Predominant Expression of Platelet-Activating Factor Receptor in the Rat Brain Microglia

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Cellular localization of platelet-activating factor (PAF) receptor in the rat brain was determined by (1) *in situ* hybridization, (2) Northern blot analysis in primary cell cultures of neurons, microglia, astrocytes, and fibroblasts, and (3) Ca^{2+} imaging in hippocampal culture. *In situ* hybridization revealed that the PAF receptor mRNA is expressed intensely in microglia and moderately in neurons. Northern blot analysis revealed that PAF receptor mRNA is the most abundant in microglia. In primary hippocampal cultures, PAF elevated intracellular Ca^{2+} concentration in microglia and also in neurons, but to a lesser extent.

These results suggest predominant presence of PAF receptor in microglia. Cultured microglia also expressed cPLA_2 mRNA the most intensely. PAF-activated microglia released arachidonic acid in a Ca^{2+} -dependent manner and without conversion to its derivatives. We propose that microglia as well as neurons contribute to PAF-related events in the CNS by releasing arachidonic acid.

Key words: platelet-activating factor; PAF; receptor; mRNA; microglia; *in situ* hybridization; Ca^{2+} imaging; arachidonic acid

Platelet-activating factor (PAF) (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) was characterized originally as a potent activator of platelets; its actions include aggregation, morphological changes, and granule secretion (Benveniste et al., 1979; Blank et al., 1979; Demopoulos et al., 1979). This lipid mediator was shown later 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 CNS, PAF is involved in various events (Feuerstein et al., 1990; Frerichs and Feuerstein, 1990; Doucet and Bazan, 1992; Bazan, 1994). This factor is produced in the nervous system through application of acetylcholine (Sogos et al., 1990), dopamine (Bussolino et al., 1986), or convulsant electrical stimuli (Kumar et al., 1988). PAF receptor (PAFR) (Honda et al., 1991; Bito et al., 1994) is present functionally in brain tissues (Bito et al., 1992); PAFR antagonists suppress post-ischemic neuronal injury (Panetta et al., 1989; Gilboe et al., 1991; Prehn and Kriegelstein, 1993). It is also related to human immunodeficiency virus (HIV)-associated neuronal cell death (Genis et al., 1992; Epstein and Gendelman, 1993; Gelbard et al., 1994; Lipton, 1994; Lipton et al., 1994). It enhances excitatory synaptic transmission (Clark et al., 1992), induces long-term potentiation (LTP) in the hippocampus (Wieraszko et al., 1993; Kato et al., 1994), and increases memory test performance (Izquierdo et al., 1995). It activates intracellular signaling cascades such as phosphoinositide turnover (Murphy and Welk, 1990; Yue et al., 1992;

Petroni et al., 1994), rise in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (Kornecki and Ehrlich, 1988, 1991; Willard, 1992; Yue et al., 1992), and immediate-early gene expression (Squinto et al., 1989). More recently, PAF has been shown to play a role in the development of the CNS, because a subunit of PAF acetylhydrolase, a PAF-inactivating enzyme, has 99% homology with the protein encoded by the gene causing Miller-Dieker lissencephaly, a disorder characterized by the absence of gyri and sulci in the cerebral cortex (Reiner et al., 1993; Hattori et al., 1994).

Our previous study (Bito et al., 1992) showed that PAFR is expressed ubiquitously in the rat brain and functions in both neurons and non-neuronal cells in hippocampal cultures. Other studies showed that cultured astrocytes (Petroni et al., 1994) and microglia (Rhigi et al., 1995) respond to PAF. Thus, we assumed that PAFR in glia takes part in the PAF-related events in the CNS. In the present study, we attempted to localize PAFR in identified cell types of the rat brain by combining *in situ* hybridization and immunohistochemistry. We then identified PAF-responsive glial cells in primary hippocampal cultures by measurement of PAF-elicited rise in $[\text{Ca}^{2+}]_i$, followed by immunocytochemistry. We subsequently characterized PAF-induced release of arachidonic acid from microglia, which we found express the largest amount of PAFR mRNA. Our findings suggest a possible contribution of microglia to the PAF-related events in the CNS.

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MATERIALS AND METHODS

In situ hybridization. Sprague–Dawley rats (male, ~250 gm) were anesthetized deeply with diethyl ether and decapitated. Brains were removed quickly, dissected coronally or parasagittally, embedded in OCT compound, and frozen in isopentane at -30°C . Ten-micrometer-thick coronal and parasagittal sections were made on a cryostat, thaw-mounted on poly-L-lysine (PLL)-coated slide glass, and air-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 until use. Sections of rat

whole embryos, embryonic day 18 (E18), and pup brains (P0, P7, P14, and P21) were prepared in the same procedures.

In situ hybridization was carried out as described previously (Shigemoto et al., 1992), with several modifications. Briefly, a *PvuII-AvaI* fragment (0.9 kb) of the coding region of rat PAF receptor cDNA (Bito et al., 1994) was subcloned into pBluescript II (Stratagene, La Jolla, CA) and linearized by *Bam*HI digestion. A 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 10^5 cpm/ μ l. After hybridization at 55°C for 6 hr in a humid chamber, the sections were washed in 2× SSC at room temperature overnight and in 2× SSC at 60°C for 1 hr, and they were treated with RNase A (20 μ g/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× SSC at 67°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- β max (Amersham, Buckinghamshire, UK) or dipped in 1:1 diluted NTB-2 emulsion (Kodak, Rochester, NY). The exposure periods were 10 d for the film and 6–7 weeks for the emulsion. The hybridization signals were evaluated by counting the number of silver grains per cell in the sections processed in the same procedures.

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 marker (OX-42, anti-CR-3 complement receptor antibody) or an astrocyte marker [antiglial 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.

Cryostat sections (10 μ m 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 bovine serum albumin (BSA) (Sigma) in PBS for 5 min at room temperature. Incubation with the purified primary antibodies (1:200) was carried 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 (Vectastain 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 of the immunostaining procedures were carried out under RNase-free conditions.

Primary cell culture. Microglial cells were prepared from a primary culture of neonatal rat cerebral tissue, as described previously (Nakajima et al., 1989, 1992). The purity of microglia was estimated to be >99% (data not shown) by staining with fluorescein isothiocyanate (FITC)-labeled isolectin B₄ (Sigma).

Neurons were prepared from embryonal (E16) rat neocortical tissues as described previously (Takci et al., 1991). The purity of neuronal cells was estimated to be >99.5% (data not shown) by staining with two monoclonal antibodies against neurofilament and microtubule-associated protein 2 (MAP2) (Serotec).

Astrocytes were prepared from the brain of rat pups (P3) by the previously described method (Mori et al., 1990), with modifications. Nonastroglial cells were removed by shaking (MacCarthy and de Vellis, 1980) and subculturing twice. The final purity of astrocytes was estimated to be >99.5% (data not shown) by staining with anti-GFAP antibody. The maturity of neurons and astrocytes was confirmed by immunoreactivity of neurofilament/MAP2 and GFAP, respectively.

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).

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 thiocyanate/CsCl method (MacDonald et al., 1987). The RNA samples (10 μ g each) were electrophoresed in 1.2% agarose gel containing 7% formaldehyde and were 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 *PvuII-AvaI* fragment (0.9 kb) of rat PAF receptor 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× SSC containing 0.1% SDS at room temperature for 30 min and in 1× SSC 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 1 μ g of total RNA from each cell culture. Aliquots (1/50) of the cDNA samples were amplified with PCR using rat PAFR-specific primers: CCGCTGTGGATTGTCTATTA (upstream, 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.

Fluorometric imaging of $[Ca^{2+}]_i$ in 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 μ M Fura 2-AM (Dojin Chemicals, Kumamoto, Japan) in glutamate-free DMEM (Nissui, Tokyo, Japan) 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 and Cowan, 1977), with modifications. Briefly, 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 $150 \times g$ for 20 sec, and the cell pellet was resuspended in 0.3 mg/ml trypsin inhibitor (Gibco) and 40 μ g/ml DNase I in the above buffer. After gentle trituration with a Pasteur pipette, the cells were centrifuged at $150 \times g$ for 3 min. The cell pellet was resuspended in glutamate-free DMEM/10% horse serum supplemented with 0.1 mg/ml transferrin (Sigma), 16 μ g/ml putrescine (Sigma), 5 μ g/ml insulin (Wako Chemicals, Osaka, Japan), 20 nM progesterone (Sigma), and 30 nM Na_2SeO_3 (Sigma) (Bottenstein, 1985) and plated on polyethylene imine-coated coverslips with four-well silicon chambers (Flexiperm disk, Heraeus Biotechnology, Hanau, Germany) at 1.0×10^6 cells/well. The culture was maintained for 2 d in this medium and subsequently for 10–13 d 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 d. Fluorometric Ca^{2+} imaging was carried out as described above. Before PAF application, the cells were pretreated with 0.5 μ M 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 μ M NMDA (Wako Chemicals) in the HEPES-Tyrode buffer/0.1% BSA to identify neuronal cells.

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 0.1 mM each of $CaCl_2$, $MgCl_2$, and $MnCl_2$ at room temperature for 10 min and then in the same solution containing 10 μ g/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 washes with PBS, the samples were incubated with tetramethylrhodamine isothiocyanate (TRITC)-labeled anti-mouse IgG

Figure 1. Film-autoradiographic images showing *in situ* hybridization of PAF receptor mRNA in coronal (*a*, *b*) and parasagittal (*c*, *d*) sections of adult rat brain. Fresh-frozen rat brain sections (10 μ m) were hybridized with a 35 S-labeled antisense cRNA probe synthesized from a *Pvu*I-*Ava*I fragment (0.9 kb) of rat PAF receptor cDNA in the absence (*a*, *c*) or presence (*b*, *d*) of 100-fold excess unlabeled cRNA. The hybridized sections were autoradiographed to x-ray film for 8 d. For details, see Materials and Methods.

antibody (1:50; Serotec) in 10% horse serum/PBS for 30 min at room temperature. After three brief washes with PBS, the samples were then mounted in 50% glycerol containing 0.5% 1,4-diazabicyclo-[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 amoeboid, ramified, and reactive microglia and thus are suitable markers for detection of a wide variety of microglia (Thomas, 1992; Nakajima and Kohsaka, 1993).

Northern blot analysis for cPLA₂ in primary cell culture. One of the important outputs of PAFR activation is release of arachidonic acid (Honda et al., 1994). Its release depends mainly on activation of cytosolic phospholipase A₂ (cPLA₂) (Clark et al., 1991); however, it has not been clear which cells in the brain express cPLA₂. One report showed that astrocytes alone are stained with an anti-cPLA₂ antibody (Stephenson et al., 1994), but another showed cPLA₂ mRNA expression in neurons (Owada et al., 1994). Because PAF stimulates arachidonic acid release in astrocytes (Petroni et al., 1994) and a *myc*-immortalized microglial cell line (Rhigi et al., 1995), the presence of cPLA₂ was sought in primary cell culture systems. 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. I. Kudo, Showa University, Tokyo). The subsequent procedures were carried out as described above.

Immunoblot analysis for cPLA₂ in microglia. Microglial cells were isolated as described above (~2–4 $\times$ 10⁶ cells/75 cm² flask). The medium was aspirated, and the cells were washed with ice-cold PBS. The flasks were cooled in liquid nitrogen and stored at –80°C until use. The frozen cells were thawed and lysed by the addition of 200 μ l/flask lysis buffer containing protease inhibitors. The cell lysate was centrifuged at 15,000 $\times$ g for 10 min, and the resulting supernatant was treated with 5 $\times$ Laemmli buffer, boiled for 5 min, and subjected to 7.5% SDS-PAGE (20 mA/gel, 20 μ g protein/lane). After electrophoresis for 6 hr, the samples were transferred to a nitrocellulose membrane (Hybond-ECL, Amersham) at 5 mA/cm² for 30 min. The blot 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, Genetics 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).

Arachidonic acid release from PAF-stimulated microglia. Because microglia showed predominant expression of both PAFR and cPLA₂, the

release of arachidonic acid in PAF-stimulated microglia was characterized. Arachidonic acid release was measured in microglial culture prelabeled with [³H] arachidonic acid essentially as described (Lin et al., 1992). Microglial cells (~2 $\times$ 10⁶ cells/75 cm² flask) were incubated with 1 μ Ci/ml [³H] arachidonic acid (DuPont NEN) in DMEM/0.1% BSA for 18 hr at 37°C. After two washes with DMEM/0.1% BSA, the cells were incubated with 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 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 (Top Count, Packard, Meridian, CT), and A/(A+B) was calculated as a release ratio. Because arachidonic acid release by cPLA₂ is reported to depend on [Ca²⁺]_i (Clark et al., 1991; Kramer et al., 1991), [Ca²⁺]_i-dependence of PAF-stimulated release of arachidonic acid was evaluated in cultured microglia. The microglial cells loaded with [³H] arachidonic acid were preincubated in DMEM/0.1% BSA/1% dimethylsulfoxide (DMSO) with or without a [Ca²⁺]_i chelator, O,O'-bis(2-aminophenyl) ethylene glycol-N,N,N',N'-tetra-acetic acid, tetra-acetoxymethyl ester (BAPTA-AM, Dojin Chemicals), 20 μ M, 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 radioactivity of the medium and the cell lysate was determined in the liquid scintillation counter. Next, the cPLA₂ products released from PAF-stimulated microglia were analyzed, because arachidonic acid derivatives also constitute bioactive mediators in the CNS (Shimizu and Wolfe, 1990). Isolated microglia (~10⁷ cells) were loaded with [³H] arachidonic acid and incubated for 10 min at 37°C in the presence or absence of 10 nM PAF. Lipids were extracted from the conditioned medium and subsequently analyzed with a high-performance liquid chromatography (HPLC)/radio detector system (System Gold, Beckman, Fullerton, CA).

RESULTS

Ubiquitous expression of PAFR in rat brain

The *in situ* hybridization signals were ubiquitous but relatively intense in the cerebral cortex, olfactory bulb, pyramidal cell layer of the hippocampus, medial thalamus, hypothalamus, and granular cell layer of the cerebellum (Fig. 1). In the hippocampus, intense signals were scattered randomly, and moderate signals were found in the pyramidal cell layer and dentate gyrus (Fig. 2*a*). In the cerebral cortex, intense signals were scattered randomly in all of the layers, and moderate signals were found in layers II–VI, with relative concentration to layers III, IV, and VI (Fig. 2*b*). In

Figure 2. Dark-field photomicrographs showing *in situ* hybridization of PAF receptor mRNA in rat brain. *a*, Adult hippocampus; *b*, adult neocortex; *c*, adult cerebellum; *d*, the same photomicrograph as *c*, with a schematic showing each layer of the cerebellum; *e*, P7 hippocampus; *f*, P14 hippocampus; *g*, P21 hippocampus. *In situ* hybridization was carried out as described in the legend to Figure 1. The hybridized sections were emulsion-dipped and autoradiographed for 6–7 weeks. For details, see Materials and Methods. *W* in *b* represents the white matter. *M*, *G*, and the interrupted lines in *d* denote the molecular, granular, and Purkinje layers, respectively.

the cerebellum, intense signals were scattered randomly as in the hippocampus, and slight to moderate signals were found in the granular cell and Purkinje cell layers (Fig. 2*c,d*). The scattered distribution of the intense signals was evident throughout the whole brain in both gray and white matter. The signals were not highly concentrated in any specific brain areas or nerve nuclei. The intensity of the signals was constant in film autoradiographs among E18, P0, P7, P14, and P21 brains (data not shown). The distribution pattern of the signals was similar to that of the adult from P7 in the hippocampus (Fig. 2*e–g*) and also in the other brain regions (data not shown).

Predominant expression of PAFR in microglia in rat brain

The PAFR-expressing cells could be classified into at least two groups (Fig. 3*a,b*). One type of cells was densely labeled and had small nuclei that stained darkly with cresyl violet. Most of these nuclei were of angular or irregular shapes. The cells of this group were scattered ubiquitously and randomly in both gray and white matter. These findings suggest that microglia comprise this group (del Rio-Hortega, 1932; Vaughan, 1984). The other group of cells was labeled moderately and had larger nuclei that stained lightly. These cells were found most frequently in layers II–VI of the cerebral cortex but not in layer I. They were also found in the pyramidal cell layers of the hippocampus (Fig. 3*a*, indicated by an arrow). These findings suggest that the second group includes

neurons. No intense signals were found in cells with round and moderately stained nuclei, which characterize astrocytes (del Rio-Hortega, 1932; Vaughan, 1984). Table 1 summarizes the intensity of the hybridization signals as evaluated by the number of grains per cell. The number of grains on microglia were outstanding from P7 to adult, exceeding more than twice those on neurons.

Immunohistochemistry followed by *in situ* hybridization revealed that almost all of the densely labeled cells are OX-42-positive but anti-GFAP-negative in the adult rat brain (Fig. 3*c–f*). This indicated that microglia express PAFR mRNA the most intensely; however, because all of the OX-42-positive cells were not labeled, microglia apparently are heterogeneous in terms of PAFR mRNA expression. On the other hand, all of the anti-GFAP-positive cells were labeled only slightly, if at all. Weak or moderate signals were found in OX-42- and anti-GFAP-negative cells with large nuclei, which apparently were neurons (data not shown). The intensely labeled cells in the pup brain were also probably microglia. We could not confirm it successfully with immunohistochemistry, however, because immunostaining pup brain sections required longer incubation with the antibody, which diminished the signals of the following hybridization, possibly by degrading RNA.

Predominant expression of PAFR in cultured microglia

Northern blot analysis revealed that PAFR mRNA expression is predominant in microglia and hardly detectable in neurons, astrocytes, and fibroblasts (Fig. 4). In the RT-PCR analysis, the

Figure 3. Bright-field photomicrographs showing *in situ* hybridization of PAF receptor mRNA in adult rat hippocampal (*a*, *c*, *e*) and cortical (*b*, *d*, *f*) sections stained with cresyl violet (*a*, *b*), OX-42 (*c*, *d*), or anti-GFAP antibody (*e*, *f*). *In situ* hybridization and immunostaining were carried out as described in Materials and Methods. Intense labeling is found in cells with small and darkly stained nuclei that have angular or irregular shapes (*a*, *b*), and in OX-42-positive cells (*c*, *d*) but not in anti-GFAP-positive cells (*e*, *f*). The arrow in *a* shows the pyramidal cell layer. Scale bars: *a*, *b*, 20 μ m; *c*-*f*, 10 μ m.

Table 1. Expression levels of PAF receptor mRNA in the rat brain

Tissue	Number of grains per cell			
	P7	P14	P21	Adult
Microglia	++++	++++	++++	++++
Neuron				
Neocortex	+–++	++	++	++
CA1 (pyramidal cell layer)	++	++	++	++
Dentate gyrus	+	+	+	+

Number of grains per cell: +, <10; ++, 10–19; +++, 20–39; +++++, 40–80. P7, 7-d-old; P14, 14-d-old; P21, 21-d-old. Microglia and neurons were identified by the Nissl-staining characteristics as described in Materials and Methods.

specific PCR product (~0.38 kb) apparently was detected in the RNA from microglia (≥ 25 cycles), astrocytes (≥ 30 cycles), and neurons (35 cycles) but not in fibroblasts (35 cycles) (Fig. 5). The specificity of PCR amplification was confirmed by complete digestion of the products with *EcoRV*, whose restriction site was mapped in the middle of the amplified region of the PAF receptor cDNA (data not shown).

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

Isolated microglia

PAF (10 nM) elevated $[Ca^{2+}]_i$ in almost all (>95%) of the isolated microglial cells. Figure 6*a,b* shows imaging of $[Ca^{2+}]_i$ in four PAF-treated microglial cells and their temporal profiles, respectively.

Hippocampal culture

PAF (10 nM) elevated $[Ca^{2+}]_i$ in a small population of the cultured hippocampal cells. Not more than a few PAF-responsive cells were found in one observation field ($\sim 1 \times 1$ mm). This

Figure 4. Northern blot analysis of PAF receptor mRNA expression in primary cell culture of microglia, neurons, astrocytes, and fibroblasts prepared from rat brain. Total RNA (10 μ g) from cultured cells was loaded in each of the left four lanes, and poly (A)⁺ RNA (5 μ g) from adult rat spleen was loaded in the right lane. The blot membrane was hybridized with a [³²P] dCTP-labeled probe synthesized from the *PvuI-AvaI* fragment (0.9 kb) of rat PAF receptor cDNA. The bottom shows control hybridization with a β -actin probe. For details, see Materials and Methods.

Figure 5. RT-PCR analysis of PAF receptor mRNA in primary cell culture of microglia, neurons, astrocytes, and fibroblasts prepared from rat brain. The first-strand cDNA was synthesized from 1 μ g of total RNA from each cell culture. Aliquots (1/50) of the cDNA samples were amplified with PCR using rat PAFR-specific primers: CCGCTGTGGATTGTCTATTA (upstream, 5'-3') and AGGAGGTGATGAAGATGTGG (downstream, 5'-3') for the indicated number of cycles with a thermal cycle of 58°C, 72°C, 94°C (each for 1 min). The PCR products were electrophoresed in agarose gel and visualized by ethidium-bromide staining. ϕ X174 DNA-*HaeIII* digest was used as a DNA size marker.

finding was reproducible in >10 samples prepared from different embryos on different occasions. Immediately after fluorometry, the culture was stained simultaneously with isolectin B₄ for microglia and with anti-MAP2 antibody for neurons or an anti-GFAP antibody for astrocytes. More than 90% of the PAF-responsive cells were stained with isolectin B₄ but not with the anti-GFAP antibody, suggesting that they are mostly microglia. On the other hand, not all of the isolectin B₄-positive cells responded to PAF, suggesting that the microglia in these conditions are heterogeneous in terms of PAFR expression. This is compatible with the results of immunohistochemistry followed by *in situ* hybridization. Figure 6*c–e* shows an example of the fluorometric and immunocytochemical results. Isolectin B₄-negative cells only occasionally responded to PAF. These isolectin B₄-negative and PAF-responsive cells partly responded to NMDA and showed MAP2 immunoreactivity (Fig. 6*f–h*), suggesting that some neurons express functional PAFR. Also, a small portion of these cells showed GFAP immunoreactivity (data not shown), suggesting that astrocytes also express functional PAFR, albeit to a much smaller degree.

Predominant expression of cPLA₂ in cultured microglia

Northern blot analysis revealed that microglia predominantly express cPLA₂ mRNA (Fig. 7*a*). The hybridization signals for neurons, astrocytes, and fibroblasts are scarcely visible in the figure but were detectable in the original autoradiograph. Additionally, the immunoblot analysis demonstrated that cPLA₂ protein is present in microglia (Fig. 7*b*).

Arachidonic acid release from PAF-stimulated microglia

The arachidonic acid release from microglia was dose- and time-dependent. The optimal PAF concentration was 10 nM (Fig. 8*a*),

Figure 6. Fluorometric $[Ca^{2+}]_i$ imaging of PAF-treated microglial and hippocampal cultures. Fluorometric Ca^{2+} imaging and immunostaining were carried out as described in Materials and Methods. PAF (10 nM) was bath-applied for 1 min. *a*, $[Ca^{2+}]_i$ of four isolated microglial cells 20 sec after PAF application; *b*, time courses of $[Ca^{2+}]_i$ in the cells in *a*; *c*, $[Ca^{2+}]_i$ of hippocampal culture 20 sec after PAF application; *d*, the same culture stained with isolectin B₄; *e*, the same culture stained with anti-GFAP; *f*, $[Ca^{2+}]_i$ of another hippocampal culture 20 sec after PAF application; *g*, $[Ca^{2+}]_i$ of the same culture 20 sec after NMDA application; *h*, the same culture stained with anti-MAP2. In one hippocampal culture (*c-e*), one of the two isolectin B₄-positive cells (*large arrow*) responded to PAF, whereas the other (*small arrow*) did not. Note that the former cell is anti-GFAP-negative. In another culture (*f-h*), two (*arrowheads*) of the four NMDA-responsive and MAP2-positive cells responded to PAF, whereas the other two (*asterisks*) did not. These four cells were OX-42-negative (data not shown). The corresponding $[Ca^{2+}]_i$ is indicated by the color scale shown in *b*.

Figure 7. *a*, Northern blot analysis of cytosolic phospholipase A₂ (cPLA₂) mRNA expression in primary cell cultures of microglia, neurons, astrocytes, and fibroblasts prepared from rat brain. The blot membrane used in hybridization with the PAFR probe was rehybridized with a cPLA₂ probe. *b*, Immunoblot analysis of cPLA₂ in primary cell culture of microglia. For details, see Materials and Methods.

and the net (treated minus untreated) release reached a plateau in 10 min (Fig. 8*b*). Preincubation with BAPTA-AM decreased the arachidonic acid release by ~50%, indicating that the release is partially dependent on $[Ca^{2+}]_i$ (Fig. 8*b*). Fluorometric Ca^{2+} imaging confirmed that the BAPTA-AM treatment suppressed 10 nM PAF-elicited rise in $[Ca^{2+}]_i$ in microglia prepared on a coverslip (data not shown). In the HPLC/radiodetector analysis of the conditioned media of the microglia, all the radioactivity ($\sim 2 \times 10^4$ cpm for PAF-treated microglia and $\sim 1 \times 10^4$ cpm for untreated microglia) was eluted with the same retention time as standard arachidonic acid. No radioactivity peaks corresponding to prostaglandins or hydroxyicosatetraenoic acids (HETEs) were detected in the medium of microglia incubated with or without 10 nM PAF

Figure 8. Dose–response relationship (*a*) and Ca^{2+} -dependent time course (*b*) of arachidonic acid release from PAF-treated microglia. *a*, Microglial cells prelabeled with [3H] arachidonic acid were incubated with 10 nM PAF for 10 min. *b*, Microglial cells prelabeled with [3H] arachidonic acid were preincubated with 20 μ M BAPTA-AM/1% DMSO or 1% DMSO only, and then incubated with or without 10 nM PAF for the indicated time. For details, see Materials and Methods. The mean and SD are shown ($n = 3$).

(data not shown). Because this system detects 100 cpm radioactivity peaks, these results indicate that >99% of the released arachidonic acid remained unmetabolized.

DISCUSSION

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 animal and primary cell culture systems. The results strongly suggest predominant presence of PAF receptor 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 have a large number of PAF receptors (Hayashi et al., 1991; Ring et al., 1993) and 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, 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. Another is that one microglial cell may change the level of PAFR mRNA 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- κ B 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.

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.

Predominant expression of cPLA₂ in microglia

In our primary cell culture systems, cPLA₂ was predominantly expressed in microglia, a finding in contrast to two previous

reports. One showed that anti-cPLA₂ antibody exclusively stains astrocytes in the rat brain (Stephenson et al., 1994), and the other showed by *in situ* hybridization that cPLA₂ mRNA is expressed in rat neurons after ischemia (Owada et al., 1994). These discrepancies may be ascribed to the differences between the brain and cell culture and between mRNA and protein. Nevertheless, cPLA₂ in microglia deserves further attention, and the pathophysiological relevance of its function and transcriptional regulation needs to be evaluated.

Release and metabolism of arachidonic acid in PAF-treated microglia

PAF-treated microglia efficiently released arachidonic acid, a finding compatible with the predominant PAFR and cPLA₂ expression in microglia. The optimal concentration of PAF for arachidonic acid release was 10 nM. The same concentration is also optimal for arachidonic acid release in CHO cells stably expressing the guinea pig PAFR gene (Honda et al., 1994). The release of arachidonic acid decreased by ~50% after treatment with an intracellular Ca²⁺ chelator, BAPTA-AM. The Ca²⁺-dependence of PAF-induced arachidonic acid release agrees with the finding that cPLA₂ is activated when translocated to the membrane by a rise in [Ca²⁺]_i (Clark et al., 1991). The increase in PLA₂ activity in the absence of Ca²⁺ rises might suggest the presence of Ca²⁺-independent PLA₂ in microglia, although we presently have no direct evidence for its presence. The released arachidonic acid was scarcely metabolized to its derivatives, e.g., HETEs, leukotrienes, and prostaglandins. These findings are in contrast to those obtained in peripheral macrophages and compatible with the recent report that PAF treatment of a *myc*-immortalized microglial cell line causes arachidonic acid release but no detectable production of 6-keto prostaglandin F₁α and leukotriene B₄ (Rhigi et al., 1995).

Possible involvement of microglia in PAF effects on CNS

Accumulated evidence has shown that PAF plays crucial roles in both physiological and pathological conditions in the CNS: PAF modulates synaptic transmission (Clark et al., 1992), induces LTP (Wieraszko et al., 1993; Kato et al., 1994), and contributes to neuronal cell injury (Panetta et al., 1989; Gilboe et al., 1991; Prehn and Kriegstein, 1993). These PAF effects have been assumed as direct actions of PAF on neurons. Our findings, however, suggest that PAF-stimulated microglia also may be responsible at least in part for the known effects of PAF on neurons.

Microglia are known to affect neurons by releasing mediators, including neurotransmitters and neuromodulators, by phagocytosis, and by displacement of synapses (synaptic stripping) (Banati et al., 1993; Nakajima and Kohsaka, 1993). In the case of PAF-activated microglia, one can speculate about the possible involvement of the following neurotransmitter/modulators in altering synaptic transmission. (1) Glutamate: This excitatory amino acid is released from activated microglia and causes neuronal cell death (Piani et al., 1991). Whether it is released from PAF-activated microglia should be clarified in an additional study. (2) Arachidonic acid: This lipid messenger potentiates NMDA receptor currents (Miller et al., 1992), induces LTP (Williams et al., 1989; Kato et al., 1991), and inhibits the high-affinity glutamate uptake system in synaptosomes and astrocytes (Barbour et al., 1989; Volterra et al., 1992). The present study revealed that PAF-stimulated microglia release arachidonic acid without notable conversion to its derivatives. (3) NO: This gaseous mediator

induces LTP (Dawson and Snyder, 1994) and is also involved in microglia-mediated nerve cell injury (Boje and Arora, 1992; Chao et al., 1992; Merrill et al., 1993). Whether PAF-activated microglia release NO remains to be clarified in a future study. (4) PAF: Microglia may release PAF itself, because PAF activates the cPLA₂ activity in microglia. If this were the case, PAF could serve as an autacoid messenger, thus forming a positive feedback loop. Simultaneous increase in both arachidonic acid and PAF may have synergistic effects on synaptic transmission. In addition to these mediators, there is a possibility that PAF-activated microglia release potentially cytotoxic substances such as free oxygen radicals, proteolytic enzymes, inflammatory cytokines, and unidentified neurotoxins. Indeed, microglia/macrophages, when activated by zymosan (Giulian et al., 1993b), neuronal injury (Giulian et al., 1993a), HIV infection (Giulian et al., 1990), or gp120 protein (Lipton et al., 1991; Lipton, 1992) release neurotoxic factor(s) that act presumably through activation of NMDA receptors. We are now evaluating neurotoxicity of PAF-stimulated microglia and determining whether such neurotoxins are involved. On the other hand, phagocytosis and neuronal stripping by PAF-activated microglia have not been reported. Nevertheless, PAF injection into the mouse hippocampus induces morphological activation of microglia (Andersson et al., 1992). Because PAF (Panetta et al., 1989; Gilboe et al., 1991; Prehn and Kriegstein, 1993) and phagocytosis by microglia (Lees, 1993) both contribute to delayed neuronal cell death after ischemia, it is possible that microglia act as phagocytes to neurons in response to PAF produced after ischemia.

The present findings suggest the possibility that the PAF-related events in the CNS arise not only from neuron–neuron interactions but also from a complex web of interactions between neurons and glia, in particular, microglia. To understand the roles of PAF in this web, one should first answer the following questions. (1) What are the outputs of PAFR activation in microglia and other cells? (2) Which cells, under which conditions, produce PAF? (3) How does PAF interact with other mediators, including NO and arachidonic acid? Monocytes-astroglia interactions reportedly produce PAF, arachidonic metabolites, and cytokines in HIV infection (Genis et al., 1992; Gelbard et al., 1994). Furthermore, one should also answer the question about whether there are any PAF receptors other than the PAFR that was cloned in our laboratory, because the presence of distinct PAF binding sites in intracellular membranes has been reported (Bazan et al., 1993; Bazan, 1994). Some of these critical questions will be answered shortly when PAFR-deficient mice are established and their phenotypes are analyzed.

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