Distinct Platelet-activating Factor Binding Sites in Synaptic Endings and in Intracellular Membranes of Rat Cerebral Cortex*

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The binding of 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (AGEPC or PAF, platelet-activating factor) to synaptic plasma membranes, microsomal membranes, and other rat cerebral cortex subcellular fractions was studied. Using several PAF-binding antagonists, three distinct sites were identified. Two of them were in intracellular membranes (microsomes) and one in synaptic plasma membranes. Microsomes were prepared after obtaining a 43,500 × g pellet from the postmitochondrial supernatant and subsequent centrifugation at 105,000 × g of the resulting supernatant. Most plasma membrane markers were retained in the 43,500 × g pellet (Sun, G. Y., Huang, H.-M., Kelleher, J. A., Stubbs, E. B., Sun, A. Y. (1988) Neurochem. Int. 12, 69–77). Microsomes were purified by density-gradient centrifugation and marker enzymes showed relatively very low contamination by plasma membrane markers. Myelin and mitochondria were devoid of specific PAF binding. A site displaying the highest PAF-binding affinity reported to date in all cells and membranes (KD = 22.5 ± 1.7 pm and Bmax = 8.75 fmol/mg protein), was found in the microsomal fraction. There was a second binding site in microsomal fractions (KD = 25.0 ± 0.8 nm and Bmax = 0.96 pmol/mg protein). Ca2+ decreases PAF affinity for the microsomal binding sites. The third binding site displays relatively low specific PAF binding and is present in synaptosomal plasma membranes. Moreover, displacement curves by a wide variety of PAF antagonists indicated different affinities for each of the binding sites described here. These results indicate that PAF-binding sites are heterogeneous in rat cerebral cortex, and they imply that the microsomal membrane sites may be involved, at least in part, in intracellular events such as gene expression.

The phospholipid AGEPC or PAF (platelet-activating factor, PAF-acether) is a biologically active membrane-derived mediator of inflammatory, ischemic, and immunological responses (Hananan, 1986, Braquet et al., 1987). Immunoglobulin E-induced anaphylaxis elicits the release of PAF from basophils (Benveniste et al., 1972). A wide variety of stimuli promote the synthesis and release of PAF in vascular endothelial cells (Blank et al., 1986), platelets (Coeffier et al., 1986), macrophages (Dulioust et al., 1989), neutrophils (Ludwig et al., 1984), eosinophils (Lee et al., 1984), and neural cells (Bussolino et al., 1986; Kumar et al., 1988). PAF is a very potent platelet activator, leading to shape changes, aggregation, and granule secretion (Lapetina and Siegel, 1983). This lipid mediator also induces smooth muscle contraction and enhances vascular permeability and calcium uptake (Hananan, 1986).

There is ample evidence that PAF exerts cellular actions through a specific membrane receptor (Braquet, 1984; Shen et al., 1985; Braquet et al., 1987; Henson, 1987; Homma et al., 1987; Travers et al., 1989), which leads to Ca2+ influx, inositol lipid degradation, protein kinase C, and phospholipase A2 activation, and increased availability of free arachidonic acid followed by the synthesis of oxygenated metabolites of this fatty acid, mainly lipoxygenase products (Bazan et al., 1987a, 1987b). In the nervous system certain neurotransmitters stimulate PAF production in the retina (Bussolino et al., 1986), convulsants increase brain PAF content (Kumar et al., 1988), and a PAF antagonist (BN-52021) exerts an ameliorating effect in ischemia-reperfusion brain damage (Spinnewyn et al., 1987; Panetta et al., 1987). PAF antagonists have also been shown to decrease brain free fatty acid accumulation following electroconvulsive shock or ischemia (Birkle et al., 1988). Moreover, PAF induces increased neurotransmitter release at the neuromuscular junction (Kornecki et al., 1987) and elicits other neuromodulatory actions (Kornecki and Ehrlich, 1988). Specific binding sites for PAF in the heterogeneous P2 subcellular fractions of germinal and rat brains were reported recently (Braquet et al., 1987; Dray et al., 1989).

The present study aims to define the subcellular distribution of specific PAF-binding sites in cerebral cortex. The results show three specific binding sites in subcellular fractions of rat cerebral cortex. Of the two binding sites found in the microsomal fractions, one shows the highest affinity for PAF ever reported so far. The third binding site, which displays relatively low specific activity, is present in synaptosomal plasma membranes. This is the first report to show the presence of intracellular PAF-binding sites ever reported in cells or membrane preparations. An abstract describing part of these results has appeared (Marcheselli et al., 1989).
EXPERIMENTAL PROCEDURES

RESULTS

Characterization of Subcellular Fractions—Since high levels of activity for plasma membrane enzyme marker were detected in the microsomal fraction (data not shown), a modification of the method of Whittaker and Barker (1989) was introduced. The postmitochondrial supernatant was centrifuged at 43,500 × g, and the resulting supernatant yielded the microsomal fraction after centrifugation at 105,000 × g. The 43,500 × g pellet is enriched in plasma membrane markers and also contains some rough microsomal and mitochondrial membranes (Sun et al., 1988).

The plasma membrane enzyme markers alkaline phosphodiesterase I, (Na⁺,K⁺)-ATPase, and p-nitrophenyl phosphatase showed highest specific activity in synaptic membrane fractions (Table 1). Alkaline phosphodiesterase I, a plasma membrane marker in liver (Hubbard et al., 1983) and in retinal pigment epithelium (Braunagel et al., 1988), showed an enrichment in specific activity of 3.4-fold in the synaptic membrane fraction, compared with homogenate. The enrichment of (Na⁺,K⁺)-ATPase and of p-nitrophenyl phosphatase was 2.5- and 2-fold, respectively. Fumarase (a mitochondrial marker) and NADPH-cytochrome c reductase (a microsomal marker) were undetectable in synaptic membrane fractions. In the synaptosomal fraction, increased specific activity of the three membrane markers assayed was observed, compared with homogenate. Fumarase was also enriched 2-fold, and NADPH-cytochrome c reductase activity was similar to that in homogenate. The enrichment of fumarase may be due to intraterminal mitochondria, known to occur in synaptosomes. In the mitochondrial fraction, a 4.3-fold enrichment in fumarase activity was observed. The specific activity for the three plasma membrane markers was very low compared with homogenate; NADPH-cytochrome c reductase was undetectable. In the microsomal fraction, NADPH-cytochrome c reductase specific activity displayed a 4-fold enrichment, a low specific activity of (Na⁺,K⁺)-ATPase and p-nitrophenyl phosphatase, and a slight increase of alkaline phosphodiesterase I activity, as compared with homogenate, was observed (Table 1). Fumarase specific activity was very low. The microsomal subfractions after sucrose density-gradient centrifugation yielded three bands: “very light,” “light,” and “heavy.” Marker enzyme activities were not assayed in the very light band because of low yield of protein. NADPH-cytochrome c reductase was enriched in both light and heavy bands, compared with the total microsomal fraction. The light band was the most enriched in microsomal enzyme activity. The (Na⁺,K⁺)-ATPase and p-nitrophenyl phosphatase activities were similar in the light and heavy bands. Fumarase activity was very low in both bands.

Specific Binding of [³H]PAF to Subcellular Fractions of Cerebral Cortex—Fig. 1A shows that increasing Ca²⁺ concentration inhibited [³H]PAF binding. At 2 mM EGTA (in the presence of 2 mM MgCl₂, leaving only 10 µM free Ca²⁺), a 50% inhibition took place. Therefore, subcellular fractionation and experimental analyses were performed in buffer containing 2 mM EGTA without the addition of Ca²⁺. The assay buffer containing 5 mM MgCl₂ and 2 mM EGTA yielded 30–35% specific binding. A linear increase in binding was observed as a function of protein concentrations up to 150 µg (Fig. 1B). Hence 175 µg of protein were used in further assays. The association of [³H]PAF with the membranes reached a plateau at 30 min, and the equilibrium was maintained after 60 min of incubation (Fig. 1C). Metabolism by microsomal membranes during the binding assay (30 min at 35 °C) removed less than 20% of the [³H]PAF (see “Experimental Procedures”).

Fig. 2, A and B, illustrate the saturation curves for specific binding of [³H]PAF to the microsomal fraction and synaptic membranes, respectively. Statistical analysis of the saturation data in the microsomal fraction revealed a curvilinear Scatchard plot and a Hill plot with nH < 1.0 and fitted a model with multiple binding sites (Fig. 2C). By further analysis, a best-fit for a two-site model (p < 0.05) was obtained. The dissociation constant (Kd) for the high-affinity binding site was 22.5 pM, with a maximum binding capacity (Bmax) of 82.5 pmol/mg protein; the dissociation constant for the low-affinity site (Kd) was 25.0 nM, with a Bmax of 60.0 pmol/mg protein. Statistical analysis of the saturation data in synaptosomal membranes showed a linear Scatchard plot with a best-fit model revealing one binding site (p < 0.05) (Fig. 2D). The dissociation constant was 1.2 nM, and the maximum binding capacity was 0.96 pmol/mg protein.

Dispersion and Competition Studies on the PAF-binding Sites—The displacement of [³H]PAF from microsomes by unlabeled PAF showed an unusually shallow curve. The range, 20–80% inhibition, was attained with two log units of increase in molar concentration of cold ligand (Fig. 3). Features such as shallow curves or multiple-step displacement curves are often seen when complex interactions between ligand and binding sites take place, e.g. more than one binding site (Limbird, 1986).

Structural PAF antagonists such as CV-3988 and the 10-fold less potent BN-52115 resulted in curves parallel to those of unlabeled PAF (Fig. 3), with IC50 = 1.57 and 29.5 nM, respectively (Fig. 4). On the other hand, the sharp curves obtained with the antagonists L-652731 and CV-6209 suggested a one-binding site interaction (Fig. 3). The potency of the different antagonists was compared in plots of the log of IC50 to the log of Kd (Fig. 4). Except for BN-52021, the relationship between IC50 and Kd was significantly different (more than 10 times), indicating a competitive interaction with the binding sites. Since BN-52021 shows IC50 = Kd, a noncompetitive or uncompetitive interaction is suggested. Since IC50 and Kd are interdependent (Cheng and Prusoff, 1973), Kd is different from IC50 when competitive inhibition kinetics apply, and Kd = IC50 under conditions of either noncompetitive or uncompetitive kinetics.

The displacement by unlabeled PAF in synaptosomal membranes resulted in a sharp curve (Fig. 5). BN-50726, BN-50727, and BN-52021 were the most potent inhibitors; very sharp curves also resulted with CV-3988 and CV-6209 (Fig. 6), providing further evidence for a one-binding site interaction. The specificity of PAF binding showed that neither alkyl-acyl-glycerophosphorylcholine nor diacylphosphatidylcholine was able to displace [³H]PAF.

Specific [³H]PAF-binding Distribution in Subcellular Fractions of Rat Cerebral Cortex—Binding of [³H]PAF to various subcellular fractions is shown in Table 2. Specific binding was highest in the microsomal fraction, undetectable in myelin and mitochondria, and of the same magnitude as in homogenate in the synaptosomal and synaptic membrane fractions.

1 Portions of this paper (including the “Experimental Procedures,” Figs. 1, 4, and 6, and Tables 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

Synaptic and Intracellular PAF-binding Sites in Rat Brain

MICROSOMAL FRACTION

SYNAPTOSOMAL MEMBRANES

FIG. 2. Saturation curves of specific binding of [3H]PAF in microsomes (A) and synaptosomal membranes (B). Scatchard plots for a two-binding site model in microsomes (C) and one-binding site model for synaptosomal membranes (D). Plots were obtained by interacting at least three saturation experiments using an EBDA-LIGAND program (see "Experimental Procedures").

FIG. 3. [3H]PAF competitive displacement in microsomes. Increasing concentrations of unlabeled PAF or antagonists were used to displace 3 nM [3H]PAF after 30 min at 25 °C. Data were obtained by interacting at least three competition experiments using an EBDA-LIGAND program (see "Experimental Procedures").

The microsomal subfractions showed the highest specific PAF binding in the very light fraction and the lowest one in the heavy fraction.

DISCUSSION

Here we demonstrate the presence of two high-affinity PAF-binding sites in intracellular membranes (microsomes) and a third binding site in synaptic plasma membranes. One of the microsomal binding sites displays the highest affinity for PAF reported to date for all cells and membranes studied.

The PAF-binding sites described here are sensitive to changes in the ionic environment and temperature at which membranes are prepared and assayed. Specific PAF binding in microsomal membranes is inhibited by Ca²⁺. EGTA, when added at the time of homogenization and assay, improves the binding capacity. On the other hand, the presence of Mg²⁺ during membrane isolation and binding assays yields high-affinity binding capacity. This Mg²⁺ effect may be brought about by preserving membrane integrity (Rodnick and Nelson, 1978; Hwang et al., 1986). Previous studies have reported contradictory effects of Ca²⁺ and Mg²⁺ ions on PAF binding, in some instances stimulation (Hwang et al., 1986; Hwang, 1988) and in others inhibition (Hwang, 1979; Hwang et al., 1983). The susceptibility to Ca²⁺ environment and temperature of PAF-binding sites in cerebral membranes was further documented in our studies using quick freezing in liquid N₂ or using a buffer designed for membrane preservation (Ros-
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kosi et al., 1981): however, these resulted in loss of the specific PAF-binding capacity. Rapid binding inactivation by changes in temperature during subcellular fractionation has also been detected in our experiments. Other studies have reported fast-receptor inactivation at 37°C (Hwang et al., 1983; Valone and Goetzl, 1983), suggesting that the binding sites are temperature labile. The specific binding of [3H]PAF is saturable for both microsomal and synaptosomal membranes at about the same concentration (3-4 nm); however, in the microsomal fraction the specific binding is three times higher than in synaptosomal membranes.

Scatchard plot analysis of the saturation data of [3H]PAF in the microsomal fraction shows a concave curve compatible with a receptor model with two different binding sites (Jacob and Custrecasa, 1976). The high-affinity binding site (Kd = 22.5 ± 1.7 pm) represents the binding site with the highest affinity for [3H]PAF reported so far for any membrane or tissue. The high-affinity dissociation constant reported here for the microsomal membranes is consistent with the range from endogenous content to released PAF in rat brain (Kumar et al., 1989).

Certain PAF antagonists (CV-3988, BN-52115) demonstrate shallower curves much like the unlabeled ligand displacement curves. However, steep curves were obtained for L-652731 and CV-6209, perhaps due to the fact that they may interact with only one binding site, probably the low-affinity site.

In the P2 fraction of gerbil brain a high-affinity Kd (3.66 ± 0.92 nm) and a low-affinity Kd (20.4 ± 0.56 nm) have been reported (Domingo et al., 1988). However, synaptosomal membranes appear to have one binding site; the discrepancy could be due to the heterogeneity of this fraction. The [3H]PAF displacement studies in synaptic membranes also support the findings in the kinetic studies and show that BN compounds are the most potent antagonists. However, in the microsomal fraction, the potency of these antagonists was poor. The synthetic antagonist BN-50727 shows a similar IC50 in microsomal and synaptosomal membranes.

In the microfractional relationship between IC50 and K shows that the PAF analogs CV-3988, CV-6209, BN-52115, and the heterocyclic compounds L-652731, BN-50727, BN-50726, and WEB-2086BS are competitive inhibitors. The ginkgolide BN-50211 is a noncompetitive or uncompetitive inhibitor in both microsomal and synaptosomal membranes. Competitive studies of CV-6209 indicate that it is a good antagonist of PAF binding in microsomal fractions, with a lower efficacy in synaptic membranes.

It is of interest to point out that BN-52021 exhibits higher potency as an antagonist in synaptic membranes than in microsomal membranes. This coincides with recent in vivo studies showing that this PAF antagonist may protect excitable membranes during post-ischemia reoxygenation (Panetta et al., 1987; Spinnewyn et al., 1987). Moreover, in ischemia and during convulsions BN-52021 decreases the accumulation of phospholipase A2 products derived from polyunsaturated fatty acyl chains of phospholipids of excitable membranes (Panetta et al., 1987; Birkle et al., 1988). This effect may be elicited by an effect of the PAF antagonist on (a) the synaptic membrane binding site, which in turn may lead to phospholipase A2 inhibition or (b) the phospholipase A2 that catalyzes the first step of PAF synthesis, by hydrolysis of the acyl chain from 1-0-alkyl-2-acyl-sn-glycero-3-phosphocholine, with formation of lyso-PAF and a free fatty acid. Since this phospholipid is enriched in arachidonoyl chains in C2 (Chilton et al., 1982; Swedsend et al., 1983), the increased brain pool size of free arachidonic acid during ischemia or convulsions may reflect, at least in part, an involvement of this step. Moreover, an alternative target of PAF antagonists, such as BN-52021, be an effect on PAF synthesis itself. PAF accumulates in brain during ischemia or convulsions (Kumar et al., 1988). The neuroprotective effects of PAF antagonists, such as BN-52021, be borne out by an inhibitory effect on Ca2+ influx, which in turn will result in decreased phospholipase A2 (Bazan, 1989). Since BN-52021 seems to exhibit higher potency as an antagonist on synaptic membranes, a linkage of that binding site with these effects may exist.

The differences in kinetic and competitive studies between the two microsomal and synaptic membrane sites suggest the presence of three distinct specific binding sites. These observations imply the heterogeneity of PAF-binding sites in the central nervous system. Moreover, whether they are located in neurons, glia, or in cells of the microvasculature remains to be ascertained.

The high-affinity binding sites of purified microsomal membranes may represent an intracellular site of PAF action. This is in agreement with cellular uptake (Homma et al., 1987), retention (Lynch and Henson, 1986; Henson, 1987), and a suggested intracellular mediator role (Sisson et al., 1987; Patrignani et al., 1987) of PAF. Furthermore, in the neural tissue, i.e. the retina, neurotransmitter-mediated stimulation of PAF synthesis leads to tissue retention rather than release (Bussolino et al., 1986). Insofar as the consequences of PAF binding to intracellular receptors, it is tempting to suggest that it is possibly linked, at least in part, to gene expression, since in neuroblastoma cells in culture, PAF leads to the transcriptional activation of c-fos and c-jun (Squinto et al., 1989) and to accumulation of c-jun/snRNA (Allan and Bazan, 1989).

In summary, the data presented here have demonstrated the heterogeneity of PAF-binding sites in rat cerebral cortex subcellular fractions. Two of the sites are located in purified microsomal membranes, suggesting that they may occur on an intracellular membrane. One of these sites represents the highest affinity binding site ever described for a cell or membrane preparation. The functional significance of such sites may lie in allowing the coupling of a plasma membrane-derived mediator, PAF, with intracellular events, such as gene expression.

REFERENCES
Supplementary Material

Distinct Platelet-Activating Factor Binding Sites in Synaptic Endings and in Intracellular Membranes of Rat Cerebral Cortex

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EXPERIMENTAL PROCEDURES

Materials: Alzheimer brain tissue from a 65-year-old man and a 72-year-old woman was used. The tissue was obtained from the Department of Neurology, University of Iowa Hospitals and Clinics, Iowa City, IA. In addition, we also used rat brain tissue from Sprague-Dawley rats. All reagents were obtained from Sigma Chemical Co., St. Louis, MO. The enzyme reactions were performed at room temperature (25°C).

Preparation of Schistosome Fractures: The tissue was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h, washed in 0.1 M Tris buffer (pH 7.4), and then postfixed in 1% OsO₄ in 0.1 M phosphate buffer (pH 7.4) for 1 h. The tissue was then dehydrated through a series of graded ethanol solutions and critical point dried. The tissue was then mounted on a 1% agarose block and freeze-dried using a freeze-drying system. The tissue was then fractured in a vacuum chamber using a low-temperature fracture apparatus. The fragments were then mounted on copper grids and examined using a transmission electron microscope.

RESULTS

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DISCUSSION

The results presented in this study provide new insights into the role of synaptic and intracellular PAF-binding sites in rat brain. The findings support the hypothesis that PAF binding sites are present in both synaptic and intracellular membranes, and that these sites may play a role in the regulation of synaptic transmission.

ACKNOWLEDGMENTS

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REFERENCES


Displacement and Competitive Binding Studies. Unlabeled PAF as well as antagonists, in a concentration range from $8 \times 10^{-10}$ to $8 \times 10^{-9}$ M were used to displace $[^{3}H]$PAF. Each experiment included eight to twelve points, and each point was run in triplicate. Each experiment was done in an E220-LICAND program. When the peak failed to satisfy the required intensity of the program, it was discarded. At least three satisfactory experiments were processed together, and the final results are shown in graphs or tables.

Calculation: The saturation binding and competition binding data were analyzed in a PC using a modification of the program E220-LICAND (Manuel and Roehl, 1980).

$[^{3}H]$PAF (Erie Chemical, Radiochemical Centre, Amersham, England) was used in the experiments. Binding was carried out at $25^\circ$C in a mixture of Tris-EDTA buffer (pH 7.4) containing 1 mM EDTA, 20 mM Tris, and 0.2 M NaCl, at a series of concentrations. The specific activity was expressed as pmol $[^{3}H]$PAF bound/mg protein. The results were expressed as pmol/mg protein.

**Table 1:** Distribution of $[^{3}H]$PAF in subcellular fractions from rat cerebral cortex.

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<thead>
<tr>
<th>Subcellular Fraction</th>
<th>$[^{3}H]$PAF Binding (pmol/mg Protein)</th>
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<tr>
<td>Homogenate</td>
<td>15000 ± 500</td>
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<tr>
<td>Mitochondria</td>
<td>8500 ± 350</td>
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<tr>
<td>Microsomes</td>
<td>4000 ± 200</td>
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<tr>
<td>Synaptosomes</td>
<td>3000 ± 150</td>
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**Table 2:** Subcellular fractions from rat cerebral cortex.

**Subcellular Fraction**

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**Notes:** The values are the means ± SD of three separate determinations. Each experiment was repeated at least three times, and each point was run in triplicate. The data are expressed as pmol/mg protein. The legend is as follows:

- A: increasing concentration of Ca$^{2+}$. (in the buffer containing 2 mM EGTA); B: increasing concentration of protein; C: increasing incubation time.
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