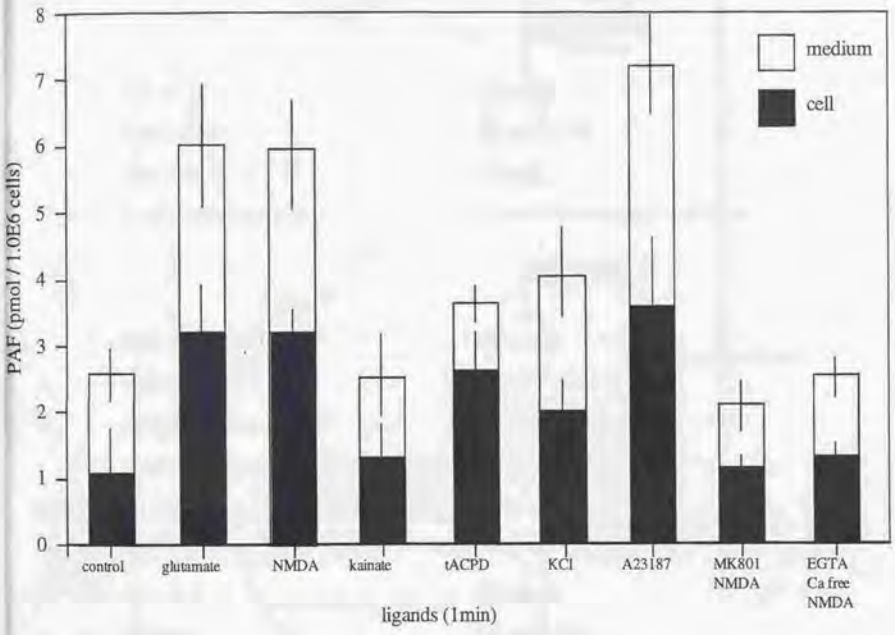


Fig. 5 PAF synthesis in neuron mediated by NMDA receptor and calcium elevation
Rat hippocampal neurons were stimulated by various ligands; 10 μM glutamic acid, 50 μM NMDA, 100 μM kainate, 100 μM *t*ACPD, 50 mM KCl and 1 μM A23187. 1 μM MK-801, NMDA receptor antagonist, was applied 30 min before NMDA application. PAF was synthesized mainly by NMDA receptor mediated stimulation and intracelllular calcium elevation. Columns and vertical bars denote the mean and SD, respectively.

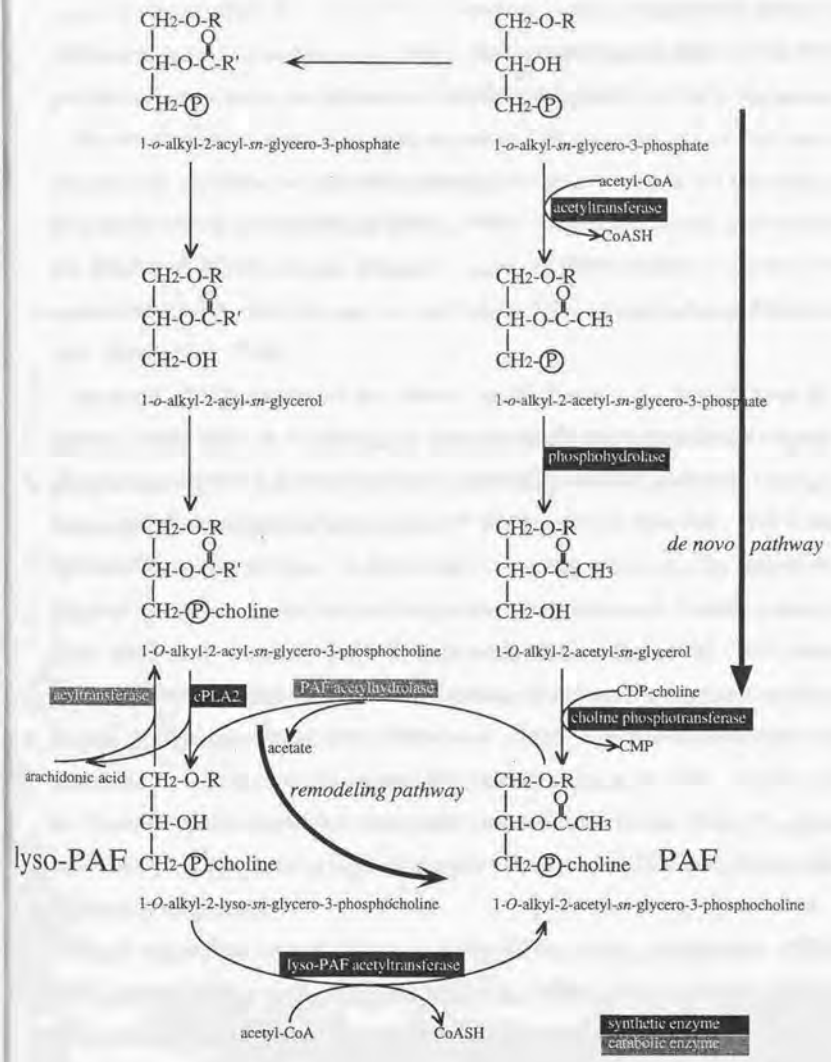


Fig. 6 Metabolic pathway of PAF

form agreed with the previous results using guinea-pig neutrophils (Aoki et al., 1995). Other neurotransmitters might be involved in PAF synthesis, such as acetylcholine (Sogos et al., 1990)and dopamine (Bussolino et al., 1986). However, the present study for the first time provided a direct evidence that glutamic acid stimulated the synthesis of PAF in the neuron.

The fact that calcium influx is necessary to produce PAF can account for the PAF elevation in ischemia and convulsion because these pathological events subsequently evoke long-lasting intracellular calcium rise (Nishida and Markey, 1996). Furthermore, our data agree with the fact that MK801, an NMDA receptor antagonist, suppresses PAF elevation by murine or human aquired immunodeficiency virus infection and reduces following neuronal death (Gelbard et al., 1994; Nishida et al., 1996).

Synthesis of PAF can involve two different routes (Bazan et al., 1991) (**Figure 6**). One pathway stems from alkyl-acetyl-glycerophosphocholine which deacylated by cytoplasmic phospholipase A2 (cPLA2) to form lyso-PAF and mostly arachidonic acid (AA). Then lyso-PAF acetyltransferase introduces an acetyl group at C-2 of lyso-PAF to form PAF. PAF is degraded by PAF-AH to lyso-PAF again. In this *remodeling* pathway, cPLA2 is a key enzyme which is triggered by intracellular calcium rise through many neuronal events. Another pathway stems from alkyl-acetyl-glycerol which is transfered phosphocholine of CDP-choline by cholinephosphotransferase. In this *de novo* pathway, this enzyme is expressed in microsomal fraction of immature cerebral cortex (Baker et al., 1993). It is still unknown which route is predominantly working and what signals evoke these pathway in the CNS. In this report, we demonstrated intracellular calcium rise evoked PAF synthesis in neuron. This fact supports that at least the *remodeling* pathway is possibly related to PAF synthesis in neuron, because cPLA2 is activated by calcium ion.

Rapid degradation of PAF (**Figure 2**) is due to the acitvity of PAF-AH. PAF-AH is predominantly present in the cytosol (Hattori et al., 1993) and its activity is related to the neuronal migration (Albrecht et al., 1996) and development of the CNS (Hattori et al., 1994). In an intact brain tissue, PAF is possibly functioning as a neuronal messenger in a short range of time and space. In fact, it was reported that PAF evoked a long-term potentiation in hippocampus and is one of candidates of retrograde messengers related to neuronal plasticity (Kato et al.,

1994). Under various pathological conditions such as ischemia, convulsion and infection, the balance between PAF synthesis and PAF-AH activity may collapse by long-lasting elevation of intracellular calcium concentration, and PAF will increase enough to induce various responses to adjacent neuron and glia. Further studies are needed to elucidate the pathway to synthesize and decompose PAF in neuronal cells.

B. Localization and expression of PAF receptor (PAFR) in CNS

B-1 Experimental procedures

B-1-1* in situ *hybridization

Sprague-Dawley rats were anesthetized with diethylether and decapitated. Brains were removed, dissected coronally or parasagittally, embedded in an OCT-compound (Tissue-Tek, Miles, Erkhart, IN) and frozen in isopentane at -30 °C. Coronal and parasagittal sections of 10 μm thick were prepared on a cryostat, mounted on poly-L-lysin coated slide glass (Matsunami, JAPAN), and dried for 10 min at room temperature. The sections were fixed in 4 % paraformaldehyde buffered with 0.1 M phosphate buffer for 10 min, acetylated with 0.25 % acetic anhydride, dehydrated in ethanol of ascending concentrations (70 %, 95 %, 100 % and 100 %), and stored at -80 °C. Sections of rat whole embryos, embryonic day 18 (E18), and pup brains (P0, P7, P14, P21) were prepared in the same procedure.

In situ hybridization was carried out as described previously (Shigemoto et al., 1992), with modifications. Briefly, a *Pvu*I-*Ava*I fragment (0.9 kb) of the coding region of rat PAFR cDNA [Bito, 1994 #20] was subcloned into pBluescript II (Stratagene, La Jolla, CA) and linearized by *BamH*I digestion. An ^{35}S-labeled cRNA probe was made with a cRNA synthesizing kit (Maxiscript, Ambion, Austin, TX). The final radioactivity of the probe solution was adjusted to 105 cpm/μl. After hybridization at 55 °C for 6 hr in a humid chamber, the sections were washed in 2 × SCC at room temperature overnight and in 2 × SCC at 60 °C for 1 hr, and they were treated with RNaseA (20 μg/ml) at 37 °C for 30 min in 0.5 M NaCl/10 mM Tris-Cl/1 mM EDTA. The final wash was carried out in 0.1 ×SCC at 37 °C for 1 hr. The sections were dehydrated in ethanol of

ascending concentrations (30 %, 90 % 100 % and 100 %). The air-dried sections were autoradiographed on Hyperfilm-betamax (Amersham, Buckinghamshire, UK) or dipped in 1:1 diluted NTB-2 emulsion (Kodak, Rochester, NY). The exposure periods were 10 days for the film and 6-7 weeks for the emulsion.

B-1-2 Immunohistochemistry followed by in situ *hybridization*

To identify the most intensely labeled cells, the brain sections from adult rats were immunostained with a macrophage/microglia maker (OX-42; anti-CR-3 complement receptor antibody) or an astrocyte marker (anti-glial fibrillary acidic protein (GFAP) antibody) before *in situ* hybridization. OX-42 (Serotec, Oxford, UK) and anti-GFAP antibody (G-3893, Sigma, St.Louis, MO) were affinity-purified twice with protein-G columns (Pharmacia, Uppsala, Sweden), because incubation with the crude antibodies substantially decreased mRNA signals of the subsequent *in situ* hybridization, probably due to the RNase in the crude preparations.

Cryostat sections (10 μM thick) of adult rat brains were fixed serially in 4 % paraformaldehyde buffered with 0.1 M phosphate buffer, 50 % aqueous acetone, 100 % acetone, and 50 % aqueous acetone (each for 2 min), and blocked with 0.1 % nuclease-free BSA (Sigma) in PBS for 5 min at room temperature. Incubation with the purified primary antibodies (1:200) was carrried out for 10 min at room temperature with 0.1 % BSA/PBS. After three brief washes with PBS, the sections were incubated with biotinylated anti-mouse IgG antibody (1:200; Vector, Burlingame, CA) with 0.1 % BSA/PBS for 10 min and subsequently with avidin-biotin-horseradish peroxidase complex (Vectastatin Elite; Vector) for 10 min, according to the manufacturer's instructions. Immunostaining was visualized with 1 mg/ml diaminobenzidine tetrahydrochloride (DAB) in PBS containing 0.02 % H_2O_2. The stained sections were then acetylated , dehydrated, and subjected to *in situ* hybridization, as described above. All the immunostaining procedures were carried out under RNase-free conditions.

B-1-3 Primary cell cultures

Neuron, astroglia and microglia were prepared as described above. Fibroblasts were prepared from pooled meninges of neonatal rat brains, as described previously (Saitoh et al., 1992). The

purity of fibroblastic cells was estimated to be >90 % from the staining with anti-human fibronectin antiserum (Cappel-Organon Teknika, Belgium). Microglia derived from PAFR knock-out mice (Ishii and Shimizu, 1998) was also purified with the same procedure.

B-1-4 Preparation of RNA and Northern blot/RT-PCR analyses for PAFR in primary cell culture

Total cellular RNA was isolated from each culture by the guanidium thocyanate/CsCl method (MacDonald et al., 1987). The RNA samples (10 μg each) were electrophoresed in 1.2 % agarose gel containing 7 % formaldehyde and alkaline-transferred onto a Hybond-N+ membrane (Amersham). The blot membrane was incubated at 65 °C in 10 ml rapid hybridization buffer (Amersham) for 30 min. A [^{32}P] dCTP-labeled probe was synthesized from the *Pvu*I-*Ava*I fragment (0.9 kb) of rat PAFR cDNA with a random-primed DNA labeling kit (Ready-Prime, Amersham) and added to the hybridization buffer. After incubation on a shaker at 65 °C for 2 hr, the membrane was washed in 50 ml of 2 × SCC containing 0.1 % SDS at room temperature afor 30 min and in 1 × SCC at 65 °C for 30 min. The signals were detected by autoradiography on X-ray film. PAFR mRNA expression was evaluated further by reverse transcription-PCR (RT-PCR). The first-strand cDNA was synthesized from 1μg of total RNA from each cell culture. Aliquots (1/50) of the cDNA samples were amplified with PCR using rat PAFR-specific primers: CCGCTGTGGATTGTCTATTA (upsteam, 5' -> 3') and AGGAGGTGATGAAGATGTGG (downstream, 5' -> 3') for 25, 30, or 35 cycles with a thermal cycle of 58 - 72 - 94 °C (each for 1 min). The PCR products were electrophoresed in agarose gel and visualized by ethidium-bromide staining.

B-1-5 Fluorometric imaging of $[Ca^{2+}]_i$ primary cultures of rat microglia and hippocampal cells

Because PAFR mRNA expression was detected in the brain and primary cell culture systems, the functional presence of the receptor was then determined in cultured cells. Fluorometric measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was carried out using primary cell cultures of (1) microglia isolated from culture of neonatal rat cerebrum and (2) hippocampal cells

from E16 rat embryos. PAFR protein itself could not be detected immunohistochemically because no anti-PAFR antibodies were available for this purpose.

(1) Microglia were prepared as described above and maintained on PLL-coated coverslips for 12-24 hr. The cell culture was loaded with 5 μM Fura 2-AM (Dojin Chemicals, Kumamoto, Japan) in glutamate-free DMEM containing fatty acid-free 0.1 % BSA (Sigma) at 37 °C for 1 hr. Fluorometric imaging was carried out with an inverted microscope (TMD300, Nikon, Tokyo, Japan) equipped with fluorescence lenses (Fluor-10 and -40, Nikon) and an ICCD camera/image analysis system (Argus-50; Hamamatsu photonics, Hamamatsu, Japan) at 22-25 °C. Eight frames (frame/0.03 sec) were integrated, and ratio images (340/380 nm) were acquired every 10 sec. PAF (10 nM) was bath-applied in the HEPES-Tyrode buffer (140 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 12 mM $NaHCO_3$, 5.6 mM D-glucose, 0.49 mM $MgCl_2$, 0.37 mM NaH_2PO_4, 25 mM HEPES, pH 7.4) containing 0.1 % BSA.

(2) Primary culture of hippocampal tissues was prepared from rat embryos (E16) as described previously (Banker et al., 1977), with modifications. Briefly, hippcampal tissues were removed and treated with 1 mg/ml trypsin (Gibco, Gaithersburg, MD) and 0.5 mg/ml DNase I (Boehringer Mannheim, Germany) in 120 mM NaCl, 5 mM KCl, 25 mM D-glucose, 20 mM PIPES, pH 7.0, for 5 min at 37 °C. The tissue suspension was centrifuged gently at 150 × g for 20 sec, and the cell pellet was resuspended in 0.3 mg/ml trypsin inhibitor (Gibco) and 40 μg/ml DNase I in the above buffer. After gentle trituration with a Pasteur pepette, the cells were centrifuged at 150 × g for 3 min. The cell pellet was resuspended in glutamate-free DMEM/10 % horse serum supplemented with 0.1 mg/ml transferrin (Sigma), 16 μg/ml putrescine (Sigma), and 5μg/ml insulin (Wako Chemicals, Osaka, Japan), 20nM progesterone (Sigma), and 30 nM $NaSe_2O_4$ (Sigma) (Bottenstein, 1985) and plated on polyethylene imine-coated coverslips with four-well silicon chambers (Flexiperm disc, Heraeus Biotechnology, Hanau, Germany) at 1.0×10^6 cells /well. The culture was maintained for 2 days in this medium and subsequently for 10-13 days in the medium containing 0.1 % fatty acid-free BSA (Pentex, Miles, Kankakee, Illinois) in place of horse serum. The medium was changed every 3 days. Fluorometric Ca^{2+} imaging was carried out as described above. Before PAF application, the cells were pretreated with 0.5 μM tetrodotoxin (Wako Chemicals) in the HEPES-Tyrode buffer containing 0.1% BSA. After $[Ca^{2+}]_i$ returned to

the baseline level, Ca^{2+}imaging was repeated in some of the samples with application of 50 μM NMDA in the HEPES-Tyrode buffer/0.1 % BSA to identify neuronal cells.

B-1-6 Immunocytochemical examination of PAF-responsive hippocampal cells

Immediately after the fluorometric imaging, the hippocampal cultures on the coverslips were washed briefly three times with PBS, fixed in 2% paraformaldehyde/PBS at room temperature for 5 min, washed three times with PBS, fixed in 100% methanol at -25°C for 30 sec, and rinsed with PBS. Subsequently, the samples were incubated in 10 % horse serum/PBS containing 0.1mM each of $CaCl_2$, $MgCl_2$ and $MnCl_2$ at room temperature for 10 min and then in the same solution containing 10μg/ml FITC-labeled isolectin B4 and monoclonal anti-GFAP (1:200, Sigma) or anti-MAP2 antibody (1:500, Sigma) for 45 min at room temperature. After three brief washed with PBS, the samples were incubated with tetramethylrhodamine isothiocyanate (TRITC)-labeled anti-mouse IgG antibody (1:50, Serotec) in 10 % horse serum/ PBS for 30 min at room temperature. After three brief washed with PBS, the samples were then mounted in 50% glycerol containing 0.5 % 1,4-diazobicycle-[2.2.2.]octane (DABCO, Sigma). The stained samples were observed using the fluorescence microscope/ARGUS system described above. FITC and TRITC were visualized under 470 and 540 nm emission light, respectively, and with appropriate absorption filters. Isolectin B4 and OX-42 (used in immunostaining the sections) both bind to ameboid, ramified, and reactive microglia and thus are suitable markers for detection of a wide variety of microglia (Thomas, 1992, Nakajima, 1993 #105).

B-2 Results

B-2-1 Ubiquitous expression of PAFR in rat brain by in situ *hybridization*

The *in situ* hybridization signals of PAFR were ubiquitously expressed in rat brain, and relatively intense in the cerebral cortex, olfactory bulb, pyramidal cell layer of the hippocampus, medial thalamus, hypothalamus, and granular cell layer of the cerebellum (**Figure 7**). In the hippocampus, intense signals were scattered randomly, and moderate signals were found in the pyramidal cell layer and dentate gyrus (**Figure 8**). In the cerebral cortex, intense signals were found

in all the layers and moderate signals were detected in layers II-V (**Figure 9a**). In the cerebellum, intense signals were scattered randomly as in the hippocampus and cortex, and moderate signals were found in the granular cell layer and Purkinje cell layers (**Figure 9b**). These scattered distributions of intense signals were common in all the brain areas. The intensity and distribution pattern of signals were constant among P7, P14 and P21 brains and similar to that of the adult (**Figure 10**).

B-2-2 Predominant expression of PAFR in microglia in rat brain

The PAFR-expressing cell types were classified into at least 2 groups by *in situ* hybridization and nuclear staining with cresyl violet (**Figure 11**). One type of cells was densely labeled and had small nuclei of irregular and anglar shapes with dense staining (**Figure 11. small arrow**). These cells were ubiquitously distributed in the gray and white matter of the whole brain. These findings suggest that this type of cells are microglia [del Rio-Hortega, 1932 #37, Vaughan, 1984 #137]. The other type of cells was moderately labeled and had large nuclei of round shapes with light staining (**Figure 11. large arrow**). These cells were distributed in pyramidal cell layer of the hippocampus and layers II-V in the cerebral cortex. These findings suggest that this type of cells are neurons.

To certify the cell types expressing PAFR, immunohistochemistry followed by *in situ* hybridization were performed. It revealed that almost all of the densely labeled cells are OX-42 positive but anti-GFAP negative in the adult rat brain (**Figures 12.13.**). This indicates that microglia predominantly expressed PAFR mRNA in rat brain. However, all of the OX-42 labeled cells were not positive for mRNA signals. It means that microglia heterogeneously expressed PAFR mRNA. On the other hand, weak and moderately signals were found in OX-42 and anti-GFA P negative cells with large and light-stained nuclei. These cells were apparently neurons. We tried immunohistochemistry for pup brain but without success because it required longer incubation time with antibody and caused signal of labeled cRNA probe weaker possibly by degrading RNA.

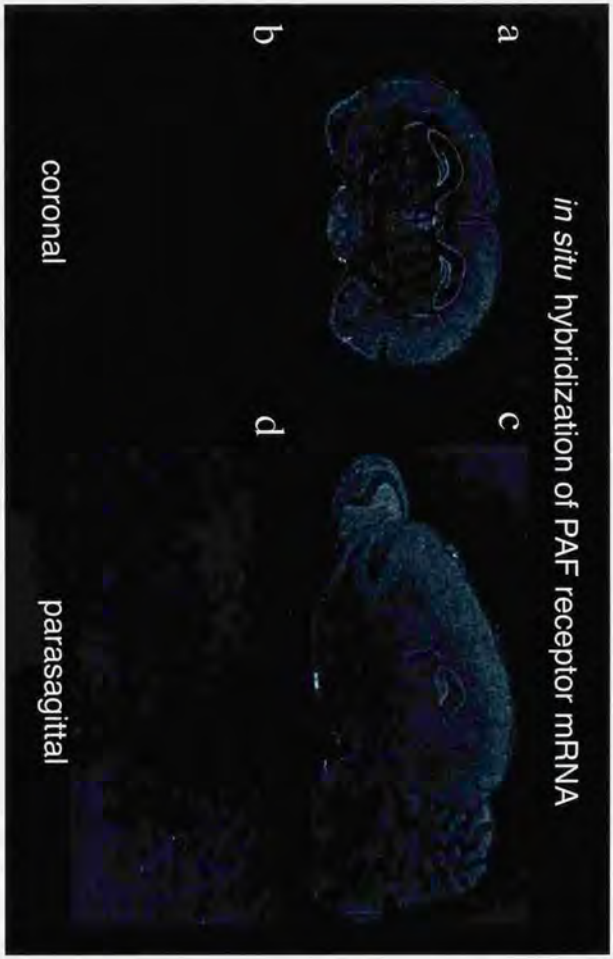


Fig. 7 *In situ* hybridization of PAFR mRNA in adult rat brain

Fresh-frozen rat brain coronal (a.b) and parasagittal (c.d) sections were hybridized with a ^{35}S-labeled antisense cRNA probe synthsized from a *Pvu*I-AvaI fragment of rat PAFR cDNA in the absence (a.c) or presence (b.d) of 100-fold cold excess unlabeled cRNA. The signals were ubiquitous but relative intense in the cerebral cortex, olfactory bulb, pyramidal cell layer of hippocampus, medial thalamus, hypothalamus, and granular cell layer of cerebellum.

Fig. 8 *In situ* hybridization of PAFR mRNA in adult rat brain hippocampus

In situ hybridization was performed as described in the legend of fig. 7. In the hippocampus, intense signals were scattered randamly, and moderate signals were found in the pyramidal cell layer and dentate gyrus.

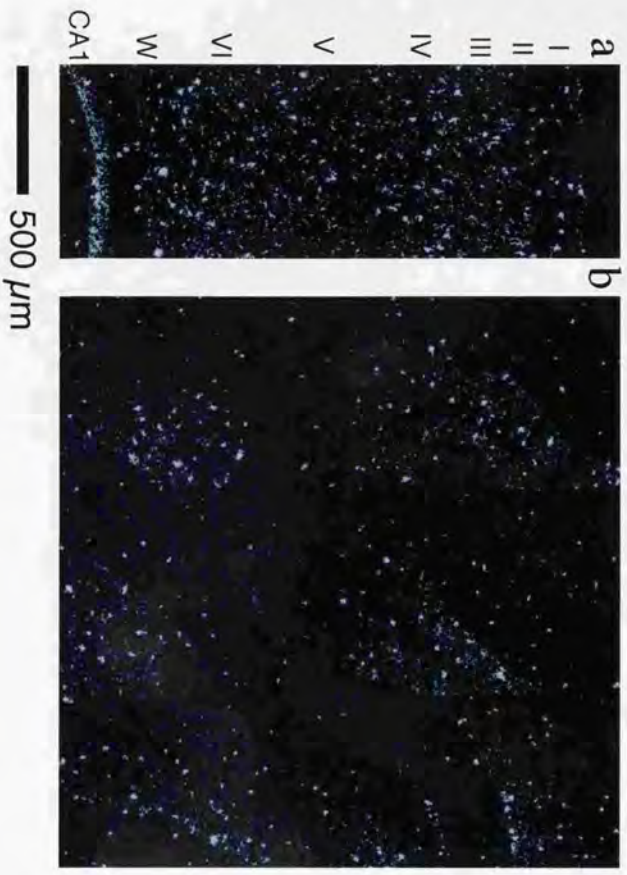


Fig. 9 *In situ* hybridization of PAFR mRNA in adult neocotex and cerebellum

In situ hybridization was performed as described in the legend of fig. 7. In the neocotex (a), intense signals were scattered randamly, and moderate signals were found in the II-IV layers. In the cerebellum, (b) intense signals were scattered randamly, and moderate signals were found in the granular and Purkinje cell layers.

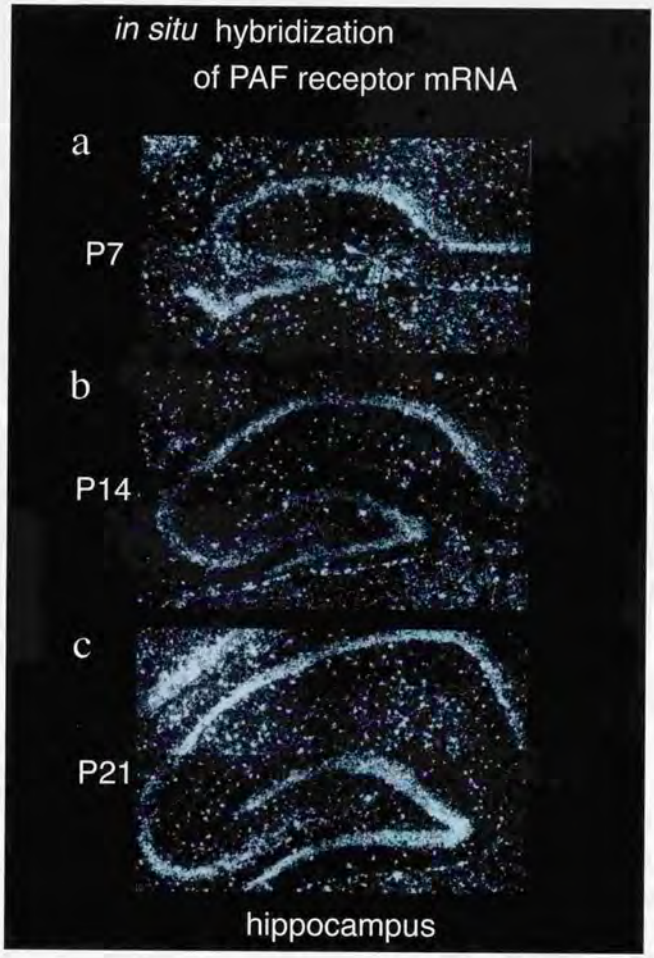


Fig. 10 developmental changes of PAFR mRNA expression in rat hippocampus
In situ hybridization was performed as described in the legend of fig. 7. The intense of signals was constant among P7 (a), P14 (b) and P21(c).

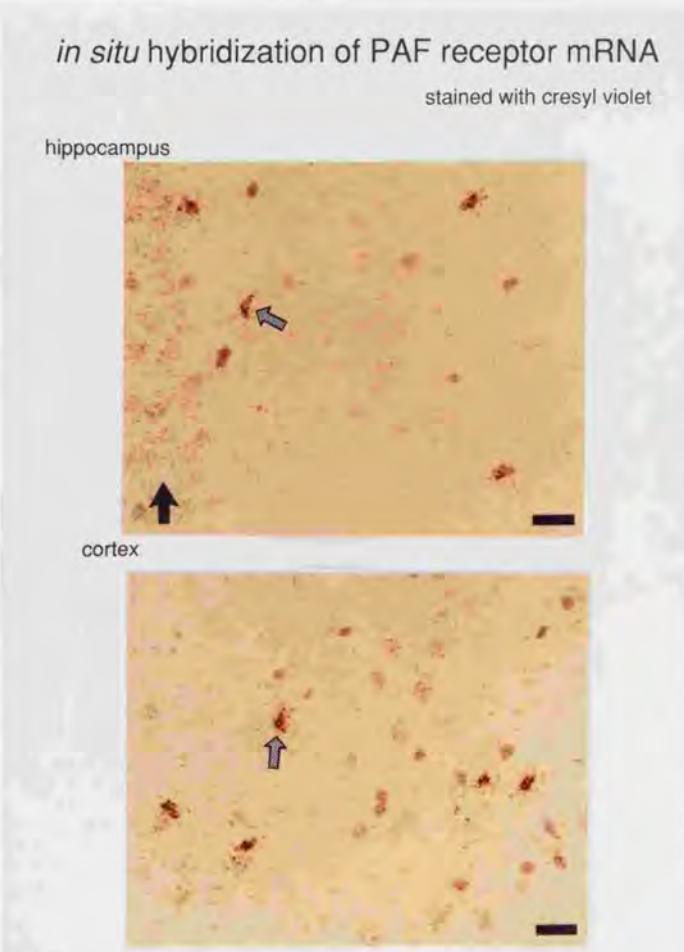


Fig. 11 *In situ* hybridization of PAFR mRNA and detection of cell types by nulear staining

In situ hybridization and immunohistochemistry were carried out as described in Experimental Procedures. Cresyl violet staining was performed in adult rat hippocampal (a) and neocortical sections (b). Intense labelled cells have dense stained small nuclei with irregular and anglar shapes, which were distributed ubiquitously in hippocampus and neocortex. Moderately labelled cells have light stained large round nulcei, which were distributed in pyramidal cell layer of hippocampus and layer II-IV of cortex.(large arrow)

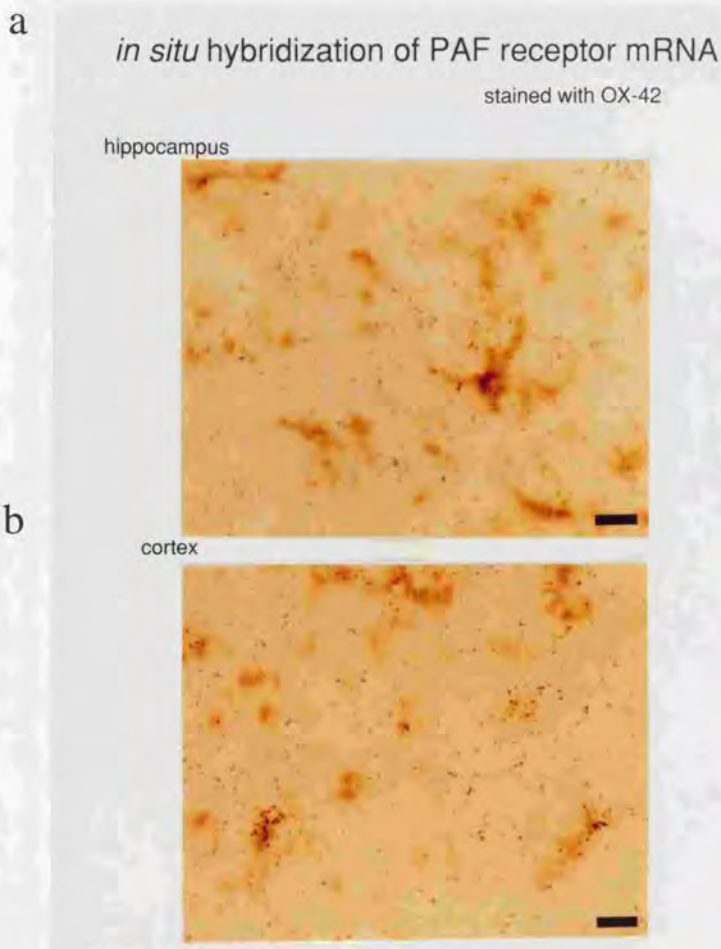


Fig. 12 *In situ* hybridization of PAFR mRNA and detection of cell types by microglial marker
In situ hybridization and immunohistochemistry were carried out as described in Experimental Procedures. OX-42, microglial surface marker was used in adult rat hippocampal (a) and neocortical sections (b). Densely labelled cells were almost OX-42 positive. However all of OX-42 positive cells did not indicate the PAFR mRNA signal.

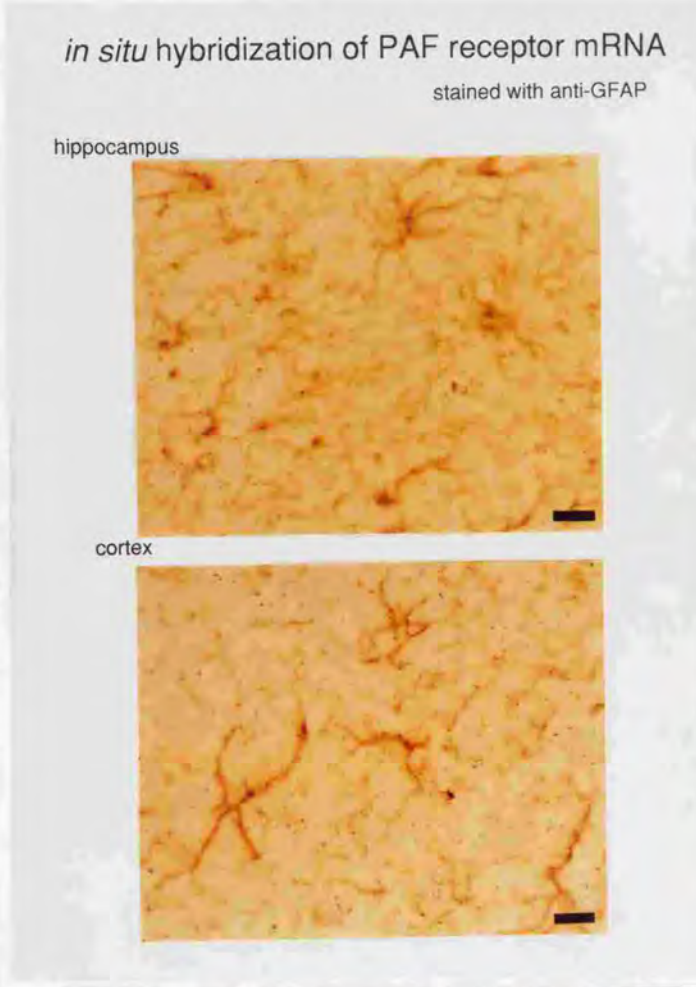


Fig. 13 *In situ* hybridization of PAFR mRNA and detection of cell types by astroglial marker
In situ hybridization and immunohistochemistry were carried out as described in Experimental Procedures. Anti-GFAP, astroglial surface marker was used in adult rat hippocampal (a) and neocortical sections (b). Densely labelled cells were GFAP negative.

B-2-3 Predominant expression of PAFR in cultured microglia by Northern blotting and RT-PCR

Northern blot analysis revealed that PAFR mRNA is predominantly expressed in microglia and hardly detectable in neuron, astrocyte and fibroblast (**Figure 14**). In the RT-PCR analysis, the specific PCR product (0.38 kb) was apparently detected in the RNA of microglia (≧25 cycles), astrocytes (≧30 cycles) and neurons (≧35 cycles) but not in fibroblasts (≧35 cycles) (**Figure 15**). The identity of PCR product was confirmed by Southern blot hybridization (data not shown).

B-2-4 PAF-elicited $[Ca^{2+}]_i$ response in cultured cells

In isolated microglial culture, almost all the cells responded to PAF 10 nM and caused $[Ca^{2+}]_i$ elevation. **Figure 16a** shows imaging of $[Ca^{2+}]_i$ in PAF-treated microglial cells. **Figure 16b** shows its temporal profile. Rapid elevation and slow decrease of $[Ca^{2+}]_i$ were observed. To investigate the signal to raise $[Ca^{2+}]_i$, 100 ng/ml PTX treatment was performed. PTX-treated microglia showed no response to PAF (**Figure 17ab**). Additionally, to clarify that this $[Ca^{2+}]_i$ elevation is mediated by PAFR activated by PAF, the same procedure was applied to PAFR knock-out mouse microglia. $[Ca^{2+}]_i$ was not increased in *pafr* -/- microglia (**Figure 18ab**).

On the other hand, in cultured hippocampal cells, a small population of the cells responded to PAF(**Figure 19ab**). After calcium imaging, this culture was stained with isolectin B4 for microglia and anti-GFAP for astroglia. Most of the PAF-responsive cells (≧90 %) were stained with isolectin B4 but not with anti-GFAP antibody, suggesting that they are mostly microglia (**Figure 19cd**). Not all of the isolectin B4 positive cells responded to PAF (**Figure 19bd**), suggesting that microglia in such mixed neuronal culture are heterogeneous in terms of PAFR expression. This result is compatible with the results of immunohistochemisty after *in situ* hybridization using hippocampal slice.

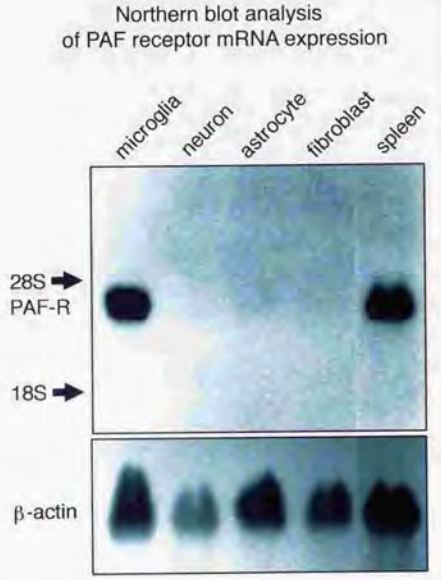


Fig.14 Northern blot analysis of PAFR mRNA expression in primary brain cell culture
Total RNA (10 μg) of rat brain primary cell cultures; microglia, neuron, astroglia, fibroblasts and poly A RNA (5 μg) of spleen were loaded on each lane. The membrane was hybridized with a [^{32}P] dCTP-labelled probe synthesized from a *Pvu*I-AvaI fragment of rat PAFR cDNA and control probe of ß-actin.
PAFR mRNA was predominantly expressed in rat brain microglia.

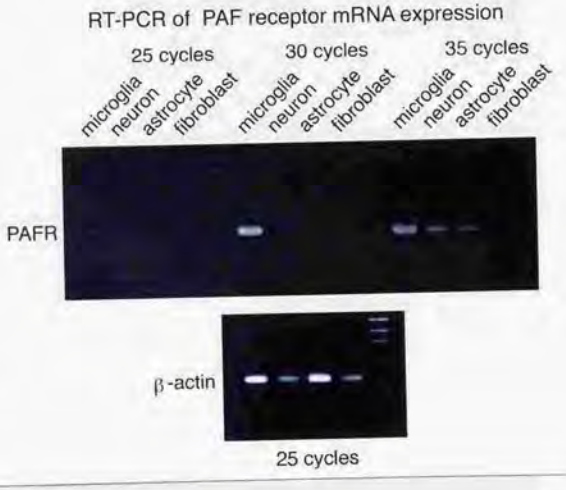


Fig. 15 RT-PCR analysis of PAFR mRNA in primary cell culture of rat brain
The first strand cDNA was synthesized from 1 μg of total RNA from each cell culture of microglia, neuron, astroglia and fibroblast. Aliquots (1/50) of the cDNA samples were amplified with PCR using rat PAFR-specific primers: CCGCTGTGGATTGTCTATTA (upsteam, 5'->3') and AGGAGG-TGATGAAGATGTGG (downstream, 5'->3') for 25, 30, or 35 cycles with a thermal cycle of 58-72-94 °C (each for 1 min). The PCR products were electrophoresed in agarose gel and visualized by ethidium-bromide staining.
PAFR mRNA was predominantly expressed in microglia and slightly expressed in neuron and astroglia.

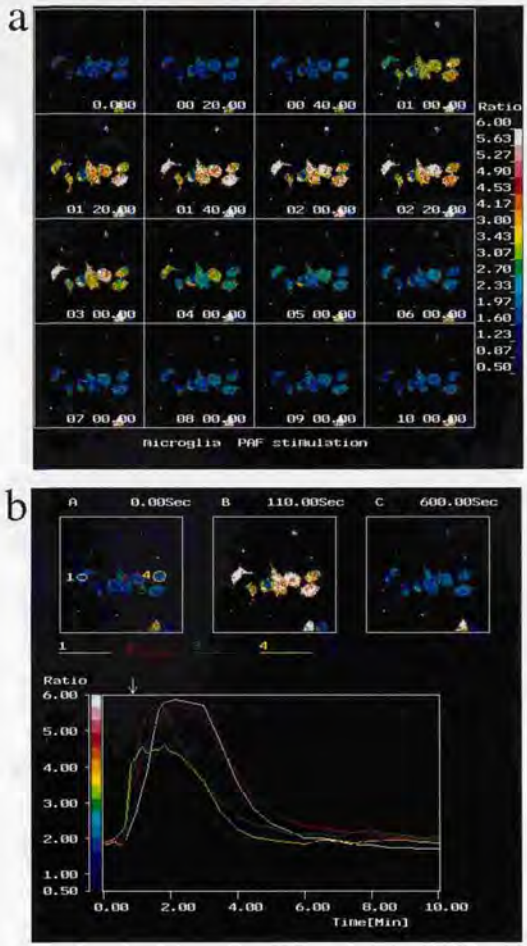


Fig. 16 PAF-elicited $[Ca^{2+}]_i$ response in microglia

Microglial culture was carried out as described in Experimental Procedures. The cell culture was loaded with 5 μM Fura 2-AM at 37 °C for 1 hr. Fluorometric imaging was carried out with an inverted microscope equipped with fluorescence lenses and an ICCD camera/image analysis system (Argus-50) at 22-25 °C. PAF (10 nM) was bath-applied in the HEPES-Tyrode buffer.

(a) Color imaging of $[Ca^{2+}]_i$ elevation. (b)Temporal profile of $[Ca^{2+}]_i$ rise.

PAF elicits prominent $[Ca^{2+}]_i$ rise in microglia.

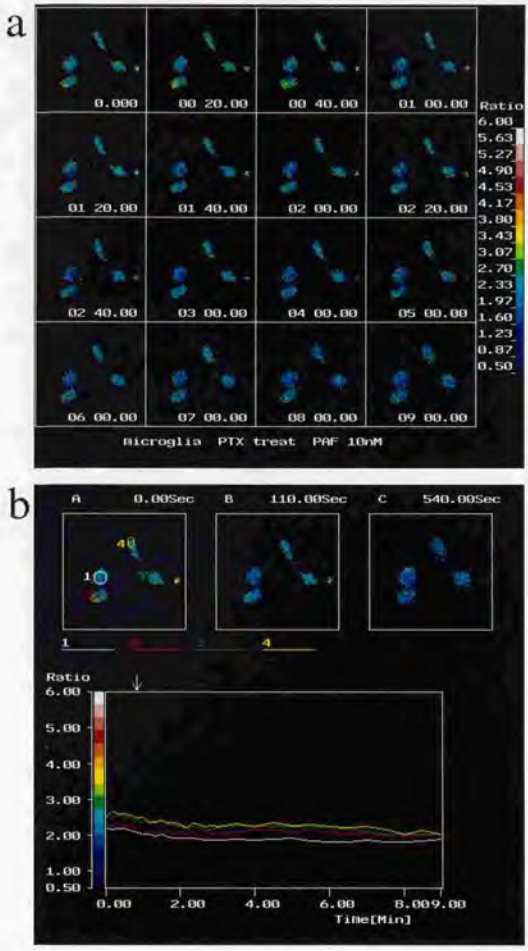


Fig. 17 The effect of PTX treatment to PAF-elicited $[Ca^{2+}]_i$ elevation in microglia
100 ng/ml PTX overnight treatment was carried out before PAF application.
(a) Color imaging of $[Ca^{2+}]_i$ elevation. (b) A temporal profile of $[Ca^{2+}]_i$ rise.
PTX inhibits $[Ca^{2+}]_i$ rise completely, indicating that PAFR might be coupled with Gi-like G protein.

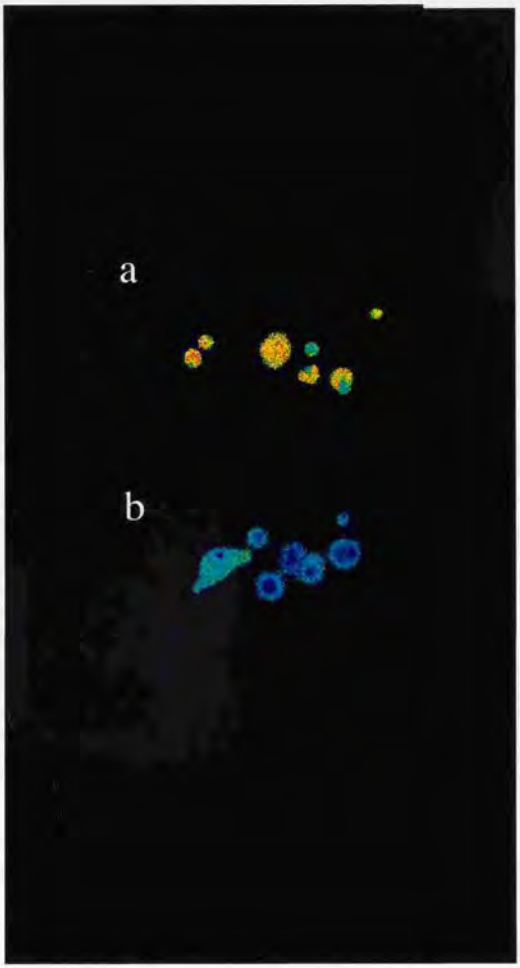


Fig. 18 PAF-elicited $[Ca^{2+}]_i$ elevation in PAFR knock out mouse microglia
Microglial cell culture was derived from *pafr* +/+ and -/- mice. Calcium imaging was carried out with the same procedure described in Fig.16. PAFR-mediated $[Ca^{2+}]_i$ elevation was appeared in *pafr* +/+ microglia (a), but not in *paf* -/- microglia (b).

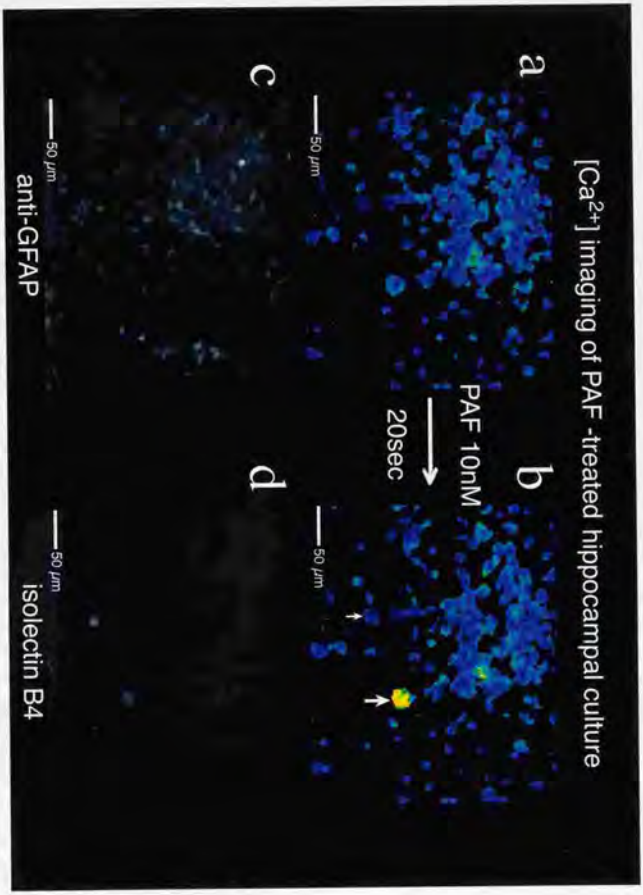


Fig. 19 PAF-elicited $[Ca^{2+}]_i$ elevation in rat hippocampal mixed culture
Rat hippocampal cell culture and fluorometric calcium imaging were carried out as described in Experimental Procedures. After calcium imaging, cell culture was stained with isolectin-B4 for microglia or anti-GFAP antibody for astroglia. Some of culture cells responded to 10 nM PAF to cause $[Ca^{2+}]_i$ elevation (a, b). Most of the PAF-responsive cells were stained with isolectin B4 (d) but not with anti-GFAP antibody (c). Not all of isolectin B4 positive cells responded to PAF (b, d), suggesting that microglia in such mixed neuronal culture are heterogeneous in terms of PAFR expression.

B-3 discussion

B-3-1 Predominant PAFR expression in microglia

Predominant expression of PAFR mRNA in microglia was demonstrated by *in situ* hybridization and Northern blot/RT-PCR analyses. PAFR mRNA expression in neurons was more apparent in *in situ* hybridization than in Northern blot analysis. This discrepancy may be ascribed to differences between the whole brain and primary cell culture systems. The results strongly suggest the predominant presence of PAFR in microglia, although mRNA levels do not necessarily parallel the protein amount.

Fluorometric $[Ca^{2+}]_i$ imaging revealed that PAF-responsive cells in primary hippocampal cultures were mostly microglia. We also confirmed the results of our previous report that a small number of neurons also respond to PAF (Bito et al., 1992). The present results, taken together, show that microglia constitute the major population in the hippocampal cells that functionally express PAFR.

Predominant PAFR expression in microglia is a reasonable finding, because microglia are related to macrophages (Thomas, 1992; Nakajima and Kohsaka, 1993), which express PAFR mRNA intensely (Ishii et al., 1996). This finding also seems relevant to the fact that in humans the leukocytes and brain express only PAFR transcript 1 (leukocyte type), one of the two different species of PAFR mRNA (Mutoh et al., 1993; Mutoh et al., 1996), although splice variants for PAFR have not been studied in rats.

Immunohistochemistry followed by *in situ* hybridization revealed that PAFR is expressed in some OX-42-positive cells. In primary hippocampal cultures, PAF also elicited calcium response in some isolectin B4 positive cells. The cause of this heterogeneity is presently unclear. One possibility is that microglia are categorized into several subclasses of cells with different levels of PAFR expression, depending on certain factors. In fact, transcription of human PAFR transcript 1 in the brain is regulated by activation of protein kinase C (PKC) through NF-kB or by PAF itself (Mutoh et al., 1994). Thus it is likely that various pathophysiological stimuli that activate PKC may induce PAFR mRNA expression in microglia.

B-3-2 PAFR expression in CNS development

PAFR expression in the rat brain was constant, at least from E18. The characteristic pattern of *in situ* hybridization signals, i.e., scattered distribution of intensely labeled cells with small and dark-stained nuclei, was observed from P7. The involvement of PAF in CNS development has been suggested, because deficiency of a subunit of a PAF-inactivating enzyme results in Miller-Dieker lissencephaly or agyria (Reiner et al., 1993; Hattori et al., 1994), a disorder attributable to incomplete migration of immature neurons to the cerebral cortex. There is also a hypothesis that microglia contribute to eliminating cells that die through development-associated cell death (Thomas, 1992). In light of all of these events, PAF-activated microglia may play a role in developmental events in the CNS such as neuronal selection and migration.

C.Function of PAF in microglia

C-1 Experimental procedure

C-1-1 Cell culture

Microglia were prepared from neonatal rat cerebral tissues, as described previously (Nakajima et al., 1989; Nakajima et al., 1992). Isolated microglia were cultured in DMEM with 0.1 % BSA (PENTEX, Miles Inc., Kankakee, IL) and used within 24 hr. The purity of microglia was estimated to be >99.5 % (data not shown) by staining with FITC-labelled-isolectin B4 (Sigma), a microglial surface marker.

C-1-2 Treatment with PTX , WEB2086 and PD98059

To characterize the signal molecule of PAFR downstream, Gi-coupled receptor inhibitor, PTX (Kaken Seiyaku, Tokyo, Japan) and MEK1-inhibitor, PD98059 (New England Biolabs, Beverly, MA) were applied. The isolated microglia were incubated overnight with 100 ng/ml PTX in culture media. PAFR antagonist, WEB2086 in ethanol and PD98059 in DMSO solution were dissolved into DMEM containing BSA 0.1 % by sonication. The final concentrations of ethanol and DMSO were less than 0.1 % which have no cellular effects alone. The varying

concentrations of WEB 2086 and PD98059 were added into microglial cultures 30 min before assay.

C-1-3 MAPK gel mobility shift assay

Isolated microglia were incubated overnight in the serum-deprived media and stimulated by 10 nM PAF. After indicated stimulation time, the culture medium was aspirated and the reaction was terminated with 200μl of the lysis buffer; 20 mM Tris-HCl pH 7.5, 20 mM β-glycerophosphate (Sigma), 1 mM sodium orthovanadate, 2 mM EGTA, 2 mM DTT, 100 μM PMSF (Sigma), 0.1 % Triton-X100 and 10 μg/ml aprotinin (Sigma). Total cell lysates were collected, centrifuged at 15,000×*g* for 10 min, and the resulting supernatant was boiled at 100°C for 10 min in SDS-PAGE buffer containing 2-mercaptoethanol. 10 μg protein of microglial lysate was electrophoresed on 12.5 % SDS-PAGE gel (Daiichi-Kayaku, Tokyo) at the 30 mA constant current. After electrophoresis, proteins were transfered to Hybond-ECL (Amersham, Tokyo) nitrocellulose membrane. The membrane was blocked with Block-Ace (Dainihon Seiyaku, Tokyo) for 3 hr in room temperature, washed with 0.1% Tween 20 and incubated with 1:5000 anti-ERK1 & 2 mouse monoclonal antibody (Zymed, San Francisco, CA) for 3 hr. Then it was incubated with anti-mouse IgG conjugated with horseradish peroxidase (1:10000, Amersham) for 1 hr. After washing for 1 hr, signals were visualized with an ECL solution (Amersham) and ECL film (Amersham).

C-1-4 MAPK phosphorylation assay

The activation of MAPK was quantified with highly-sensitive and specific method developed in our laboratory; p42/p44 MAPK enzyme assay system (Amersham), with modifications (Waga et al., 1996). Total cell lysates and its supernatant were prepared with the same procedure as manufacturer's instruction except for the lysis buffer containing 100 mM NaCl. Each samples were mixed with Q-Sepharose equilibrated with the same lysis buffer/100 mM NaCl for 30 min at 4 °C in 96 well multiscreen (Millipore, Bedford, MA). MAPK-bound beads were washed twice with lysis buffer/100 mM NaCl and mixed well with lysis buffer/400 mM NaCl for 30 min. Elution was collected through the filter by vaccum. Aliquots of elution containing MAPK

were incubated with an Erk1/2 specific peptide derived from EGF receptor in [^{32}P]ATP/$MgCl_2$ buffer for 30 min at 30 °C. The reaction was terminated by orthophosphoric acid and the total reaction reagent were loaded onto peptide binding paper discs. The paper were washed 2 times with 1% phosphoric acid and 2 times with water. The radioactivity of ^{32}P-incorporated peptide binding on air-dried paper discs were counted by Fuji imaging plate and Fuji BAS2000 system (Fuji Film, Tokyo, Japan).

C-1-5 Chemotaxis of microglia

Polycarbonate filters with 8 μm pores (Neuroprobe, Cabin John, MD) were coated with 10 μg/ml fibronectin (Sigma) in PBS for 60 min. A briefly dried coated filter was placed on the 96-well chamber (Neuroprobe) containing the standard PAF solution or extracted samples from NMDA-stimulated neuronal cell cultures. In the upper part of well separated by the filter, isolated microglia was seeded at the number of 10^4 per well. The cell suspension medium and ligand solutions were prepared with the same buffer, DMEM containing 0.1 % BSA. After incubation at 37 °C in a humidified atomosphere of 5 % CO2 in air for 4 hr, the filter was removed from chamber. The cells on the filter were fixed with methanol and stained with 3 solutions of a Diff-Quick staining kit (International Reagents Corp. Kobe, Japan) each for 30 sec. Then the upper side of the filter was well wiped to remove cells. The number of migrated cells on the lower surface of the filter was counted by transillumination microscopy. Assays were repeated four times.

C-1-6 Northern blot analysis of cPLA2

After sufficient time for decay of radioactivity, the membrane used in the analysis of PAFR mRNA expression was rehybridized with a probe synthesized from the coding region (~2.2 kb) of the mouse cPLA2 cDNA (kindly provided by Dr.Kudo, Showa University, Tokyo). The subsequent procedures were carried out as described above.

C-1-7 Western blot analysis of cPLA2

Microglial cell lysate for immunobloting method were prepared as described in the above

chapter of MAPK gel shift assay. The samples were loaded on 7.5 % SDS-PAGE (20 mA/gel, 20 μg protein/lane). After electrophoresis for 6 hr, the samples were transferred to a nitrocellulose membrane (Hybond-ECL, Amersham) at 5 mA/cm^2 for 30 min. The membrane was blocked with 0.1 % Tween 20 containing 10% horse serum, washed with 0.1 % Tween 20, incubated with 1:5000 rabbit anti-human cPLA2 (kindly provided by Dr. J. D. Clark, Genetic Institute, Cambridge, MA) for 1 hr and then with peroxidase-conjugated goat anti-rabbit IgG antisera (1:5000; Cappel, West Chester, PA) in 0.1 % Tween 20 containing 10 % horse serum for 30 min. The signal was visualized by chemiluminescence (ECL Western blotting reagents, Amersham).

C-1-8 Detection of AA release

Isolated microglia were incubated with 1 μCi/ml [^{3}H]AA (DuPont NEN) in DMEM with 0.1 % BSA for 18 hr at 37 °C. After washing 2 times with DMEM/0.1 % BSA, the cells were stimulated by 0, 0.1, 1, 10, 100, or 1000 nM PAF in 5 ml DMEM/0.1 % BSA at 37 °C for 10 min. The conditioned medium was carefully collected not to aspirate cells, and the remaining cells were lysed with 1 % Triton X-100. The radioacitivity of the medium (A) and the cell lysate (B) was determined in a liquid scintillation counter (Top Count, Packard, Meridian, CT), and A/(A+B) was calculated as a release ratio. Because AA release by cPLA2 is reported to depend on $[Ca^{2+}]_i$ (Clark et al., 1991; Kramer et al., 1991), calcium dependency was evaluated in microglial culture. Before loading with [^{3}H]AA, cells were incubated in DMEM/0.1 % BSA/0.1 %DMSO with or without a $[Ca^{2+}]_i$ chelator, BAPTA-AM (Dojin Chemicals), 20 μM, at 37 °C for 2 hr and then incubated with 10 nM PAF in 5 ml DMEM/0.1 % BSA for 0, 5, 10, or 20 min. The redioactivity of the medium and the cell lysate was determined as described above.

C-1-9 Determination of glutamic acid release

We analyzed the release of glutamic acid as one of candidates of neurotoxic factors. Microglial cells were isolated as described above (5.0×10^5/ well) and incubated at 37°C overnight in glutamic acid-free DMEM/0.1% BSA. After washing twice with glutamic acid-free DMEM/0.1% BSA, cells were incubated with or without 10nM PAF for 0, 2, 6 hr. The conditioned medium were collected and stored at -20C° until use. Two methods to detect glutamic acid were used. (1)

Enzyme assay method was carried out with Farb-test kit for L-glutamic acid (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's instruction. (2) HPLC method was performed using an HP1000 system (Packerd). Briefly, 1μl of samples was injected automatically, simultaneously labeling with *o*-futalaldehyde. Labeled amino acids chlomatographed with HP aminoquant culumn (Packerd) were analyzed by a chemiluminescence detector.

C-2 Results

C-2-1MAPK activation in chemotaxis of microglia

Our previous study has shown that MAPK is activated by PAF in neutrophils or macrophages (Ferby et al., 1994; Ferby et al., 1996). Furthermore, in microglia, PAF raises the intracellular calcium concentration. Thus, we carried out a MAPK gel shift assay with immunoblotting to Erk1/2 of microglia. After 10 nM PAF stimulation in microglia, Erk2 was phosphorylated within 1 min and the activation was maximal at 3 min. After 3 min, the activity subsided at 10 min (**figure 20**). Additionally, we examined the signaling pathway from PAFR to MAPK activation. A MEK1 inhibitor, 50 μM PD98059 and a PAFR antagonist, 1 μM WEB2086 completely suppressed PAF-stimulated MAPK activation. Moreover, pretreatment of 100ng/ml pertussus toxin (PTX) for 12 hour affected the MAPK activation (**figure 21**). These data indicate that PAF activates Erk1/2 MAPK and MEK1, and PAFR may be coupled at least with Gi-like G protein in microglia.

C-2-2 PAF induced chemotaxis of microglia

We have shown that PAFR is predominantly expressed in the rat brain microglia (**Section B**). PAF is a potent proinflammatory mediator and microglia play a central role in inflammatory response in the rat brain. Thus, we examined PAF-mediated chemotaxis of microglia by the Boyden chamber assay. Microglia demonstrated a prominent chemotactic response toward PAF. **Figure 22** demonstrates the cells migrated to the back surface of the filter through 8 μm pore in the medium containing 10 nM PAF. The chemotactic response of microglia was dose-dependent

with the optimal concentration as 10 to 100 nM (**Figure 23a**). To exclude the non-specific response, microglia were pretreated with a PAFR antagonist, 1 μM WEB2086 for 30 min. This compound suppressed chemotaxis completely, indicating that chemotaxis of microglia is mediated by a cell surface PAFR. Additionally, microglia responded to the lipid-extracted sample of NMDA-stimulated neuron and its culture medium, suggesting again that PAF was present in the neuronal cells **(Figure 23b).**

Furthermore, we examined the relationship of MAPK activation and chemotaxis. Microglia preincubated with 100ng/ml PTX overnight showed no chemotactic response to PAF (**Figure 24**). **Figure 24b.c** showed migrated microglial cells. PD98059 inhibited chemotaxis and Erk1/2 activation of microglia by dose-dependent manner **(Figure 25)**. These effects of two signal blockers to chemotaxis were paralleled MAPK activation. Therefore, we propose that Erk1/2 MAPK activation is necessary to the chemotactic response of microglia.

C-2-3 Presense of cPLA2 and production of arachidonic acid

One of the important outputs of PAFR activation is the release of AA (Honda et al., 1994). Its release depends mainly on activation of cPLA2 (Clark et al., 1991); however, it has not been clear which cells in the brain express cPLA2. Since PAF stimulates AA release in astrocytes (Petroni et al., 1994) and a myc-immortalized microglial cell line (Rhigi et al., 1995), the presence of cPLA2 was explored in primary cell culture systems. Northern blotting reveals that microglia predominantly express cPLA2 mRNA (**Figure 26a**) and Western blotting indicates that cPLA2 protein is present in microglia (**Figure 26b**).

Because of the prominent expression of cPLA2 and the activation of Erk1/2 by PAFR stimulation, AA release by cPLA2 phosphorylated by Erk1/2 was expected in microglia. The release was dose and time-dependent (**Figure 27ab**). The optimal PAF concentration was 10nM and the net release reached a plateau in 10 min. Preincubation with BAPTA-AM decreased the release by ~50 %, indicating that this AA release was partially $[Ca^{2+}]_i$ dependent.

C-2-4 Release of glutamic acid in PAF-stimulated microglia

PAFR antagonists are reported to suppress postischemic neuronal death (Panetta et al., 1989; Gilboe et al., 1991; Prehn et al., 1993) and PAF is related to human immunodeficiency virus-associated neuronal cell death (Genis et al., 1992; Epstein et al., 1993; Gelbard et al., 1994; Lipton, 1994; Lipton et al., 1994). We analyzed the release of glutamic acid as one of candidates of neurotoxic factors. Enzyme asssay revealed that glutamic acid was present in PAF-stimulated conditioned medium in 6 hr (**Figure 28a**). Amino-acid analysis with HPLC also demonstrated that glutamic acid was released about >5 times more in PAF-stimulated microglia than in control cells (**Figure 28b**). These data demonstrated that glutamic acid might be released from microglia by PAF.

C-3 Discussion

C-3-1 PAF-induced MAPK activation and chemotaxis of microglia

In this report, we demonstrate the prominent chemotactic response of microglia to PAF (**Figure 22-25**). To our knowledge, this is the first report of chemotaxis of microglia to an intrinsic factor which can be synthesized in neuron. The expression of chemokine receptors; C5a, CXC type and CC type receptor, in microglia were already reported (Nolte et al., 1996; Peterson et al., 1997; Tanabe et al., 1997). However the presence of these ligands in the CNS were not so far determined. The other chemoattractants for microglia were epidermal growth factor (EGF) (Nolte et al., 1997) and macrophage colony stimulating factor (M-CSF) released from ß-amyloid-stimulated neuron (Du et al., 1997). PAF-mediated chemotaxis of microglia appears more important than other chemoattractants because PAF is the first ligand to be synthesized in neuron.

This response may account for the activation and accumulation of microglia to the pathophysiological lesions such as ischemic brain injury (Bruce et al., 1996) and Alzheimer's disease (Davis et al., 1992). Furthermore, it is proposed that PAF has a possible role of an neuron-microglial mediator of neuronal death, synaptic plasticity and development, in addition to inflammation and wound repair. In fact, it was reported that microglia is activated in response to

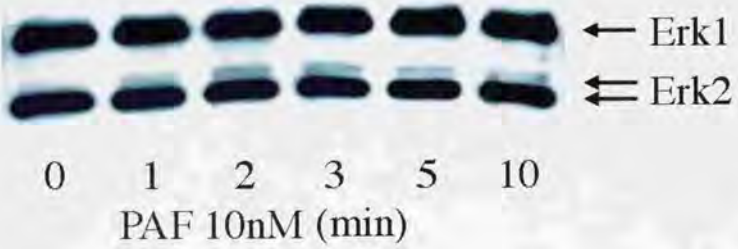


Fig.20 PAF-elicited MAPK activation in rat brain microglia
Isolated microglia was incubated overnight in the serum-deprived media and stimulated by 10 nM PAF. After indicated time, the culture medium was aspirated off and the reaction was terminated with 200 μl of the lysis buffer. 10 μg protein of microglial lysate was electrophoresed on 12.5 % SDS-PAGE gel and transfered to a Hybond-ECL nitrocellulose membrane. The membrane was incubated with 1:5000 anti-ERK1 & 2 mouse monoclonal antibody for 3 hr. Then, it was incubated with anti-mouse IgG conjugated with horseradish peroxidase (1:10000, Amersham) for 1 hr. After washing for 1 hr, signals were visualized with a ECL solution (Amersham) and an ECL film (Amersham).
Phosphorylation of Erk 1&2 MAPK was maximal at 3 min and subsided to 10 min.

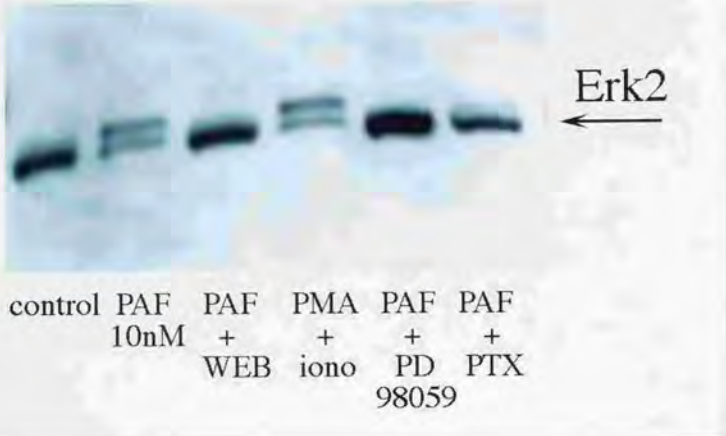


Fig.21 The effect of PTX, WEB2086 and PD98059 to PAF-elicited MAPK activation
Microglial culture was preteated with 100 ng/ml PTX overnight, 1 μM WEB2086 for 30 min and 50 μM PD98059 for 30 min before 10nM PAF application. Western blotting method was carried out as described in Fig. 20.
Phosphorylation of Erk 1/2 MAPK was inhibited by these modifications, suggesting that PAF activates Erk1/2 MAPK through PAFR, Gi-like G protein and MEK1.

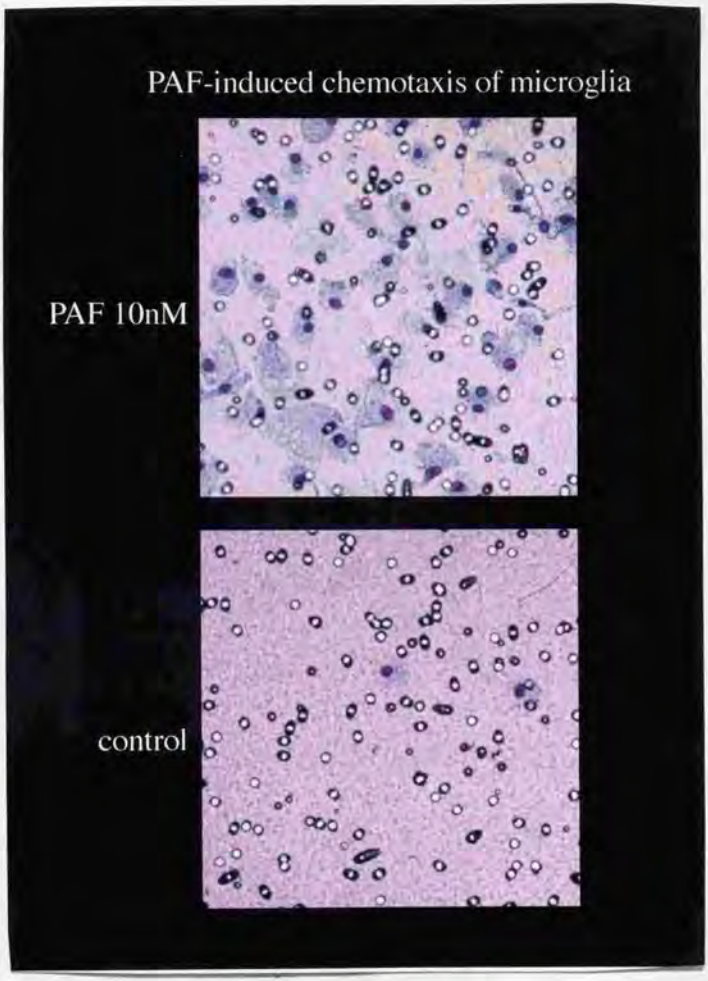


Fig. 22 PAF induced chemotaxis of microglia
Polycarbonate filters with 8 μm pores were coated with 10 μg/ml fibronectin in PBS for 60 min. A briefly dried coated filter was placed on the 96-well chamber containing the standard PAF solution. In the upper part of well separated by the filter, isolated microglia was seeded at the number of 10^4 per well. After incubation at 37 ˚C in a humidified atomosphere of 5 % CO_2 in air for 4 hr, the filter was removed from chamber. The cells on the filter were fixed with methanol and stained with 3 solutions of a Diff-Quick staining kit. Then the upper side of the filter was well wiped to remove cells. The number of migrated cells on the lower surface of the filter was counted by transillumination microscopy. Assays were repeated four times. This photographs showed migrated microglia responded to PAF (b). Very few cells were seen in control medium (a).

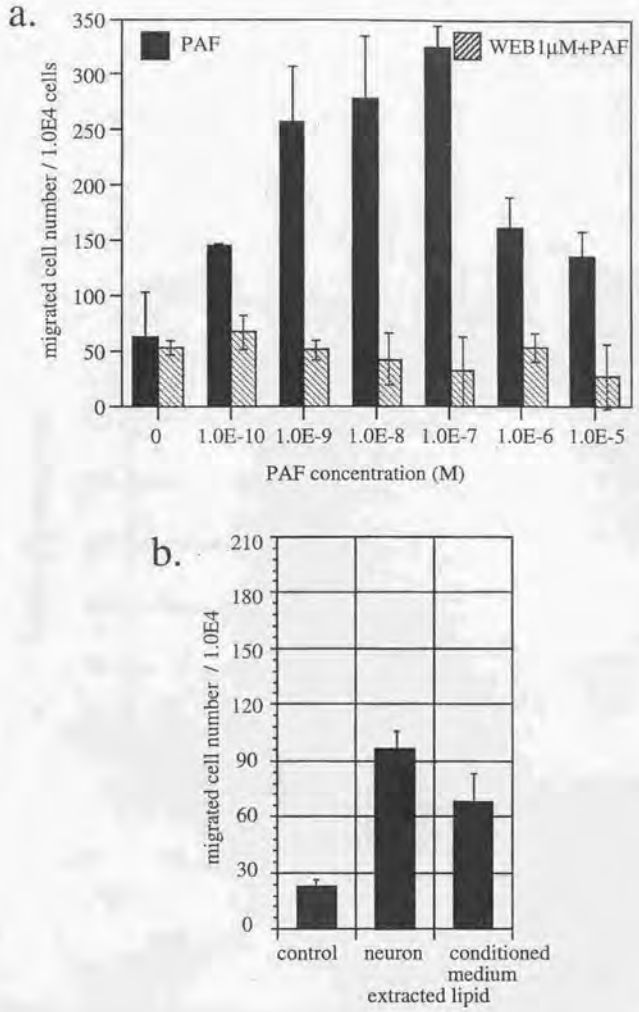


Fig. 23 Chemotactic response of rat microglia to PAF
Figure indicates the migrated cell number of microglia. Dose-dependent chemotactic response were shown in PAF-stimulated microglia and the optimal concentration of PAF was 10-100 nM. A PAFR-antagonist, WEB2086 inhibited the chemotactic response (a). Chemotaxis of microglia was seen to the extracted PAF from neuron stimulated by 50 μM NMDA for 1 min and its conditioned medium, but not in control medium (b).

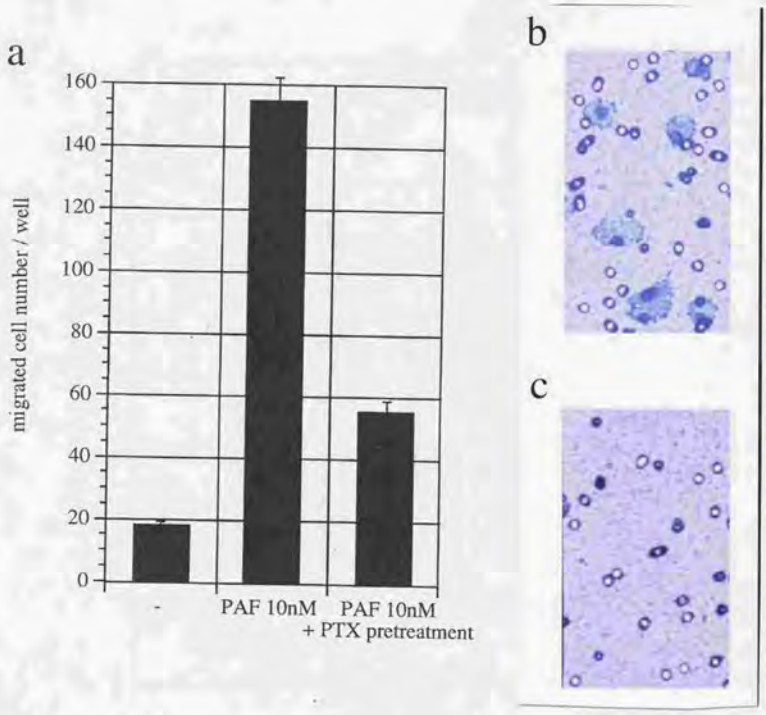


Fig.24 PTX sensitivity of PAF-induced chemotaxis of microglia
Microglia pretreated with 100 ng/ml PTX overnight showed decreased chemotactic response to 10 nM PAF (a).
Light microscopic photographs showed migrated microglia pretreated with (b) or without PTX (c).
Gi-like G protein might be related to the signal of microglial chemotaxis.

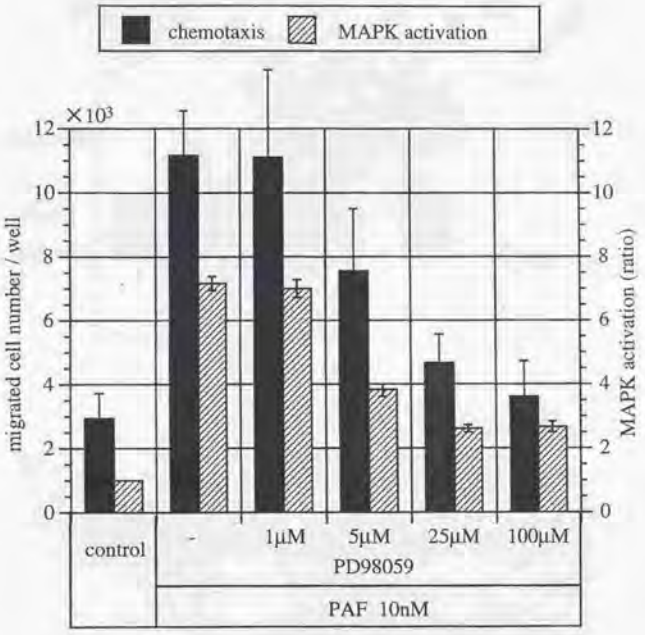


Fig. 25 PD98059 inhibits microglial chemotaxis and MAPK activation
Chemotactic response and pretreatment of PD98059 were carried out as described in Experimental Procedures. MAPK activation was analysed with p42/p44 MAPK enzyme assay system with modifications. Aliquots of elution containing MAPK from Q-sepharose beads were incubated with Erk1/2 specific peptide derived from EGF receptor in [^{32}P]ATP/$MgCl_2$ buffer for 30 min at 30°C. This reaction was terminated by orthophosphoric acid and total reaction reagent were loaded onto peptide binding paper discs. The paper were washed 2 times with 1 % phosphoric acid and 2 times with water. Radioactivity of ^{32}P-incorporated peptide binding on air-dried paper discs were counted by Fuji imaging plate and Fuji BAS2000 system. PD98059 inhibits chemotaxis and Erk1/2 activation by dose dependent manner, suggesting that MAPK activation is required to induce chemotaxis of microglia.

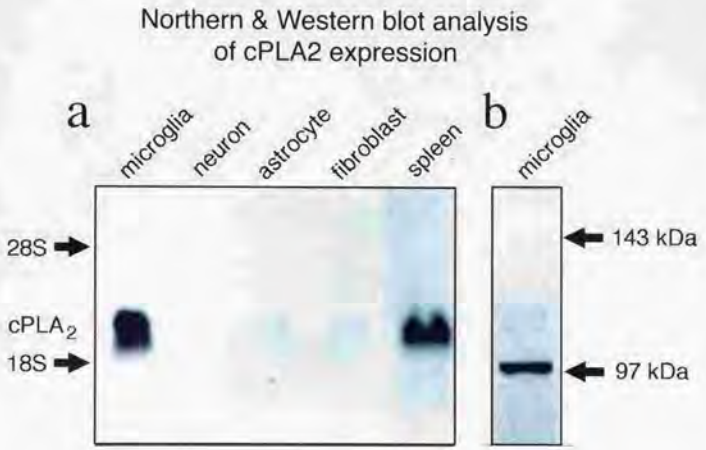


Fig. 26 Presence of cPLA2 in rat brain microglia
(a) Northern blot analysis of cPLA2 mRNA
The membrane filter was hybridized with a probe synthesized from the coding region (~2.2 kb) of the mouse cPLA2 cDNA. The subsequent procedures were carried out as described above. cPLA2 mRNA was predominantly expressed in microglia.
(b) Western blot analysis of cPLA2
Microglial cell lysates for immunobloting method were prepared as described in the above chapter of MAPK gel shift assay. The samples were loaded on 7.5 % SDS-PAGE. After electrophoresis for 6 hr, the samples were transferred to a nitrocellulose membrane. The blot membrane was incubated with 1:5000 rabbit anti-human cPLA2 for 1 hr and then with peroxidase-conjugated goat anti-rabbit IgG antisera. The signal was visualized by chemiluminescence. cPLA2 protein was present in microglia.

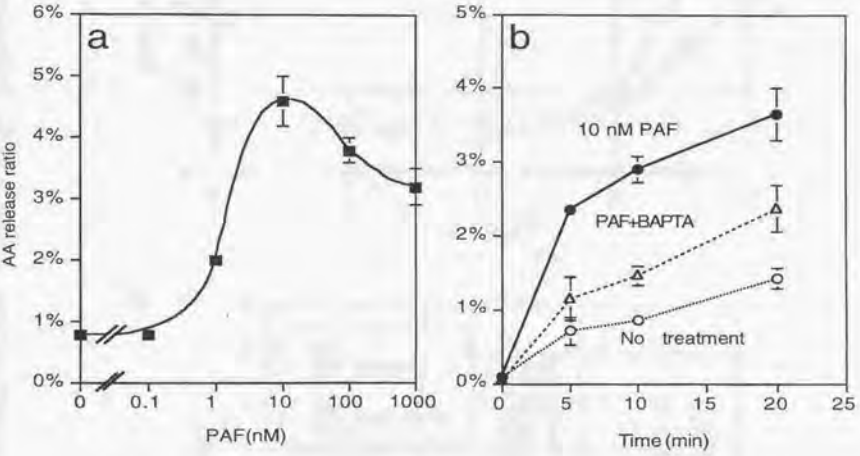


Fig. 27 Release of AA in PAF-stimulated microglia

Isolated microglia as were incubated with 1μCi/ml [^{3}H]AA for 18 hr at 37 ˚C.
(a) Dose dependency of AA release. After washing 2 times, the cells were stimulated with 0, 0.1, 1, 10, 100, or 1000 nM PAF for 10 min. The conditioned medium was collected, and the cells were lysed with 1% Triton X-100. The radioactivity of the medium (A) and the cell lysate (B) was determined in a liquid scintillation counter and A/(A+B) was calculated as a release ratio. AA was released dose-dependently and the optimal concentration was 10nM.
(b) Time course of AA release. Before loading with [^{3}H]AA, cells were incubated with or without a $[Ca^{2+}]_i$ chelator, BAPTA-AM , 20 μM, at 37 ˚C for 2 hr and then incubated with 10 nM PAF for 0, 5, 10, or 20 min. Net release of AA reached a plateau in 10 min and preincubation with BAPTA-AM decreased the release by ~50 %.

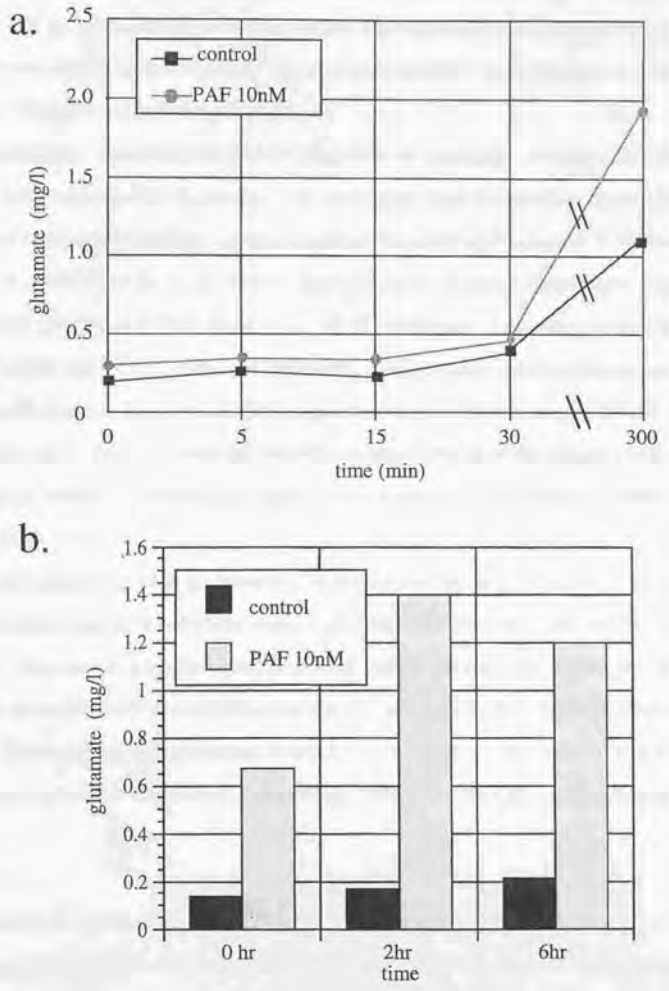


Fig. 28 glutamic acid release from PAF-stimulated microglia
Conditioned medium of PAF-stimulated microglial culture was collected and analysed by two methods to detect glutamic acid as described under Experimental Procedures.
(a) By an enzyme assay, glutamic acid was released in microglial conditioned medium for 6 hr incubation with 10 nM PAF.
(b) By an amino-acid analyser with an HPLC, glutamic acid was detected about over 5 times more in PAF-stimulated microglia than in control.
Glutamic acid might be released from microglia by PAF stimulation.

NMDA-mediated excitotoxicity in the immature rat brain (Acarin et al., 1996). This excitotoxicity may be caused by neuro-microglial interaction via PAF-activated microglia. However, in this report, we were unable to show the direct chemotactic response of microglia in the mixed culture of NMDA-stimulated neuron and microglia.

PTX overnight treatment affected the migration of microglia. It means that PAFR in microglia can couple Gi-like G protein. This fact agrees with the previous report about C5a receptor and CXC-type chemokine receptor mediated chemotaxis of microglia is PTX-sensitive (Nolte et al., 1996; Tanabe et al., 1997). Leucotriene B4 activates chemotactic response in PTX-sensitive G-protein(s) (Yokomizo et al., 1997). Microglial chemotaxis to PAF might be elicited by MEK1 and ERK1/2 pathway (**Figure25**). Little is known about molecular mechanisms underlying chemotaxis, human neutrophils appear to respond to PAF via p38 MAPK pathway (Nick et al., 1997). On the other hand, Erk1/2 activation influences fibroblastic cell migration (Klemke et al., 1997). The conflicting results of these reports may be caused by differences of spieces and cell types.

The next question is what the function of activated and migrated microglia is in response to PAF. Microglial migration can evoke direct interaction with other cells via membrane surface molecules. Because microglia functions as professional phagocytes, PAF synthesized in neuron can be one of candidates of intercellular messengers to neuronal death or reconstruction of neural networks. Besides phagocytic functions, microglia are relevant to intercellular crosstalk through various messengers such as cytokines (Kreutzberg, 1996) or lipid mediators, and various surface molecules.

C-3-2 arachidonic acid release

In microglia, cPLA2 was dominantly expressed (**Figure 26**) and AA was synthesized from membrane phospholipid mainly by the activation of cPLA2 (**Figure 27**). Previous reports have shown that astroglia was exclusively stained by anti-cPLA2 antibody in the rat brain (Stephenson et al., 1994), and other have showen that rat neuron was highly stained by *in situ* hybridization of cPLA2 mRNA (Owada et al., 1994). This difference is due to the differeces between the brain and cell culture, and between mRNA and protein.

AA release was suppressd by ~50% after treatment of the $[Ca^{2+}]_i$ chelator. This effect is compatible with the fact that cPLA2 is activated and translocated to the membrane by $[Ca^{2+}]_i$ elevation. The residual release of AA might be induced by the presence of $[Ca^{2+}]_i$ independent PLA2s in microglia. AA release from microglia may be a trigger of cascade to produce many inflammatory factors such as prostagrandins and leukotrienes, proposing that one of functions of PAF-activated microglia is an inflammatory mediator in CNS. Moreover, activated microglia may have opposing functions as cytotoxic and protective tissue guardians. Therefore, PAF-mediated effects on production of cytokines, NO or proteases must be examined to clarify neuro-microglial interaction.

C-3-3 Glutamic acid release from PAF-stimulated microglia

Previous reports demonstrated that microglia have a neurotoxic effect and release glutamic acid as one of several neurotoxic factors (Giulian et al., 1990; Giulian et al., 1993; Giulian et al., 1993; Lipton, 1994). It is unclear which factor stimulates glutamate release from microglia besides LPS (Patrizio and Levi, 1994). PAF induced glutamic acid release but it took a long time that the concentration reached the level of detection by our methods. Taken together, our data has revealed first that glutamic acid induces the synthesis of PAF in neuron, and in turn, PAF induces the release of glutamic acid from microglia. This PAF-mediated neuro-microglial interaction might explain the previous reports that NMDAR antagonists supressed the brain injury after ischemia or neuronal injury co-cultured with microglia (Piani et al., 1991; Piani et al., 1992; Streit et al., 1992). In brief, in a brain injury like ischemia or virus infection, glutamic acid release from neuron or $[Ca^{2+}]_i$ elevation are evoked to trigger the synthesis PAF. Then released PAF induces chemotaxis and releases glutamic acid to damage neuronal cells. This hypothesis is a summay of our *in vitro* study but only one of many routes evoked in such a brain lesion. Of course, many inflammatory factors like cytokines or toxic factors such as nitoric oxide will be related to these events.

Conclusion

(A) PAF is predominantly synthesized in neuronal cells by the stimulation of glutamic acid, especially through NMDA receptor.

(B) PAFR mRNA is expressed in microglia and hippocampal neuron as determined by *in situ* hybridization and immunohistochemisty. PAFR expressed in microglia functionally elicited the intracellular calcium mobilization.

(C) PAF induces Erk 1/2 MAPK activation through Gi-like G protein and MEK1, and elicits arachidonic acid release through cPLA2 in microglia. Microglia shows the chemotactic response toward PAF, which is synthesized in neuron by glutamic acid stimulation.

In this report, we propose that PAF functions in the CNS as one of intercellular messenger molecules from neuron to microglia, to mediate inflammatory response, cytotoxic effect to neuronal cells, chemotaxis, phagocytosis or apoptosis (**Figure 29**). Ultimately, further studies about PAF-mediated neuro-microglial crosstalk are needed to elucidate the pathogenesis of various neurologic disorders such as ischemia, convulsion and virus infection as well as neurodegenerative disorders such as Alzheimer' disease or multiple sclerosis.

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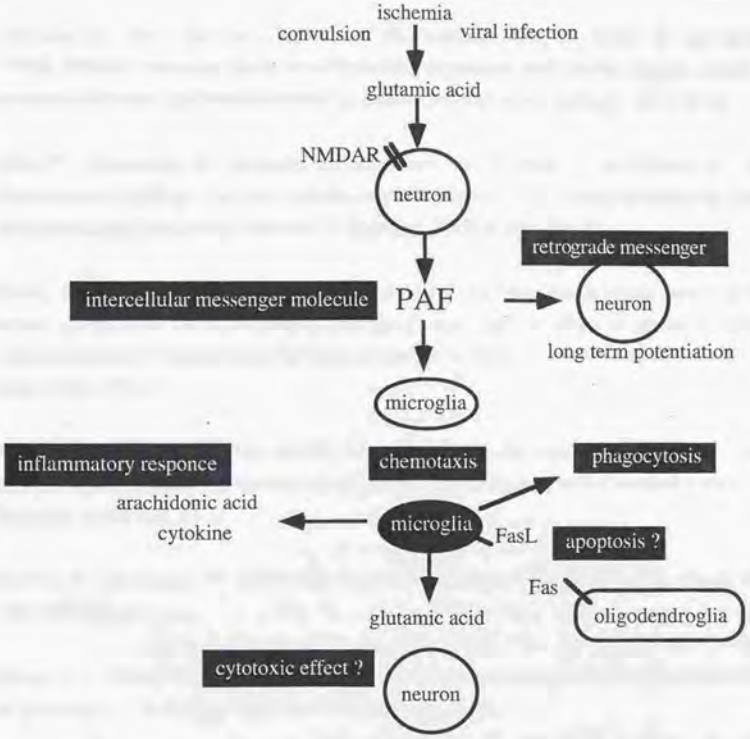


Fig.29 PAF-mediated neuro-microglial interaction in the CNS

References

Acarin, L., Gonzalez, B., Castellano, B. and Castro, A.J. (1996). Microglial response to N-methyl-D-aspartate-mediated excitotoxicity in the immature rat brain. J. Comp. Neurol. *367*, 361-74.

Albrecht, U., Abu, I.R., Ratz, B., Hattori, M., Aoki, J., Arai, H., Inoue, K. and Eichele, G. (1996). Platelet-activating factor acetylhydrolase expression and activity suggest a link between neuronal migration and platelet-activating factor. Developmental Biology *180*, 579-93.

Aoki, Y., Nakamura, M., Kodama, H., Matsumoto, T., Shimizu, T. and Noma, M. (1995). A radioreceptor binding assay for platelet-activating factor (PAF) using membranes from CHO cells expressing human PAF receptor. J. Immunol. Method *186*, 225-31.

Baker, R.R. and Chang, H.Y. (1993). The potential for platelet-activating factor synthesis in brain: properties of cholinephosphotransferase and 1-alkyl-sn-glycero-3-phosphate acetyltransferase in microsomal fractions of immature rabbit cerebral cortex. Biochim. Biophys. Acta *1170*, 157-64.

Baker, R.R. and Chang, H.Y. (1994). MgATP inhibits the synthesis of 1-alkyl-2-acetyl-sn-glycero-3-phosphate by microsomal acetyltransferase of immature rabbit cerebral cortex. Biochim. Biophys. Acta *1213*, 27-33.

Banker, G. and Cowan, W. (1977). Rat hippocampal neurons in dispersed cell culture. Brain Res *126*, 397-425.

Bazan, N.G. (1994). Platelet-activating factor is a synapse messenger and an intracellular modulator of gene expression. J. Lipid Mediat. Cell Signal. *10*, 83-86.

Bazan, N.G., Squinto, S.P., Braquet, P., Panetta, T. and Marcheselli, V.L. (1991). Platelet-activating factor and polyunsaturated fatty acids in cerebral ischemia or convulsions: intracellular PAF-binding sites and activation of a fos/jun/AP-1 transcriptional signaling system. Lipids *26*, 1236-1242.

Bito, H., Nakamura, M., Honda, Z., Izumi, T., Iwatsubo, T., Seyama, Y., Ogura, A., Kudo, Y. and Shimizu, T. (1992). Platelet-activating factor (PAF) receptor in rat brain: PAF mobilizes intracellular Ca2+ in hippocampal neurons. Neuron *9*, 285-94.

Bligh, E.C. and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. *37*, 911-917.

Bottenstein, J. (1985). Growth and differentiation of neural cells in defined media. Cell culture in the neurosciences (New York: J. Bottenstein and G. Sato), pp. 3-43.

Braquet, P., Touqui, L., Shen, T.Y. and Vargaftig, B.B. (1987). Perspectives in platelet-activating factor research. Pharmacol. Rev. *39*, 97-145.

Bruce, A.J., Boling, W., Kindy, M.S., Peschon, J., Kraemer, P.J., Carpenter, M.K., Holtsberg, F.W. and Mattson, M.P. (1996). Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors. Nature Med. *2*, 788-94.

Bussolino, F., Gremo, F., Tetta, C., Pescarmona, G. and Camussi, G. (1986). Production of platelet-activating factor by chick retina. J. Biol. Chem. *261*, 16502-16508.

Clark, G.D., Happel, L.T., Zorumski, C.F. and Bazan, N.G. (1992). Enhancement of hippocampal excitatory synaptic transmission by platelet-activating factor. Neuron *9*, 1211-1216.

Clark, J.D., Lin, L.-L., Kriz, R.W., Ramesha, C.S., Sultzman, L.A., Lin, A.Y., Milona, N. and Knopf, J.L. (1991). A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. Cell *65*, 1043-1051.

Davis, J.B., McMurray, H.F. and Schubert, D. (1992). The amyloid beta-protein of Alzheimer's disease is chemotactic for mononuclear phagocytes. Biochem Biophys Res Commun *189*, 1096-100.

Doucet, J.P. and Bazan, N.G. (1992). Excitable membranes, lipid messengers, and immediate-early genes. Alteration of signal transduction in neuromodulation and neurotrauma. Mol. Neurobiol. *6*, 407-424.

Du, Y.S. et al (1997). Amyloid-beta peptide-receptor for advanced glycation endproduct interaction elicits neuronal expression of macrophage-colony stimulating factor: a proinflammatory pathway in Alzheimer disease. Proc Natl Acad Sci U S A *94*, 5296-301.

Epstein, L. and Gendelman, H. (1993). Human immunodeficiency virus type 1 infection of the nervous system: pathogenetic mechanisms. Ann. Neurol. *33*, 429-436.

Ferby, I., Waga, I., Kume, K., Sakanaka, C. and Shimizu, T. (1996). PAF-induced MAPK activation is inhibited by wortmannin in neutrophils and macrophages. Adv Exp Med Biol *416*, 321-6.

Ferby, I.M., Waga, I., Sakanaka, C., Kume, K. and Shimizu, T. (1994). Wortmannin inhibits mitogen-activated protein kinase activation induced by platelet-activating factor in guinea pig neutrophils. J. Biol. Chem. *269*, 30485-8.

Feuerstein, G., Yue, T.L. and Lysko, P.G. (1990). Platelet-activating factor. A putative mediator in central nervous system injury? Stroke *21 (Suppl III)*, III 90-94.

Francescangeli, E., Domanska, J.K. and Goracci, G. (1996). Relative contribution of the de novo and remodelling pathways to the synthesis of platelet-activating factor in brain areas and during ischemia. J. Lipid Med. Cell Signal. *14*, 89-98.

Frerichs, K.U., Lindsberg, P.J., Hallenbeck, J.M. and Feuerstein, G.Z. (1990). Platelet-activating factor and progressive brain damage following focal brain injury. J. Neurosurg. *73*, 223-233.

Gelbard, H.A. et al (1994). Platelet-activating factor: a candidate human immunodeficiency virus type 1-induced neurotoxin. J. Virol. *68*, 4628-4635.

Genis, P. et al (1992). Cytokines and arachidonic metabolites produced during human immunodeficiency virus (HIV)-infected macrophage-astroglia interactions: implications for the neuropathogenesis of HIV disease. J. Exp. Med. *176*, 1703-1718.

Gilboe, D.D., Kintner, D., Fitzpatrick, J.H., Emoto, S.E., Esanu, A., Braquet, P.G. and Bazan, N.G. (1991). Recovery of postischemic brain metabolism and function following treatment with a free radical scavenger and platelet-activating factor antagonists. J. Neurochem. *56*, 311-319.

Giulian, D., Corpuz, M., Chapman, S., Mansouri, M. and Robertson, C. (1993). Reactive mononuclear phagocytes release neurotoxins after ischemic and traumatic injury to the central nervous system. J. Neurosci. Res. *36*, 681-693.

Giulian, D., Vaca, K. and Corpuz, M. (1993). Brain glia release factors with opposing actions upon neuronal survival. J. Neurosci. *13*, 29-37.

Giulian, D., Vaca, K. and Noonan, C. (1990). Secretion of neurotoxins by mononuclear phagocytes infected with HIV-1. Science *250*, 1593-1596.

Hanahan, D.J. (1986). Platelet activating factor: a biologically active phosphoglyceride. Ann. Rev. Biochem. *55*, 483-509.

Hattori, M., Adachi, H., Tsujimoto, M., Arai, H. and Inoue, K. (1994). Miller-Dieker lissencephaly gene encodes a subunit of brain platelet-activating factor acetylhydrolase. Nature *370,* 216-218.

Hattori, M., Arai, H. and Inoue, K. (1993). Purification and characterization of bovine brain platelet-activating factor acetylhydrolase. J. Biol. Chem. *268,* 18748-53.

Honda, Z., Takano, T., Gotoh, Y., Nishida, E., Ito, K. and Shimizu, T. (1994). Transfected platelet-activating factor receptor activates mitogen-activated protein (MAP) kinase and MAP kinase kinase in Chinese hamster ovary cells. J. Biol. Chem. *269,* 2307-2315.

Ishii, S., Matsuda, Y., Nakamura, M., Waga, I., Kume, K., Izumi, T. and Shimizu, T. (1996). A murine platelet-activating factor receptor gene: cloning, chromosomal localization and up-regulation of expression by lipopolysaccharide in peritoneal resident macrophages. Biochem. J. *314,* 671-678.

Ishii, S. and Shimizu, T. (1998). J. Exp. Med. *in press.*

Izquierdo, I., Fin, C., Schmitz, P., Da Silvia, R., Jerusalinsky, D., Quillfeldt, J., Ferreira, M., Medina, J. and Bazan, N. (1995). Memory enhancement by intrahippocampal, intraamygdala, or intraentorhinal infusion of platelet-activating factor measured in an inhibitory avoidance task. Proc. Natl. Acad. Aci. U.S.A. *92,* 5047-5051.

Izumi, T. and Shimizu, T. (1995). Platelet-activating factor receptor: gene expression and signal transduction. Biochim. Biophys. Acta. *1259,* 317-333.

Kato, K., Clark, G.D., Bazan, N.G. and Zorumski, C.F. (1994). Platelet-activating factor as a potential retrograde messenger in CA1 hippocampal long-term potentiation. Nature *367,* 175-179.

Klemke, R.L., Cai, S., Giannini, A.L., Gallagher, P.J., de, L.P. and Cheresh, D.A. (1997). Regulation of cell motility by mitogen-activated protein kinase. J. Cell Biol. *137,* 481-92.

Kramer, R.M., Roberts, E.F., Manetta, J. and Putnam, J.E. (1991). The Ca^{2+}-sensitive cytosolic phospholipase A_2 is a 100-kDa protein in human monoblast U937 cells. J. Biol. Chem. *266,* 5268-5272.

Kreutzberg, G.W. (1996). Microglia: a sensor for pathological events in the CNS. [Review] [75 refs]. Trends Neurosci. *19,* 312-8.

Kumar, R., Harvey, S., Kester, M., Hanahan, D. and Olson, M. (1988). Production and effects of platelet-activating factor in the rat brain. Biochim. Biophys. Acta. *963,* 375-383.

Kunievsky, B. and Yavin, E. (1994). Production and metabolism of platelet-activating factor in the normal and ischemic fetal rat brain. J. Neurochem. *63*, 2144-51.

Lipton, S.A. (1994). Neuronal injury associated with HIV-1 and potential treatment with calcium-channel and NMDA antagonists. Dev. Neurosci. *16*, 145-151.

Lipton, S.A., Yeh, M. and Dreyer, E.B. (1994). Update on current models of HIV-related neuronal injury: platelet-activating factor, arachidonic acid and nitric oxide. Adv. Neuroimmunol. *4*, 181-188.

MaCarthy, K. and de Vellis, J. (1980). Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J. Cell Biol. *85*, 890-902.

MacDonald, R., Swift, G., Przybyla, A. and Chirgwin, J. (1987). Isolation of RNA using guanidium salts. Methods Enzymol. *152*, 219-227.

Mori, T., Iijima, N., Kitabatake, K. and Kohsaka, S. (1990). Alpha 2-macroglobulin is an astroglia-derived neurite-promoting factor for cultured neurons from rat central nervous system. Brain Res. *527*, 55-61.

Mutoh, H. et al (1993). Two different promoters direct expression of two distinct forms of mRNA of human platelet-activating factor receptor. FEBS Lett. *322*, 129-134.

Mutoh, H., Fukada, T., Masushige, S., Sasaki, H., Shimizu, T. and Kato, S. (1996). Tissue-specific response of the human platelet-activating factor receptor gene to retinoic acid and thyroid hormone by alternative promotor usage. Proc. Natl. Acad. Sci. U. S. A. *93*, 774-779.

Mutoh, H., Ishii, S., Izumi, T., Kato, S. and Shimizu, T. (1994). Platelet-activating factor (PAF) positively auto-regulates the expression of human PAF receptor transcript 1 (leukocyte-type) through NF-κB. Biochem. Biophys. Res. Commun. *205*, 1137-1142.

Nakajima, K., Hamanoue, M., Shimojo, M., Takei, N. and Kohsaka, S. (1989). Characterization of microglia isolated from a primary culture of embryonic rat brain by a simplified method. Biomed. Res. *10 (S3)*, 411-423.

Nakajima, K. and Kohsaka, S. (1993). Functional roles of microglia in the brain. Neurosci. Res. *17*, 187-203.

Nakajima, K., Shimojo, M., Hamanoue, M., Ishiura, S., Sugita, H. and Kohsaka, S. (1992). Identification of elastase as a secretory protease from cultured rat microglia. J. Neurochem. *58*, 1401-1408.

Nick, J.A., Avdi, N.J., Young, S.K., Knall, C., Gerwins, P., Johnson, G.L. and Worthen, G.S. (1997). Common and distinct intracellular signaling pathways in human neutrophils utilized by platelet activating factor and FMLP. J. Clin. Invest. *99*, 975-86.

Nishida, K. and Markey, S.P. (1996). Nicardipine and MK-801 attenuate platelet-activating factor increases following cerebral ischemia-reperfusion in gerbils. Brain Res. *733*, 203-10.

Nishida, K., Markey, S.P., Kustova, Y., Morse, H.3., Skolnick, P., Basile, A.S. and Sei, Y. (1996). Increased brain levels of platelet-activating factor in a murine acquired immune deficiency syndrome are NMDA receptor-mediated. J. Neurochem. *66*, 433-5.

Nolte, C., Kirchhoff, F. and Kettenmann, H. (1997). Epidermal growth factor is a motility factor for microglial cells in vitro: evidence for EGF receptor expression [In Process Citation]. Eur J Neurosci *9*, 1690-8.

Nolte, C., Moller, T., Walter, T. and Kettenmann, H. (1996). Complement 5a controls motility of murine microglial cells in vitro via activation of an inhibitory G-protein and the rearrangement of the actin cytoskeleton. Neuroscience *73*, 1091-107.

Owada, Y., Tominaga, T., Yoshimoto, T. and Kondo, H. (1994). Molecular cloning of rat cDNA for cytosolic phospholipase A_2 and the increased gene expression in the dentate gyrus following transient forebrain ischemia. Mol. Brain. Res. *25*, 364-368.

Panetta, T., Marcheselli, V.L., Braquet, P. and Bazan, N.G. (1989). Arachidonic acid metabolism and cerebral blood flow in the normal, ischemic, and reperfused gerbil brain. Inhibition of ischemia-reperfusion-induced cerebral injury by a platelet-activating factor antagonist (BN 52021). Ann. N. Y. Acad. Sci. *559*, 340-351.

Patrizio, M. and Levi, G. (1994). Glutamate production by cultured microglia: differences between rat and mouse, enhancement by lipopolysaccharide and lack effect of HIV coat protein gp120 and depolarizing agents. Neurosci Lett *178*, 184-9.

Peterson, P.K., Hu, S., Salak, J.J., Molitor, T.W. and Chao, C.C. (1997). Differential production of and migratory response to beta chemokines by human microglia and astrocytes. J Infect Dis *175*, 478-81.

Petroni, A., Salami, M., Blasevich, M., Papini, N., Galella, G., Colombo, C. and Galli, C. (1994). Eicosanoid and inositol phosphate response to platelet-activating factor (PAF) and to a PAF antagonist in rat astroglial cells. Brain Res. Dev. Brain Res. *78,* 169-174.

Piani, D., Frei, K., Do, K., Cuenod, M. and Fontana, A. (1991). Murine brain macrophages induce NMDA receptor mediated neurotoxicity in vitro by secreting glutamate. Neurosci. Lett. *133,* 159-162.

Piani, D., Spranger, M., Frei, K., Schaffner, A. and Fontana, A. (1992). Macrophage-induced cytotoxicity of N-methyl-D-aspartate receptor positive neurons involves excitatory amino acids rather than reactive oxygen intermediates and cytokines. Eur J Immunol *22,* 2429-36.

Prehn, J.H. and Krieglstein, J. (1993). Platelet-activating factor antagonists reduce excitotoxic damage in cultured neurons from embryonic chick telencephalon and protect the rat hippocampus and neocortex from ischemic injury in vivo. J. Neurosci. Res. *34,* 179-188.

Prescott, S.M., Zimmerman, G.A. and McIntyre, T.M. (1990). Platelet-activating factor. [Review] [80 refs]. J. Biol. Chem. *265,* 17381-4.

Reiner, O., Carrozzo, R., Shen, Y., Whenert, M., Faustinella, F., Dobyns, W., Caskey, C. and Ledbetter, D. (1993). Isolation of a Miller-Dieker lissencephaly gene containing G protein β-subunit-like repeats. Nature *364,* 717-721.

Rhigi, M., Letari, O., Sacerdote, P., Marangoni, F., Miozzo, A. and Nicosia, S. (1995). *myc*-Immortalized microglial cells express a functional platelet-activating factor receptor. J. Neurochem. *64,* 121-129.

Saitoh, S., Iijima, N., Ikeda, M., Nakajima, K., Kimura, M., Katsuki, M., Mori, T. and Kohsaka, S. (1992). De novo production of α2-macroglobulin in cultured astroglia from rat brain. Mol. Brain Res. *12,* 155-161.

Shigemoto, R., Nakanishi, S. and Mizuno, M. (1992). Distribution of the mRNA for a metabolic glutamate receptor (mGluR1) in the central nervous system: an in situ hybridization study in adult and developing rat. J. Comp. Neurol. *322,* 121-135.

Snyder, F. (1989). Biochemistry of platelet-activating factor: a unique class of biologically active phospholipids. Proc. Soc. Exp. Biol. Med. *190,* 125-135.

Sogos, V., Bussolino, F., Pilia, E., Torelli, S. and Gremo, F. (1990). Acetylcholine-induced production of platelet-activating factor by human fetal brain cells in culture. J. Neurosci. Res. *27*, 706-711.

Stephenson, D.T. et al (1994). Calcium-sensitive cytosolic phospholipase A_2 ($cPLA_2$) is expressed in human brain astrocytes. Brain Res. *637*, 97-105.

Streit, W.J., Morioka, T. and Kalehua, A.N. (1992). MK-801 prevents microglial reaction in rat hippocampus after forebrain ischemia. Neuroreport *3*, 146-8.

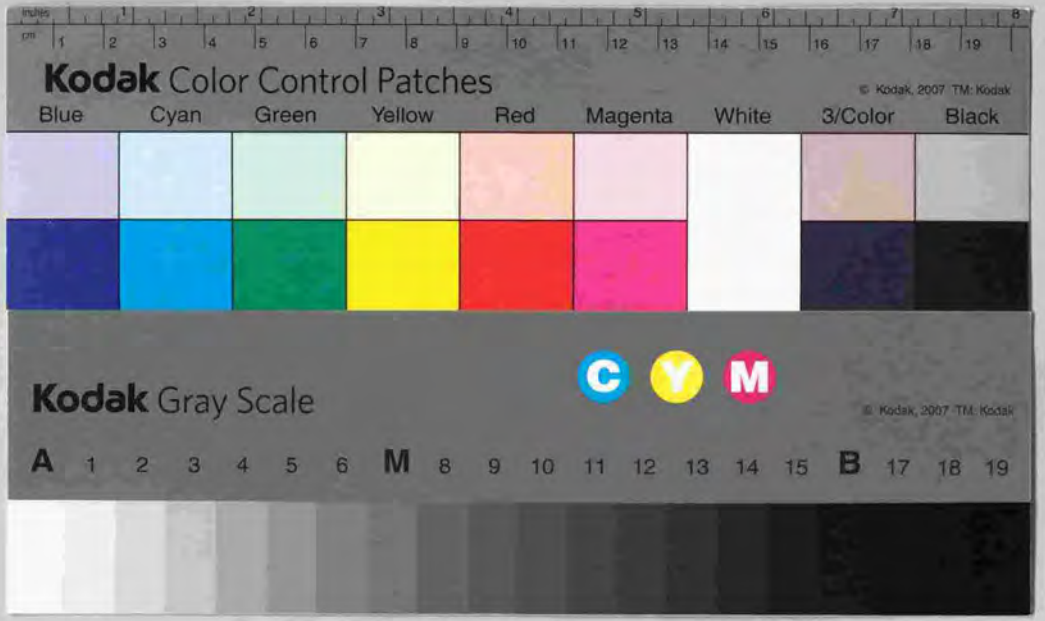
Tanabe, S. et al (1997). Functional expression of the CXC-chemokine receptor-4/fusin on mouse microglial cells and astrocytes. J Immunol *159*, 905-11.

Thomas, W. (1992). Brain macrophage: evaluation of microglia and the functions. Brain. Res. Rev. *17*, 21-74.

Waga, I., Kume, K., Ferby, I., Honda, Z. and Shimizu, T. (1996). Micro-trap phosphorylation assay of mitogen-activated protein (MAP) kinases to detect their activation by lipopolysaccharides. J. Immunol. Method *190*, 71-7.

Wieraszko, A., Li, G., Kornecki, E., Hogan, M.V. and Ehrlich, Y.H. (1993). Long-term potentiation in the hippocampus induced by platelet-activating factor. Neuron *10*, 553-557.

Yokomizo, T., Izumi, T., Chang, K., Takuwa, Y. and Shimizu, T. (1997). A G-protein-coupled receptor for leukotriene B4 that mediates chemotaxis. Nature *387*, 620-4.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