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# Production of Platelet-activating Factor by Chick Retina*

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**In the present study it is demonstrated that platelet-activating factor (PAF) was produced by chick retinas, upon stimulation with neurotransmitters such as acetylcholine (ACh), dopamine, or with calcium ionophore A23187, but not upon stimulation with γ-amino-*n*-butyric acid, L-glycine, L-glutamate, epinephrine, or histamine. PAF produced in response to ACh, dopamine, or A23187 was not released into supernatants but was extractable from retinas. The amounts of extractable PAF increased after sonication of stimulated retinas. While no PAF activity could be recovered from unstimulated retinas, small amounts of this lipid can be detected following sonication of the tissue. The amount of extractable PAF from ACh-, dopamine-, or A23187-stimulated retinas was dependent upon the incubation time and concentration of the agonists. PAF was identified on the basis of chemical and lipase treatments, biological activity with washed rabbit platelets, behavior on thin layer chromatography, and high pressure liquid chromatography. Control cell preparations (leukocytes, erythrocytes, and embryogenic fibroblasts) did not produce PAF upon neurotransmitter stimulation.**

**ACh and dopamine promoted PAF production by increasing dithiothreitol-insensitive cholinephosphotransferase activity, without affecting the acetyltransferase activity. In contrast, the A23187 ionophore stimulated the acetyltransferase activity but did not affect the dithiothreitol-insensitive cholinephosphotransferase.**

PAF,[1] a highly potent lipid mediator of inflammation, belongs to a recently discovered class of "autacoids," namely the acetylated alkyl phosphoglycerides (1, 2). PAF was originally described as a product released from antigen-stimulated IgE-sensitized rabbit basophils (3, 4). Later, it was shown that, after appropriate stimulation, PAF can be released from a variety of inflammatory cells such as neutrophils, monocytes, macrophages, platelets, and endothelial cells (5–7).

Two specific enzymatic reactions have been documented to be involved in PAF biosynthesis. 1-*O*-Alkyl-2-lyso-*sn*-glycero-3-phosphocholine(1-*O*-alkyl-2-lyso-GPC):acetyl-CoA acetyltransferase catalyzes the acetylation of inactive lyso-PAF into the bioactive PAF (8–11), whereas 1-*O*-alkyl-2-acetylglycerol (AAG):CDP-choline cholinephosphotransferase transfers the phosphobase from CDP-choline to AAG (9, 12).

Alkyl ether phosphoglycerides, which are the precursors of PAF, are distributed in all animal tissues (13). Furthermore, certain tissues (spleen, liver, kidney, and heart) are able to synthesize and release PAF (5–7). However, it is unknown whether the central nervous system shares this property, even though alkyl ether phosphoglycerides form a large portion of the total phospholipids content of the brain (13).

The chick retina is composed of only nervous and glial cells (Mueller cells) but is devoid of vascular tissue (14). Therefore, the chick retina can be used conveniently to study cells of the central nervous system. In addition, the chick retina contains considerable amounts of alkyl ether phosphoglycerides (15).

In the present study, we demonstrate that PAF is synthesized in the chick retina in response to neurotransmitters via a dithiothreitol-insensitive cholinephosphotransferase and in response to calcium ionophore A23187 via an acetyltransferase.

## EXPERIMENTAL PROCEDURES

*Buffers*—The composition of Tris-Tyrode's buffer (TT) was (mM): KCl, 2.6; $MgCl_2$, $6H_2O$, 1; NaCl, 137; $CaCl_2$, $6H_2O$, 1; Tris, 1; glucose, 5. When indicated, 0.25% bovine serum albumin (BSA) was added (TT-BSA). The solution was buffered to pH 7.4 with 1 N NaOH. Phosphate-buffered saline (PBS) was composed of 150 mM NaCl and 10 mM $H_3PO_4$, pH 7.4.

*Materials*—The chemicals used and their sources were as follows: PAF (1-*O*-octadecyl-2-acetyl-GPC) and lyso-PAF (1-*O*-octadecyl-2-lyso-GPC) from Bachem Feinchemikalien (Bubendorf, Switzerland); acetyl-CoA, BSA (fraction V), L-α-phosphatidylcholine (PC) from bovine brain, L-α-lysophosphatidylcholine (lyso-PC) from bovine liver, sphingomyelin from bovine brain, triolein standard solution, phospholipase A2 from pig pancreas, lipase A1 from *Rhizophus arrhizus*, lipase from porcine pancreas, pyruvate kinase from rabbit muscle, lactate dehydrogenase from rabbit muscle, NADH, ATP, sodium cholate, isotridecanol polyglycol ether, phosphoenolpyruvate, 2,4-dichlorophenol, dithiothreitol, butylated hydroxytoluene, deoxycholate, acetylcholine (ACh), dopamine, γ-amino-*n*-butyric acid (GABA), L-glycine, L-glutamate, histamine, epinephrine, heparin, rhodamine 6G, EDTA, and EGTA from Sigma; calcium ionophore A23187, trypsin from bovine pancreas, glycerol kinase from *Bacillus stearothermophilus*, and phospholipase D from cabbage from Boehringer Mannheim GmbH (Mannheim, Federal Republic of Germany); (*RS*)-2-methyl-3-(octadecylcarbamoxyloxypropyl-2-(3-thiazolio)ethyl phosphate (CV-3988) from Takeda Chemical Ind. (Osaka, Japan); *p*-bromodiphenacyl bromide from Farmitalia (Milano, Italy); 1-*O*-[$^3$H]alkyl-2-acetyl-GPC ([$^3$H]PAF, 120 Ci/mmol), 1-[$^{14}$C]palmitoyl-2-lyso-GPC (55 mCi/mmol), CDP-methyl-[$^{14}$C]choline, ammonium salt (59 mCi/mmol), [$^3$H]acetyl-CoA (1 Ci/mmol; the specific activity was adjusted by addition of unlabeled acetyl-CoA); 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phospho[methyl-$^{14}$C]choline (55 mCi/mmol), and OCS from Amersham Int. (Bucks, United Kingdom); pyridine,

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[1] The abbreviations used are: PAF, platelet-activating factor (1-*O*-octadecyl-2-acetyl-GPC); AAG, 1-*O*-alkyl-2-acetylglycerol; ACh, acetylcholine; BSA, bovine serum albumin; CV-3988; (*RS*)-2-methyl-3-(octadecylcarbamoyloxy)propyl-2-(3-thiazolio)ethyl phosphate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GABA, γ-amino-*n*-butyric acid; GPC, *sn*-glycero-3-phosphocholine; HPLC, high pressure liquid chromatography; lyso-PAF, 1-*O*-octadecyl-2-lyso-GPC; PC, L-α-phosphatidylcholine; lyso-PC, L-α-lysophosphatidylcholine; PBDB, *p*-bromodiphenacyl bromide; PBS, phosphate-buffered saline; TLC, thin layer chromatography; TT, Tris-Tyrode's buffer; TT-BSA, TT supplemented with 0.25% BSA.

TLC plates (60F254), Silica Gel G, acetic anhydride, and dimethyl sulfoxide from Merck (Darmstadt, Federal Republic of Germany). All culture reagents were purchased from Flow Laboratories (McLean, VA); the plastic flasks and the Petri dishes came from Falcon Labware (Division of Becton Dickinson, Oxnard, CA). All solvents used for the extraction and characterization of the lipids were graded and contained 50 mg/liter butylated hydroxytoluene. High pressure liquid chromatography (HPLC) grade solvents (Merck) were filtered before use through Millex-SR filters (0.5-$\mu$m diameter, Millipore, Bedford, MA). Before use, all lipids were chromatographed using chloroform/methanol/water (65:36:6, v/v) as solvent system. A23187 was solubilized in dimethyl sulfoxide as 5 mM stock solution and then in TT containing 0.1% BSA. PBDB was diluted as stock solution (0.01 mM) in methanol by heating at 60 °C. In preliminary experiments, the final concentration of dimethyl sulfoxide (0.2%) and of methanol (0.1%) did not interfere with the experiments. CV-3988 was solubilized immediately before use in 150 mM NaCl by heating at 60 °C and then buffered to pH 7.4 with 1 N NaOH (16, 17). AAG was prepared by phospholipase C hydrolysis of PAF according to Waku *et al.* (18). Briefly, 2 mg of PAF in 1 ml of diethyl ether and 0.1 M Tris-HCl buffer, containing 1 mM $CaCl_2$, pH 7.4 (1:1, v/v), was added to 0.3 mg of phospholipase C and incubated at 22 °C for 18 h in stirring. The ether layer was dried under $N_2$, and lipids were separated by TLC on Silica Gel G plates prepared in 4% boric acid using chloroform/methanol (98:2, v/v) as solvent. The plates sprayed with 0.025% rhodamine 6G in ethanol showed a unique spot. The lipid was eluted from the silica with hexane/diethyl ether (1:1, v/v), dried under $N_2$, and resuspended in acetone. The concentration of AAG was calculated by measuring the glycerol by a spectrophotometer method (19) at 339 nm by using a Eppendorf photometer (model 1101, Eppendorf Geratebau Netheler, Federal Republic of Germany). The reaction mixture contained: the sample (5 $\mu$l), 92.2 mM Tris, 27.7 mM $MgCl_2$, 7.4 mM sodium cholate, 1.85 g/liter, isotridecanol polyglycol ether, 2.8 mM 2,4-dichlorophenol, 0.22 mM NADH, 0.09 mM ATP, 0.28 mM phosphoenolpyruvate, 2.0 units/ml lipase, 3.0 units/ml pyruvate kinase, 2.8 units/ml lactate dehydrogenase. The reaction was started by adding 1.3 units/ml glycerol kinase. The test was read after 20 min. Triolein solution was used as standard. In order to prevent the possible isomerization of the molecule, AAG was prepared the day before use. 1-[$^{14}$C]Palmitoyl-2-acetyl-GPC was prepared by treating 200 $\mu$Ci of dried 1-[$^{14}$C]palmitoyl-2-lyso-GPC with 2 ml of acetic anhydride and 2 ml of pyridine for 16 h at room temperature under constant stirring (20, 21). The reaction was then stopped by placing the tubes in an ice bath and adding 1.8 ml of cold water and 2 ml of methanol. The organic layer was recovered, dried under $N_2$, and the purity of the preparation was checked by TLC as described above.

*Preparation of the Retinas and Other Cell Populations*—Three-day-old Leghorn chicks were used. After decapitation, retinas were dissected free of the pigmented epithelium and of the pecten in cold TT (4 °C). After centrifugation (100 × *g*, 10 min, 4 °C), the retinas were placed in cold TT-BSA (22). Fibroblasts were obtained from 11-day-old chick embryos according to Hunter (23). Briefly, after decapitation, the embryos were minced in 2–3-mm square pieces. The tissue was washed three times with TT in order to remove red cells and was then incubated at room temperature for 30 min in 10 ml of 0.25% trypsin in PBS. Pieces of tissue were allowed to settle, and the supernatant was placed in 30 ml of 199 medium containing 20% fetal calf serum. After centrifugation (300 × *g*, 10 min, 22 °C), the cells were resuspended in 199 medium containing 10% fetal calf serum and plated in a 25-cm$^2$ flask (1.5 × $10^6$ cells). Subcultures were obtained in 35-mm diameter plastic Petri dishes.

Blood was obtained from 3-day-old chicks by cardiac puncture, collected in heparin (5 units/ml blood), and then centrifuged at 50 × *g*, for 15 min at 22 °C to obtain leukocyte-rich thrombocyte-free plasma (24). The leukocyte-rich plasma was centrifuged (400 × *g*, 10 min, 22 °C), and the cells were washed three times in TT-BSA without $Ca^{2+}$ and $Mg^{2+}$ (400 × *g*, 10 min, 22 °C).

Erythrocytes were purified from the same blood by four washings in TT-BSA without $Ca^{2+}$ and $Mg^{2+}$ and removal of residual buffy coat cells from each pellet (24).

*PAF Production*—Chick retinas were stimulated at 37 °C for different intervals of time with varying doses (0.1–100 $\mu$M) of ACh, dopamine, L-glycine, L-glutamate, GABA, histamine, epinephrine, or A23187 (1 $\mu$M) in 1 ml of TT-BSA. For each experimental point, two retinas have been used (range 1.8–3.3 mg of protein). In some experiments, retinas were preincubated for 10 min at 37 °C with PBDB (100 nM) or with acetyl-CoA (10–100 $\mu$M) or in 1 ml of TT-BSA without $Ca^{2+}$ containing 0.1 mM EGTA and then stimulated as above. As controls, leukocytes (1 × $10^5$ to 1 × $10^7$), red cells (hematocrit 50%), and fibroblasts (2.1 × $10^6$ ± 2.2 × $10^4$/Petri dish, mean ± S.D. of 6 experiments) were stimulated in 1 ml of TT-BSA with the above mentioned stimuli at 37 °C for 15 min. The incubation was stopped immediately by adding 5 mM EDTA (final concentration) and placing the tubes in an ice bath followed by centrifugation (800 × *g*, 10 min, 4 °C). In the experiments with the fibroblasts, the TT-BSA was removed, and the cells were detached with a rubber policeman in the presence of 1 ml of 150 mM NaCl containing 5 mM EDTA. Lipid extraction was performed according to the procedure of Bligh and Dyer (25) in which formic acid was added to lower the aqueous phase to pH 3.0 ± 0.1 (26) on: 1) unstimulated and stimulated retinas and control cell populations before or after sonication (Labsonic 1510, B. Braun Melsungen AG, Melsungen, Federal Republic of Germany) (3 pulses, 5 s, 100 watts, in ice bath; in preliminary experiments, sonication under these conditions caused complete cell lysis as assessed by phase contrast light microscopy); in some experiments, sonication was performed during lipid extraction; 2) cell-free supernatants from stimulated and unstimulated cell populations. To check overall recovery of PAF, 0.01 $\mu$Ci of [$^3$H]PAF was added to the supernatants or to the tissues. The extracted lipid material was submitted to TLC with chloroform/methanol/water (65:35:6, v/v) as solvent system. Commercially available PAF, lyso-PC, and sphingomyelin used as standards migrated with a $R_F$ 0.21, 0.11, and 0.29, respectively. The lipid material, having an $R_F$ from 0.15 to 0.24 was extracted by incubating the silica with chloroform/methanol/water (1:2:0.8, v/v) (27) for 20 min at room temperature while rotating. This procedure was repeated five times. The extracted lipids were used for biological assay and characterization as described below. The recovery of 0.1 $\mu$Ci of [$^3$H]PAF from TLC plates was 97.2 ± 1.8% (mean ± S.D. of 3 experiments).

Alternatively, the solvents employed for the TLC analysis were: chloroform/methanol/water (65:35:4, v/v; $R_F$ of PAF, lyso-PC, and sphingomyelin was 0.10, 0.08, and 0.20, respectively), chloroform/methanol/acetic acid/water (25:15:4:2, v/v; $R_F$ of PAF, lyso-PC, and sphingomyelin was 0.17, 0.12, and 0.22, respectively), and chloroform/methanol/ammonium hydroxide 28% (65:35:4, v/v; $R_F$ of PAF, lyso-PC, and sphingomyelin was 0.15, 0.19, and 0.24, respectively) (27, 28).

*PAF Assay*—PAF was detected by aggregation of washed rabbit platelets (29). The amount of PAF was expressed in ng/mg of protein and calculated over a calibration curve of standard PAF constructed for each test. The specificity of platelet aggregation was inferred from the inhibitory effect of 5 $\mu$M CV-3988 (16, 17).

Protein concentration was measured according to Lowry *et al.* (30). The total protein content of retina was 1.2 ± 0.3 mg (mean ± S.D. of 8 experiments).

*PAF Characterization*—TLC-purified PAF from each sample was resuspended in 0.2 ml of chloroform/methanol (1:1, v/v) and further characterized by its retention time on an HPLC apparatus (Beckman Instruments, Inc., Palo Alto, CA) equipped with a $\mu$Porasil column (Millipore Chromatographic Division, Waters, Milford, MA). Elution was carried out with chloroform/methanol/water (60:55:5, v/v) at a flow rate of 1.0 ml/min (31). Standard PAF (retention time, 20 min), PC (retention time, 10 min), and lyso-PC (retention time, 26 min) was used as reference. The recovery of 0.05 $\mu$Ci of [$^3$H]PAF was 91.3 ± 2.5% (mean ± S.D. of 4 experiments). The fractions were dried under $N_2$, resuspended in TT-BSA (0.5 ml), and the activity bioassayed on washed rabbit platelets as described above.

The material, eluted from the HPLC column and active on washed rabbit platelets, was compared as to its physicochemical characteristics (27, 28) and sensitivity to lipases (33) to standard PAF and to PAF derived from IgE-sensitized rabbit basophils prepared as previously described (27, 32).

Base-catalyzed methanolysis was performed in 2 ml of 0.03 N NaOH dissolved in methanol with an incubation of 5 min at 22 °C, followed by acidification of the sample to pH 6.5 ± 0.1 with 3 N HCl. Acidic treatment of PAF was accomplished for 3 h at 22 °C by incubating the samples dissolved in 1 ml of chloroform/methanol (9:1, v/v) to which 1 volume of 0.03 N HCl was added. PAF dissolved in 1 ml of chloroform/methanol (9:1, v/v) was reacted with 0.5 volume of 28% ammonium hydroxide for 30 min at 22 °C under stirring, followed by acidification to pH 6.5 ± 0.1 with 1 N HCl. After the chemical treatment, the lipids were extracted according to Bligh and Dyer (25).

The treatment with the different lipases was performed on the dried samples. The assay mixture for phospholipase A2 contained 1 ml of Tris-buffered 150 mM NaCl, pH 8.0, containing 10 mM $CaCl_2$.

Before adding 0.03 mg of phospholipase A2, the mixture was sonicated (100 watts, 5 min, in ice bath). The reaction was performed at 37 °C for 1 h. Lipase A1 hydrolysis was accomplished by sonicating the sample (100 watts, 5 min, in ice bath) in 1 ml of 0.1 M borate buffer, pH 6.5, containing 10 mM $CaCl_2$, 1 mM deoxycholate, and 0.4% BSA, then incubating the mixture with 0.2 mg of enzyme for 22 h at 22 °C under stirring. Phospholipase C treatment was performed as described below according to Waku *et al.* (18). Phospholipase D (250 mg) was washed alternatively with cold acetone and diethyl ether (10,000 × *g*, 10 min, 4 °C), resuspended in 12.5 ml of 0.1 M sodium acetate buffer, pH 5.6, and centrifuged at 10,000 × *g*, for 15 min at 4 °C (34). The reaction mixture contained: the dried sample, 0.5 ml of the solution of phospholipase D (1 mg) in 2 ml of 0.1 M sodium acetate buffer, pH 5.6, containing 50 mM $CaCl_2$ and 4 ml of diethyl ether. The mixture was dispersed by sonication (100 watts, 5 min, in ice bath). The reaction was performed at room temperature under stirring for 6 h (34, 35). After the lipase treatment, the lipids were extracted according to Bligh and Dyer (25). As control, [$^3$H]PAF (0.5 μCi) was submitted to the chemical as well as to lipase A1, phospholipase A2 and C treatments as described above, then extracted according to Bligh and Dyer (25) and submitted to TLC (solvent system: chloroform/methanol/acetic acid/water, 50:25:8:4, v/v). Finally, radioactivity of the lipids comigrating with standard PAF ($R_F$ 0.32), lyso-PAF ($R_F$ 0.20), and AAG ($R_F$ 0.94) was counted. 1-*O*-Alkyl-2-acetyl-*sn*-glycero-3-phospho[methyl-$^{14}$C]choline (0.5 μCi) was submitted to phospholipase C and D treatment as described above. Since phospholipase C and D hydrolysis releases the phosphocholine or the choline, in water-soluble form, respectively, the radioactivity was counted both in the organic and inorganic phases, without submitting the lipids to TLC. [$^3$H]PAF was hydrolyzed (96–98%) to [$^3$H]lyso-PAF by phospholipase A2 and base-catalyzed methanolysis and to [$^3$H]AAG (93–95%) by phospholipase C. Lipase A1 as well as acidic and weak base conditions did not cause the hydrolysis of [$^3$H]PAF (93–96% radioactivity recovered at $R_F$ of standard PAF). The treatment of 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phospho[methyl-$^{14}$C]choline with phospholipase C and D allowed the recovery of 92–96% and 89–95% of radioactivity in the organic phase, respectively.

After base-catalyzed methanolysis or phospholipase $A_2$ treatment, lipids were acetylated as described by others (20, 21). After lipid extraction, the biologically active material was tested on rabbit washed platelets and compared to untreated controls (29).

*Enzyme Assay*—To test acetyltransferase activity, two retinas for each experimental point were stimulated with 50 μM ACh or dopamine, or with 1 μM A23187 for different times (see "Results"). The reaction was stopped by centrifugation (800 × *g*, 10 min, 4 °C). The tissues were placed in 1 ml of 0.25 M sucrose containing 1 mM dithiothreitol and then sonicated in an ice bath (3 pulses, 5 s, 100 watts). The standard reaction mixture (8) contained 40 μM lyso-PAF, 200 μM [$^3$H]acetyl-CoA (0.5 μCi), 40 μg of protein in 0.5 ml of 0.1 M Tris-HCl, pH 6.9. The reaction was allowed to continue for 15 min at 37 °C. The enzymatic activity was linear as a function of lysate protein (up to 90 μg) and the incubation time (up to 30 min). The reaction was stopped with 3 ml of chloroform/methanol (1:2, v/v), and the lipids extracted were submitted to TLC (solvent system: chloroform/methanol/water, 65:35:6; v/v). The layer was scraped in 0.5-mm increments, and radioactivity counted in OCS scintillation liquid. The radioactivity corresponding to the $R_F$ of standard PAF (0.21) was used to measure the enzymatic activity. The results were corrected for radioactivity losses during lipid extraction and TLC purification using 1-[$^{14}$C]palmitoyl-2-acetyl-GPC as internal standard in both cases.

To test cholinephosphotransferase activity, the retinas were processed in the same way as for acetyltransferase, with the only difference that they were placed and sonicated in 0.25 M sucrose in 10 mM Tris-HCl, pH 7.4. The reaction was performed for 20 min at 37 °C in 1 ml of 0.1 M Tris-HCl, pH 8.0, containing 0.5 mM EGTA, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 μCi of CDP-methyl-[$^{14}$C]choline in 10 μl of ethanol, 5 μM AAG, 80 μg of protein (12). The enzymatic activity was linear as a function of the concentration of lysate protein (up to 160 μg) and of the incubation time (up to 40 min).

## RESULTS

*PAF Production*—The amounts of PAF recovered from either intact or sonicated chick retinas challenged with various compounds are given in Fig. 1. PAF could be extracted from intact retinas challenged with ACh, dopamine, or A23187, but not with L-glycine, GABA, L-glutamate, epinephrine, and histamine. The amounts of PAF recovered from retinas were similar with the three different agonists (Fig. 1). When stimulated retinas were sonicated before or during the lipid extraction, the recovery of PAF increased up to 57.3 ± 6.1% (mean ± S.D., 5 experiments). There was no statistically significant difference between values obtained with sonication before or during lipid extraction ($p < 0.5$). Sonication of unstimulated retinas or retinas stimulated with GABA, L-glutamate, L-glycine, histamine, or epinephrine allowed the extraction of very small amounts of PAF (Fig. 1). No PAF activity was detected in the supernatants of stimulated and unstimulated retinas (data not shown). The recovery of added [$^3$H]PAF from the supernatants and from the intact retinas ranged from 94.3 to 96.2% after TLC purification. Sonication performed before or during the lipid extraction did not modify the recovery of [$^3$H]PAF (93.1 ± 2.3 and 95.6 ± 1.8, mean ± S.D. of 3 experiments, respectively).

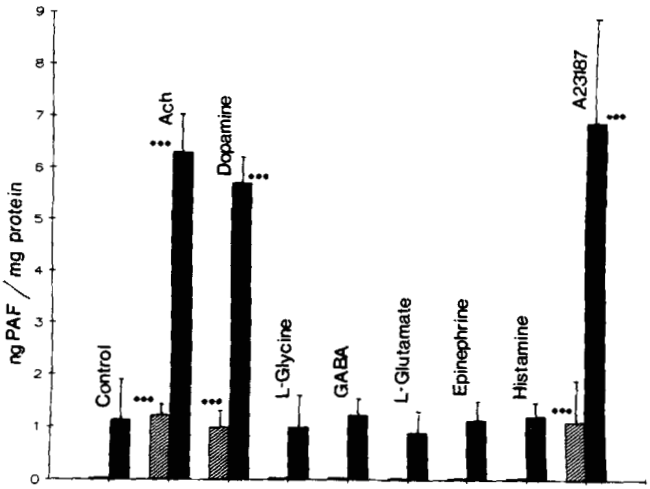


FIG. 1. **PAF production by stimulating chick retinas.** Retinas were stimulated at 37 °C for 20 min with 50 μM of different neurotransmitters and 1 μM A23187 in 1 ml of TT-BSA. Incubation was stopped by adding 5 mM EDTA followed by centrifugation (800 × *g*, 10 min, 4 °C). The lipid extraction was performed according to Bligh and Dyer (25), in which formic acid was added to lower the aqueous phase to pH 3.0 ± 0.1 (26), on intact retinas (*hatched bar*), on sonicated retina before the lipid extraction (*black bar*) (3 pulses, 5 s, 100 watts, in ice bath), and on supernatants (data not shown). The sonication was alternatively performed during the lipid extraction after the addition of the organic solvent to the aqueous mixture with the following results (ng of PAF/mg of protein): control, 1.21 ± 0.90; ACh, 5.93 ± 1.41; dopamine, 5.90 ± 0.92; L-glycine, 1.34 ± 0.53; GABA, 1.43 ± 0.90; epinephrine, 1.23 ± 0.50; histamine, 1.14 ± 0.30; A23187, 6.72 ± 2.50. No PAF activity could be recovered in the supernatants from unstimulated and stimulated intact retinas. The results are the means ± S.D. of 5 experiments. $^{**}p < 0.01$ *versus* values obtained in untreated retinas (Student's *t* test).

Fig. 2 illustrates the time course of PAF production by sonicated chick retinas stimulated with ACh, dopamine, or A23187. The addition of ACh or dopamine to the retinas resulted in a rapid production of PAF, which reached a peak after 20 min followed by a decline to basal values within 50–60 min. In contrast, A23187 induced a slower PAF production that reached its maximum at 40 min.

As shown in Fig. 3, the amounts of PAF produced by sonicated retinas were dependent upon the concentration of ACh or dopamine.

Preincubation of retinas with PBDB, a phospholipase $A_2$ inhibitor, prevented the production of PAF after A23187, but not after ACh or dopamine stimulation. EGTA completely blocked the action of the three agonists. Acetyl-CoA enhanced

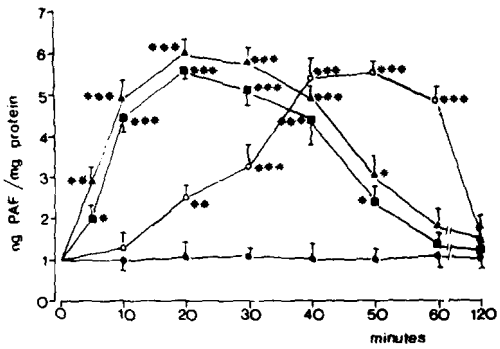


FIG. 2. **Time course of PAF production from sonicated chick retinas.** Retinas in 1 ml of TT-BSA were incubated at 37 °C for various intervals of time in the presence of ACh (50 μM, ■), dopamine (50 μM, ▲), A23187 (1 μM, ○), or without agonists. The reaction was stopped by adding 5 mM EDTA and centrifugation (800 × *g*, 10 min, 4 °C). Lipid extraction was performed after sonication (3 pulses, 5 s, 100 watts, in ice bath) as described under "Experimental Procedures." The results are the mean ± S.D. of 7 experiments. *, $p < 0.05$; **, $p < 0.02$; ***, $p < 0.01$ *versus* the control data obtained at the same time (Student's *t* test).

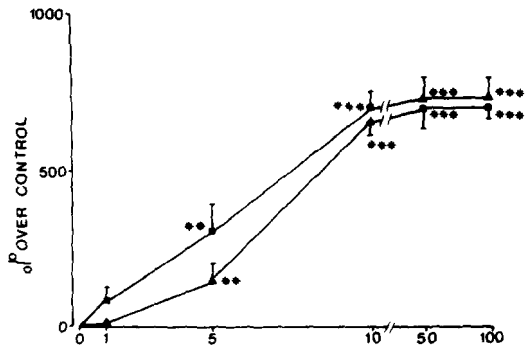


FIG. 3. **PAF production from sonicated chick retinas as a function of ACh and dopamine concentrations.** Retinas were stimulated for 20 min at 37 °C in 1 ml of TT-BSA with different concentrations of ACh (■) and dopamine (▲). Experimental conditions are as described in the legend to Fig. 1. Values for % control were based on the average of 5 untreated control (1.2 ± 0.7 ng of PAF/mg of protein). The results are the mean ± S.D. of 5 experiments. **, $p < 0.002$, ***, $p < 0.001$ *versus* the control data (Student's *t* test).

in a concentration-dependent manner the production of PAF by retinas stimulated by A23187 but not by ACh or dopamine (Table I). No such enhancement was observed when using unstimulated retinas. These results were similar to those obtained in stimulated intact retinas (data not shown).

In control cell preparations (leukocytes, red blood cells, and fibroblasts) stimulated with ACh, dopamine, L-glutamate, L-glycine, histamine, epinephrine, and GABA, PAF could not be demonstrated in the cell-free supernatants or in the cells themselves either before or after sonication. A23187 induced the PAF production only from leukocytes ($1 \times 10^7$ leukocytes/ml produced 5.1 ± 1.3 ng in the supernatants and 3.2 ± 1.6 ng in the sonicated cells; $1 \times 10^6$ leukocytes/ml produced 1.7 ± 0.9 ng in the supernatants and undetectable amounts in the cells after sonication; mean ± S.D. of 3 experiments).

*PAF Characterization*—PAF obtained from intact or sonicated retinas shared with standard PAF the same migratory properties on TLC (solvent system: chloroform/methanol/water, 65:35:6, v/v) ($R_F$ 0.21) between lyso-PC ($R_F$ 0.11) and sphingomyelin ($R_F$ 0.29). No PAF activity was detected in any other TLC fraction. Using solvent systems with different pH (neutral, acidic, and basic), the $R_F$ of PAF from retinas was similar to that of standard PAF, indicating its neutral nature. However, the $R_F$ of PAF was significantly altered by using instead of chloroform/methanol/water (65:35:6, v/v) ($R_F$ 0.21), chloroform/methanol/water (65:35:4, v/v) ($R_F$ 0.10). Thus, the behavior on TLC with solvents of different polarity indicated that PAF extracted from chick retinas was a polar lipid.

By HPLC, TLC-purified PAF was eluted in two distinct peaks, both having the same biological activity on washed rabbit platelets (*peak A*, retention time, 15 min; and *peak B*, retention time, 20 min, coeluting with standard PAF) (Fig. 4). PAF activity of both peaks was destroyed after base-catalyzed methanolysis (0–1% recovered activity of peak A and 0–3% of peak B) or after treatment with phospholipase $A_2$ (0–3% recovered activity of peak A and 1–3% of peak B) indicating the presence of an ester linkage at *sn*-2 (27–29, 33, 36). The treatment of both peaks with lipase A1 did not inhibit the PAF activity suggesting the presence of an ether bond at *sn*-1 (100% recovered activity for both peaks) (29, 33, 36). The presence of an alkenyl ether bond at *sn*-1 is unlikely, because the acidic condition had no effect on the PAF activity of peaks A and B (96–100% recovered activity of peak A and 95–100% activity of peak B) (27–29). Phospholipase C (5–12% recovered activity of peak A and 3–7% of peak B) and phospholipase D (3–6% recovered activity of peak A and 5–7% of peak B) treatments inactivated both peaks, thereby suggesting the presence of a polar head group at *sn*-3 (33, 36). In fact, in a variety of phospholipids, phospholipase C and D hydrolyze the glycerophosphate ester bond and the phosphoester bond between the phosphatidic acid and the alcoholic group, respectively. The resistance of peak A (95–97% recovered activity) and B (93–95% recovered activity) to treatment with a weak base suggested that they did not contain

TABLE I

*Effect of different chemicals on PAF production by stimulated chick retinas*

Retinas were preincubated for 10 min at 37 °C with PBDB, acetyl-CoA, and EGTA in 1 ml of TT-BSA. In the experiments with EGTA, TT-BSA did not contain $Ca^{2+}$. The retinas were then stimulated for 20 min at 37 °C with A23187 (1 μM), ACh (50 μM), and dopamine (50 μM). The lipid extraction was performed on sonicated retinas as described in the legend to Fig. 1. The numbers indicate the amount of PAF in ng/mg of protein. Mean ± S.D. of 3 experiments. *, $p < 0.01$, **, $p < 0.001$ *versus* values obtained in retinas that have not been treated with the chemicals.

| | Control | ACh | Dopamine | A23187 |
|---|---|---|---|---|
| None | 1.12 ± 0.30 | 5.67 ± 0.31 | 6.22 ± 1.15 | 6.82 ± 0.30 |
| PBDB, 100 nM | 1.00 ± 0.43 | 5.40 ± 0.76 | 6.15 ± 0.71 | 1.83 ± 0.45 |
| Acetyl-CoA | | | | |
| 10 μM | 1.23 ± 0.38 | 5.84 ± 1.00 | 6.03 ± 1.43 | 6.70 ± 0.92 |
| 50 μM | 1.36 ± 0.75 | 6.02 ± 1.57 | 5.98 ± 0.82 | 9.12 ± 1.34* |
| 100 μM | 1.30 ± 0.82 | 5.12 ± 0.72 | 6.00 ± 1.43 | 13.60 ± 2.65** |
| EGTA, 0.1 mM | 1.05 ± 0.63 | 1.72 ± 0.88** | 1.22 ± 0.51** | 0.92 ± 0.64** |

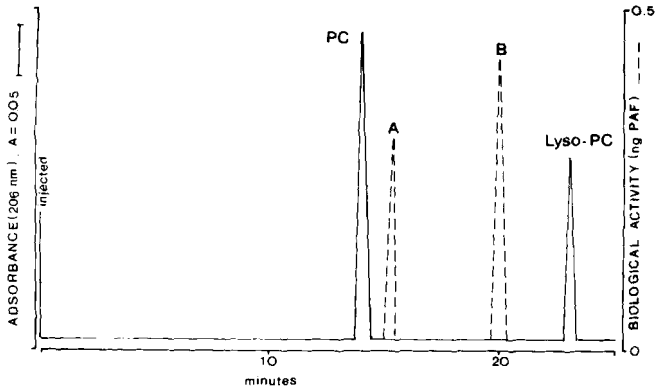


FIG. 4. **HPLC elution pattern of TLC-purified PAF obtained from sonicated retina stimulated for 20 min at 37 °C in 1 ml of TT-BSA with 50 μM ACh.** The sample was injected onto a μPorasil column and eluted with chloroform/methanol/water (60:55:5, v/v) at the flow rate of 1 ml/min. ——, ultraviolet trace (206 nm) depicting the HPLC elution of PC and lyso-PC. – – –, elution of PAF as biological activity. This chromatogram is representative of the results obtained from 3 separated experiments. Similar results have been obtained with TLC-purified PAF obtained from sonicated retinas after stimulation with dopamine (50 μM) and A23187 (1 μM) and from intact retinas stimulated by the three different agonists.

ionizable groups (27, 28). After base-catalyzed methanolysis or inactivation with phospholipase $A_2$, treatment with acetic anhydride restored (83.3 ± 7.0% and 74.0 ± 8.8%, mean ± S.D. of 3 experiments) of the biological activity of peaks A and B, respectively. The specificity of washed rabbit platelets aggregation induced by both peaks was demonstrated by the inhibitory effect of CV-3988, a specific PAF receptor antagonist (16, 17). Five μM CV-3988 completely inhibited the biological activity of PAF.

*Acetyltransferase and Cholinephosphotransferase Activity*—The time-dependent effect of ACh, dopamine, and A23187 on acetyltransferase and cholinephosphotransferase activity of chick retinas is shown in Fig. 5. It is important to note that cholinephosphotransferase but not acetyltransferase specific activity increased (up to 6-fold) only after stimulation with neurotransmitters (ACh and dopamine). Their effect on the cholinephosphotransferase activity was time dependent, being maximal 5 min after stimulation (Fig. 5*A*). In contrast, acetyltransferase was activated by A23187 after 10 min of incubation and for as long as the enzyme persisting in an activated state up to 40 min (Fig. 5*B*). Cholinephosphotransferase and acetyltransferase declined to their basal values within 20 and 50 min, respectively.

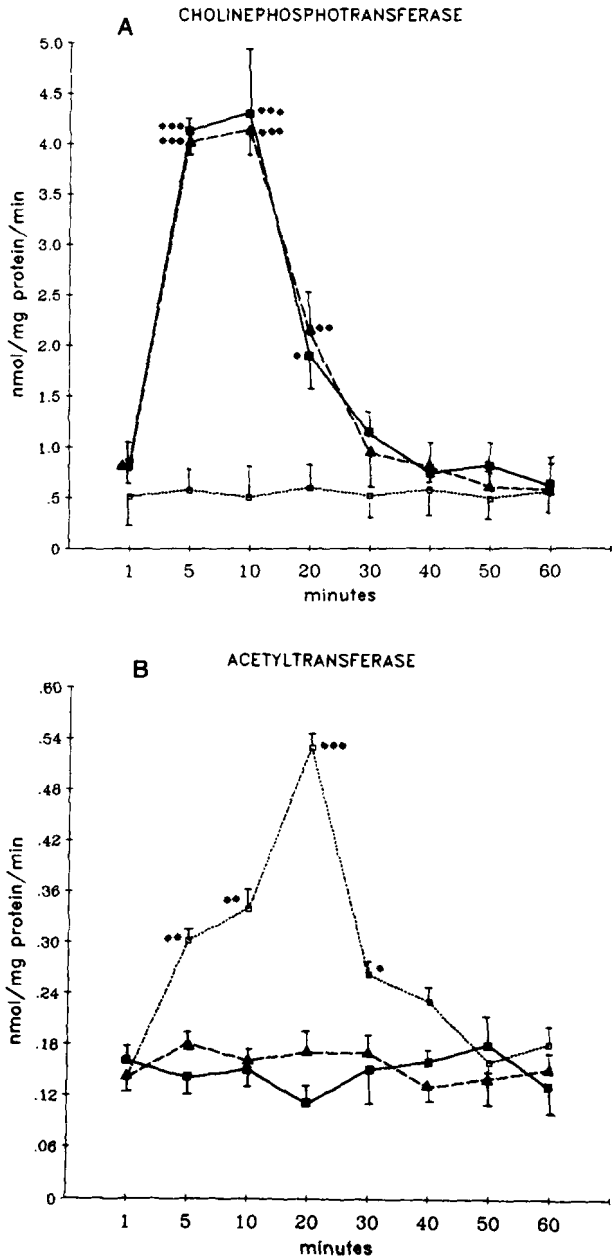


FIG. 5. **Time course of ACh-, dopamine-, and A23187-induced increase of cholinephosphotransferase (*panel A*) and acetyltransferase (*panel B*) activity.** Retinas were stimulated with ACh (■, 50 μM), dopamine (▲, 50 μM), and A23187 (□, 1 μM) in 1 ml of TT-BSA at 37 °C for the indicated times. The reaction was stopped by centrifugation (800 × *g*, 10 min, 4 °C). The tissue was placed in 1 ml of 0.25 M sucrose containing 1 mM dithiothreitol for the acetyltransferase assay and in 1 ml of 0.25 M sucrose in 10 mM Tris-HCl buffer, pH 7.4, for the cholinephosphotransferase. The lysis of the tissue was made by sonication (3 pulses, 5 s, 100 watts, in ice bath). The conditions to measure the enzymatic activities are detailed under "Experimental Procedures." The results are mean ± S.D. of 4 experiments done in duplicate. The basal values in unstimulated retinas of acetyltransferase and cholinephosphotransferase were 0.15 ± 0.05 nmol/min/mg of protein and 0.50 ± 0.04 nmol/min/mg of protein, respectively (mean ± S.D. of 4 experiments done in duplicate), and were constant for all times of incubation. *, $p < 0.02$; **, $p < 0.01$; ***, $p < 0.001$ *versus* the basal value calculated at the same time (Student's *t* test).

## DISCUSSION

The present results demonstrate the production of PAF by chick retinas stimulated by ACh, dopamine, or A23187. Identification of PAF was based on data from chemical and lipase treatments, TLC, HPLC, and bioassay. By HPLC, PAF activity was eluted in two peaks that shared with standard PAF a similar sensitivity to lipases and chemical treatments. These data may indicate a molecular heterogeneity of PAF production by stimulated chick retinas, as it has been shown for PAF recovered from basophils, neutrophils, and mast cells (4, 36–38).

PAF generated by ACh, dopamine, or A23187 was not released in the medium and was only extractable from retinal tissue. This finding is partially in agreement with data demonstrating that neutrophils and mast cells release only part of the total amount of synthesized PAF (36, 39, 40). Sonication of the retinas increased the amount of extractable PAF from stimulated retinas and allowed detection of small amounts of PAF also from unstimulated retinas. The effect of sonication did not seem to be related to tissue destruction, because sonication before or during the extraction procedure

of stimulated retinas did not influence the amount of recovered PAF. Furthermore, sonication did not seem to activate PAF production in our system since the amounts of PAF obtained from retinas sonicated before the lipid extraction did not significantly ($p < 0.5$) differ from those obtained when sonication was done during the extraction at low ($3.0 \pm 0.1$) pH that blocks any metabolic activity. PAF produced by the retinas may be complexed to tissue structures. Sonication may render this PAF more accessible to the action of the solvents used in the lipid extraction. The fact that [$^3$H]PAF could be completely recovered from the retinas independently of sonication does not undermine this hypothesis because in the present experiments, [$^3$H]PAF was not produced, but added to the retinas at the end of the incubation or during the lipid extraction.

The amounts of extractable PAF from stimulated retinas were a function of incubation time and of concentration of ACh and dopamine. PAF generation increased after challenge with ACh, dopamine, or A23187 to reach a plateau within 15 and 40 min, respectively. However, PAF production returned to basal values, probably indicating a catabolic rearrangement of the molecule. Pertinent in this respect is the recent demonstration that neutrophils (26), platelets (41), macrophages (42, 43), tumor (44), and endothelial cells (45) inactivate PAF by deacetylation to lyso-PAF that is rapidly reacylated with a long acyl residue, primarily a tetraenoic acyl species.

The highest amounts of extractable PAF were obtained after stimulation with 10–50 $\mu$M ACh or dopamine. ACh, dopamine, and A23187 promoted PAF production by acting on two distinct enzymatic pathways. ACh and dopamine increased the cholinephosphotransferase activity with a time course similar to that of PAF production. Enzyme activity increased before the peak of PAF production and declined to basal value more rapidly than PAF. This enzyme activity was shown to be dithiothreitol insensitive and $Ca^{2+}$ independent according to the criteria of Renooij and Snyder (12). However, the action of ACh and dopamine on PAF production required extracellular $Ca^{2+}$ as inferred from the inhibitory effect of EGTA. More recently, this metabolic pathway has been demonstrated to play a role in the production of PAF by rabbit platelets (46, 47). However, this is the first demonstration that physiological agents, such as ACh and dopamine, stimulate the synthesis of PAF via the dithiothreitol-insensitive cholinephosphotransferase. This pathway of PAF synthesis has been proposed to be operating in the production of PAF in rat renomedulla (48–50). In contrast, the calcium ionophore A23187 activated in chick retina the acetyltransferase route of PAF synthesis that is considered the sole pathway involved in inflammatory reactions (9, 10, 20). The time course of the acetyltransferase activity stimulated by A23187 was slower than that observed in other cell types such as neutrophils (51) and eosinophils (11). PBDB, an inhibitor of phospholipase A2 (52) and of lyso-PAF production (53), blocked the production of PAF induced by A23187, whereas acetyl-CoA enhanced this activity most probably acting as substrate for acetyltransferase (8). These data are consistent with the interpretation that A23187 induced PAF production from chick retina by activating a deacylation/reacetylation process of the alkyl ether phosphoglycerides as has been demonstrated in neutrophils and macrophages (26, 54).

Because avian retina is devoid of vascular tissue (14) and cell control preparations (leukocytes, red blood cells, and embryogenic fibroblasts) did not produce PAF in our conditions of stimulation, PAF produced by the chick retinas can only originate from nervous or glial elements responsive to neutrotransmitters. The fact that two neurotransmitters present in amacrine cells (55, 56) were able to promote PAF production suggests the neuronal origin of PAF. However, the origin of PAF from Mueller cells cannot be excluded, since cholinergic receptors are present on these cells (57).

The results of the present study demonstrate for the first time that neurotransmitters may enhance the activity of a dithiothreitol-insensitive cholinephosphotransferase and induce the production of PAF in chick retina, a tissue structure used for the investigation of cells of the central nervous system. Although we do not have information on the possible role of PAF in neurophysiology, it is interesting to note that exogenous PAF has been shown to modify the electrophysiological activity of the rat retina (58).

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