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THE DE NOVO BIOSYNTHESIS OF PLATELET-ACTIVATING FACTOR IN RAT BRAIN

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SUMMARY. Platelet-Activating Factor (PAF, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is present in nervous tissue and its function is still unknown. We have demonstrated that rat brain is able to synthesize PAF from 1-alkyl-2-acetyl-*sn*-glycerol and CDP-choline by a "DTT-insensitive" phosphocholine transferase. This represents the last step of the *de novo* pathway which apparently is the only one existing in the brain for PAF biosynthesis. The enzyme has a microsomal localization, requires Mg^{++} and is inhibited by Ca^{++} as reported for phosphocholine transferase utilizing long-chain diradylglycerols as substrates. However, other properties of PAF-synthesizing enzyme (sensitivity to DTT and dependence on pH) are different from those of phosphocholine transferase responsible for the synthesis of diacyl and long-chain alkylacyl glycerophosphocholines. These observations indicate that a specific enzyme for PAF biosynthesis might exist in rat brain.

PAF (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent lipid mediator whose properties have been recently reviewed (1,2). Two pathways are known for its biosynthesis in various cell types. The first needs another phospholipid as precursor (1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine) which is hydrolyzed by a phospholipase A_2 thus producing 1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (lyso-PAF). This is transformed into PAF by the action of an acetyltransferase. This has been called the "remodeling" pathway since it converts a phospholipid molecule into another (3). Several tissues have the capability to synthesize PAF *ex novo* and the final step of this pathway is catalyzed by a "DTT-insensitive" phosphocholine transferase which transfers the phosphocholine residue from CDP-choline to the lipid acceptor, 1-alkyl-2-acetyl-*sn*-glycerol (alkylacetylG) (4,5).

PAF has been detected in bovine brain (6) and neurotransmitters are able to induce PAF biosynthesis by phosphocholine transferase in the retina (7). This indicates a functional role of PAF in the nervous tissue where the "remodeling pathway" seems to be inoperative because the lacking of acetyltransferase activity (3). On the other hand, it has been demonstrated that brain tissue possesses all the enzymic activities for synthesizing alkylacetylglycerol (8,9), the lipid substrate for phosphocholine transferase which catalyzes the last step of the *de novo* synthesis of PAF.

This work represents the first evidence that mammalian brain can synthesize PAF by phosphocholine transferase and describes some properties of the enzyme.

Abbreviations: PAF, platelet activating factor; alkylacetylG, 1-alkyl-2-acetyl-*sn*-glycerol; diacylG, 1,2-diacyl-*sn*-glycerol; alkylacylG, 1-alkyl-2-acyl(long-chain)-*sn*-glycerol; GPC, glycerol-3-phosphocholine; DTT, dithiothreitol.

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MATERIALS AND METHODS

Materials. 1,O-hexadecyl-2-acetyl-*sn*-glycerol (alkylacetyl-G) and 1,O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) were from Novabiochem (Switzerland). Cytidine-5'-diphospho-[methyl-^{14}C] choline, ammonium salt (S.R. 51 mCi/mmol.) was from Amersham (England). Bovine serum albumin (BSA), dithiothreitol (DTT) and soybean lecithin were from Sigma (Missouri, U.S.A). Rat Fish liver oil was obtained from Western Chemical Industries, (Canada). Chemicals and solvents were from Carlo Erba, Italy. Phospholipase C (B.cereus) and HEPES were from Boehringer (Germany). AlkylacylG and diacylG were prepared from rat fish liver oil and soybean lecithin, respectively (10).

Preparation of brain subfractions. Sprague-Dowley rats (28-30 days old) were sacrificed following intracardiac perfusion under light anesthesia. Brains were homogenized in 0.32 M sucrose containing 2 mM EDTA and crude subcellular fractions were prepared according to procedures previously reported (11,12).Synaptosomes were osmotically shocked and the fraction concentrated by centrifugation (12). Protein concentrations were determined using bovine serum albumin as standard (13).

Incubation, extraction and analysis of lipids. Incubations were carried out at 39 °C in a final volume of 0.3 ml. The conditions are reported under tables and figures. Unless differently specified diradylG were dispersed by sonication in a convenient volume of 0.12% Tween-20 (14). Lipids were extracted (15) and labeled choline phosphoglycerides were isolated by TLC on silica gel G plates with chloroform/methanol/acetic acid/water (50:25:8:4 by vol) as developing solvent (5) and were identified by co-chromatography with known standards. The radioactivity of choline phosphoglycerides was measured by liquid scintillation spectrometry.

Characterization of PAF. In some samples,the radioactive product of the incubation of alkylacetylG with radioactive CDP-choline and brain microsomes, was separated by HPLC (Spherisorb S5W, 250x4.6 mm, n-hexane/isopropanol 3:2 containing 5.8% water, 1 ml min). The radioactivity was completely recovered in the fractions with identical retention time of standard PAF. Furthermore, the labeled compound purified by TLC, with the same Rf of authentic PAF, was subjected to alkaline hydrolysis (16) and the organic phase was then analyzed by a two-dimensional TLC with exposure to HCl fumes between the first and the second run both carried out using chloroform\methanol\conc.NH_4OH\water (60:35:8:2.3 v\v) as developing solvent. In this case the radioactivity was completely recovered with standard lyso-PAF. The material, isolated by TLC and with tha same Rf of PAF, induced aggregation of washed rabbit platelets and release of labeled serotonin (17). Both effects were completely abolished by BN52021, a specific antagonist of PAF receptor binding (18).

RESULTS

Brain phosphocholine transferase activity has been assayed as previously reported (14). Particularly, the lipid substrates (diradylglycerols) were resuspended by sonication using Tween-20, as detergent. Attempts for adding diradylglycerols as ethanolic solutions, as previously reported (4,5,7), failed since the presence of 2.5% ethanol in the medium (4) inhibited the synthesis of PAF, diacyl-, and alkylacyl-GPC.

The rates of the biosynthesis of PAF and diacyl-GPC by phosphocholine transferase have been determined in rat brain sub-cellular fractions (Tab. 1).

TABLE 1- BIOSYNTHESIS OF PAF AND DIACYL-GPC IN RAT BRAIN SUBCELLULAR FRACTIONS

Fraction	PAF	diacyl-CPG
Mitochondria	0.76	0.23
Myelin	0.90	0.51
Synaptosomes	1.28	1.57
Microsomes	7.61	2.41
Cytosol	1.38	0.11

Incubation conditions: 60 mM HEPES (pH 8), 20 mM $MgCl_2$, 1 mM CDP-[^{14}C]choline (Spec. Rad. 0.85 nCi/nmol), 0.02% Tween-20, 0.1 mM diradylglycerols, 0.8- 1.4 mg/ml protein from individual subcellular fractions, final volume 0.3 ml, 39 °C, 20 min. Data are expressed as nmol/mg protein/20 min and represent the average of duplicate samples.

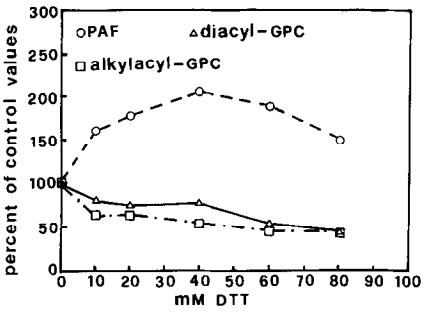

Fig.1. Effect of DTT on PAF, diacyl-CPG and alkylacyl-CPG biosynthesis.

Incubation: 50 mM Tris-HCl (pH 8), 0.5 mM EDTA, 10 mM $MgCl_2$, 10 mg/ml BSA, 0.2 mM diradylglycerol, 0.02% Tween-20, 0.1 mM CDP-[^{14}C]choline (Spec.Rad. 2.9 nCi/n mol) and protein 0.47 mg/ml. Data are expressed as nmol/mg protein/20 min and are representative of three other experiments giving similar results.

The highest specific activity was found in the microsomal fraction both with alkylacetylG and diacylG as lipid substrates. At the indicated conditions, with 0.1 mM diradylglycerols, the rate for the biosynthesis of PAF was about 2.5 fold higher than that of diacyl-GPC.

The effect of DTT on the synthesis of PAF and other choline phosphoglycerides have been studied (Fig. 1). Under the indicated experimental conditions and without DTT in the incubation mixture, the specific activities were 19.3, 2.9 and 1.8 nmol/mg prot/20 min for the synthesis of PAF, diacyl-GPC and alkylacyl-GPC, respectively. The addition of DTT to the medium caused an increase of the rate of the synthesis of PAF whereas those of diacyl- and alkylacyl-GPC were both inhibited.

Phosphocholine transferase activity was measured at different pH values using various buffers with alkylacetylG and diacylG as substrates. As shown in Fig. 2, the pH curve for PAF biosynthesis clearly showed a maximum at pH 8 whereas the rate for the biosynthesis of diacyl-GPC was maximal in the range of pH 7-8.5.

Other experiments have indicated that phosphocholine transferase for PAF synthesis required Mg^{++} or Mn^{++}. The addition of 1 mM Ca^{++} to the medium containing 10 mM Mg^{++} inhibited the rate of the synthesis of PAF by more than 80%.

The effect of CMP (1 mM) on the rate of PAF biosynthesis has been evaluated since it is known that the relative concentrations of CDPcholine and CMP are important on the regulation of the biosynthesis of long-

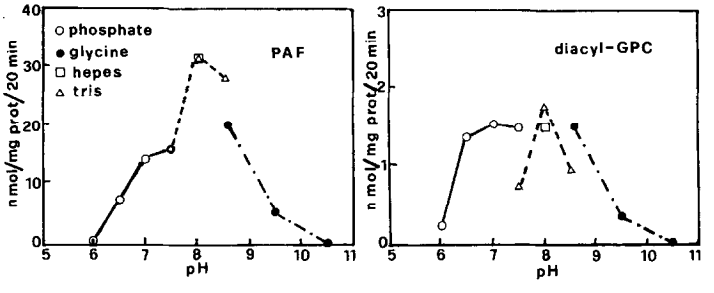

Fig.2. Effect of pH on PAF and diacyl-GPC biosynthesis in rat brain microsomal fraction.

Incubation: 50 mM Tris-HCl, potassium phosphate, HEPES or glycine, 0.02% Tween-20, 10 mg/ml BSA, 0.5 M EDTA, 0.2 mM diradylglycerols, 0.1 mM CDP- [^{14}C]choline (Spec. Rad. 1.3 nCi/nmol.) and 0.26 mg microsomal protein, 0.3 ml final volume, 39 °C, 20 min. Data are reported as nmol/mg prot/20 min.

TABLE 2 - EFFECT OF THE CONCENTRATION OF DIRADYLGLYCEROL ON PHOSPHOCHOLINE TRANSFERASE ACTIVITY

Diradylglycerols	PAF	Diacyl-GPC
[mM]		
0	0.04	0.97
0.1	2.60	0.93
0.2	4.87	0.97
0.5	8.13	0.80
1.0	11.31	1.87
2.0	11.82	10.69

Incubation: 60 mM HEPES (pH8), 20 mM $MgCl_2$, 0.02% Tween-20, 1 mM CDP-[^{14}C]choline (Spec.Rad. 0.54 nCi/nmol), diradylglycerols at the indicated concentrations, 1.5 mg/ml microsomal protein, 10 min., 37 °C, 0.3 ml final volume. 60 mM DTT was present when PAF biosynthesis was measured. Results are expressed as nmol/mgprot/10 min and represent the average of duplicate samples with a maximal variation of 10%.

chain diacyl-GPC by brain phosphocholine transferase (19-20). The incubation was carried out at the conditions indicated in Fig.1 and contained 1mM AMP to reduce CMP hydrolysis (20). Under these conditions, PAF biosynthesis was reduced by about 75%.

The activities of phosphocholine transferase, using various concentrations of alkylacetylG or diacylG as lipid substrates, are reported in Tab.2. Under the indicated conditions, at relative low concentrations of the substrates (0.1 - 0.5 mM), the rate of PAF biosynthesis was much higher than that measured for diacyl-GPC. No differences were found at higher concentrations (1 - 2 mM). Without addition of diradylglycerols, a detectable amount of radioactivity was found in the area of TLC plate corresponding to standard PAF but this was much lower than that of diacyl-GPC.

DISCUSSION

It is well established that nervous tissue is able to synthesize diacyl and ether types of choline phosphoglycerides, having a long-chain fatty acid in the carbon-2 of glycerol moiety, by the phosphocholine transferase reaction (14,21-23). Our results demonstrate that the lipid mediator 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) can be synthesized by a rat brain microsomal enzyme from CDP-choline and alkylacetylG. The enzyme requires Mg^{++} for its activity and it is inhibited by Ca^{++} as reported for the synthesis of choline phosphoglycerides with long-chain fatty acids (14,21).

The presence of DTT in the incubation medium has a stimulatory effect on the utilization of alkylacetylG for the synthesis of PAF whereas it inhibits the production of diacyl- and alkylacyl-GPC from the corresponding diradylglycerols, having long-chain fatty acids, by about 50% in the presence of 80 mM DTT. These results indicate that the phosphocholine transferase activities are differently affected by DTT when alkylacetylG or long-chain diradylglycerols are used as substrates. However it must be pointed out that brain phosphocholine transferase, synthesizing long-chain choline phosphoglycerides, is less sensitive to DTT than the enzymes from liver (5) or inner renal medulla (4), which are completely inhibited by 5-10 mM DTT.

The sensitivity to DTT is a criterion utilized for distinguishing the PAF-synthesizing phosphocholine transferase from the enzymic activity involved in the production of diacyl-GPC (4,7). On the basis of our results, it is not possible to state whether brain microsomes possess two separate enzymes. However, the differences on the effects exerted by DTT and pH on the utilization of alkylacetylG and long-chain diradylglycerols might be considered as an indication for the presence of a specific enzyme for PAF biosynthesis in the brain. The possi-

bility that DTT might cause changes on the affinity of a unique enzyme for diradylglycerols with short- or long-chain fatty acids cannot be ruled out.

At relatively low concentrations of diradylglycerols, the rate of PAF biosynthesis was certainly much higher than that measured for long-chain diacyl-GPC. This might reflect the different physical properties of the corresponding lipid precursors (alkylacetylG and long-chain diacylG).

The physiological significance of PAF in brain it is not known but it has been suggested a role on the regulation of neuronal function since it is able, at low concentration, to induce neuronal differentiation whereas, at high concentration, it is neurotoxic (24). Furthermore, it has been recently reported that convulsive agents increase the levels of PAF in rat brain and this mediator is of cerebral origin (25). In this connection, it should be pointed out that the demonstration that brain tissue is able to synthesize PAF by a phosphocholine transferase activity assumes a particular importance since it provides the evidence that the nervous tissue possesses the capability of synthesizing this lipid mediator by the *de novo* pathway. In fact, it has been already reported the presence of both 1-alkyl-2-lyso-*sn*-glycero-3-phosphate : acetyl-CoA acetyltransferase (8) and 1-alkyl-2-acetyl-*sn*-glycero-3-phosphate phosphohydrolase in the rat brain (9). Both enzymes have high specific activities if compared to other tissues and seem to be specific for the production of alkylacetylG, the lipid substrate for phosphocholine transferase.

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