Novel molecular analogues of phosphatidylcholines in a lipid extract from bovine brain: 1-long-chain acyl-2-short-chain acyl-*sn*-glycero-3-phosphocholines

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Abstract A vasodepressor phospholipid fraction named Depressor-IA from a bovine brain lipid extract was analyzed by capillary gas–liquid chromatography–mass spectrometry as *tert*-butyldimethylsilyl derivatives after hydrolysis with phospholipase C. Results show that Depressor-IA is a mixture of 3 platelet-activating factors and 17 1-acyl analogues. Three platelet-activating factors having *sn*-1-O-hexadecyl, octadecyl, and octadecenyl groups were suggested to account for the hypotensive activity of Depressor-IA, although the total amount of 1-acyl analogues of platelet-activating factor was much more than that of platelet-activating factor in the purified Depressor-IA. 1-Long-chain acyl-2-short-chain acyl-glycero-3-phosphocholines identified in Depressor-IA included novel molecular analogues having *sn*-2-propionyl, acryloyl, butyryl, valeryl, caproyl, and hepatonoyl groups.—**Tokumura, A., K. Takauchi, T. Asai, K. Kamiyasu, T. Ogawa, and H. Tsukatani.** Novel molecular analogues of phosphatidylcholines in a lipid extract from bovine brain: 1-long-chain acyl-2-short-chain acyl-*sn*-glycero-3-phosphocholines. *J. Lipid Res.* 1989. **30:** 219–224.

Supplementary key words PAF • acyl PAF • short-chain acyl group • molecular composition • *tert*-butyldimethylsilyl derivatives • positional isomer of glycerides • GLC–MS

Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a phospholipid mediator that is synthesized by a variety of animal tissues (1–3). Because of the very low cellular content of PAF, bioassays based on its in vitro platelet-activating effect or its in vivo vasodepressive effect have been used in many studies to demonstrate its existence in natural sources (1–3). Thus PAF has been characterized chemically in only limited kinds of cells (4–8) or tissue (9–11). Molecular heterogeneity of the *sn*-1-alkyl moiety of PAF (in chain length and degree of unsaturation) has been demonstrated in stimulated and unstimulated neutrophils (5–8), lesional scales of psoriatic patients (9), and isolated rat uterus (10). Furthermore, 1-acyl-2-acetyl-*sn*-glycero-3-phosphocholine, an acyl analogue of PAF (acyl PAF) has been reported to coexist with PAF in rabbit (12) and human (13) neutrophils activated with Ca^{2+}-ionophore A23187. Using conventional GLC–MS, we have recently identified more than 10 acyl PAFs including 1-long-chain acyl-2-propionyl-GPC and 1-long-chain acyl 2-butyryl-GPC together with PAF as the major components of a vasodepressor substance, which we tentatively named Depressor-IA in a lipid extract from bovine brain (14). In the present study, we have successfully applied high-resolution capillary GLC–MS for the analysis of Depressor-IA, and found an additional seven compounds of acyl PAF, which have not previously been detected in natural samples. Our results demonstrate the coexistence with PAF of a family of biologically active choline phospholipids with different combinations of *sn*-1-long-chain acyl groups and *sn*-2-short-chain acyl groups.

MATERIALS AND METHODS

1-O-Hexadecyl-2-acetyl-GPC, 1-O-octadecyl-2-acetyl-GPC, and their lyso compounds were obtained from Bachem Feinchemikalien AG, Bubendorf, Switzerland. 1-O-Octadecenyl-2-acetyl-GPC, 1-myristoyl-2-lyso-GPC, 1-palmitoyl-2-lyso-GPC, 1-stearoyl-2-lyso-GPC, 1-oleoyl-2-lyso-GPC,

Abbreviations: PAF, platelet-activating factor; acyl PAF, acyl analogue of platelet-activating factor; lysoPC, lysophosphatidylcholine; GPC, *sn*-glycero-3-phosphocholine; GLC–MS, gas–liquid chromatography–mass spectrometry; tBDMS, *tert*-butyldimethylsilyl; TLC, thin-layer chromatography; EI–MS, electron impact–mass spectrometry. The nomenclature used to designate various analogues of acyl PAF indicates the total number of carbons in the acyl chain at either *sn*-1 or *sn*-2 and the degrees of unsaturation, e.g., 16:0/3:1 represents 1-palmitoyl-2-acryloyl GPC.

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1,2-dipalmitoyl-GPC, 1,2-distearoyl-GPC, and 1,2-dioleoyl-GPC were purchased from Sigma Chemical Co., St. Louis, MO. Phospholipase C from *Bacillus cereus*, phospholipase A_2 from bee venom and lipase from *Rhizopus arrhizus* were also from Sigma. Acryloyl chloride and *n*-heptanoic anhydride were from Aldrich Chemicals, Milwaukee, WI. Acetic anhydride, *n*-propionic anhydride, *n*-butyric anhydride, *n*-valeric anhydride, and *n*-caproic anhydride were from Wako Pure Chemicals, Osaka, Japan. Perdeuterated acetic anhydride was from E. Merck, Darmstadt, West Germany. Other chemicals and solvents used were of analytical grade.

Preparation of standard PAF and acyl PAF

Various PAFs and acyl PAFs were prepared by treatment of the corresponding lyso compound with appropriate acid anhydride or acid chloride, essentially as described by Kumar et al. (15). LysoPCs with *sn*-2-palmitoyl, stearoyl, and oleoyl groups were prepared by treatments of 1,2-dipalmitoyl-GPC, 1,2-distearoyl-GPC, and 1,2-dioleoyl-GPC, respectively, with lipase from *Rhizopus arrhizus*, as described by Slotboom et al. (16) except for an addition of 4 ml diethylether to the reaction mixture (1 ml) to minimize acyl migration of 2-acyl lysoPC.

The following acyl PAFs were prepared by the method described above: 1-myristoyl-2-acetyl(14:0/2:0)-GPC, 1-palmitoyl-2-acetyl(16:0/2:0)-GPC, 1-acetyl-2-palmitoyl(2:0/16:0)-GPC, 1-stearoyl-2-acetyl(18:0/2:0)-GPC, 1-acetyl-2-stearoyl(2:0/18:0)-GPC, 1-oleoyl-2-acetyl(18:1/2:0)-GPC, 1-acetyl-2-oleoyl(2:0/18:1)-GPC, 1-palmitoyl-2-propionyl(16:0/3:0)-GPC, 1-palmitoyl-2-acryloyl(16:0/3:1)-GPC, 1-palmitoyl-2-butyryl(16:0/4:0)-GPC, 1-stearoyl-2-propionyl(18:0/3:0)-GPC, 1-stearoyl-2-acryloyl(18:0/3:1)-GPC, 1-stearoyl-2-butyryl(18:0/4:0)-GPC, 1-oleoyl-2-butyryl(18:1/4:0)-GPC, 1-palmitoyl-2-valeryl(16:0/5:0)-GPC, 1-palmitoyl-2-caproyl(16:0/6:0)-GPC, 1-palmitoyl-2-heptanoyl(16:0/7:0)-GPC, 1-palmitoyl-2-perdeuterated acetyl-GPC, and 1-O-hexadecyl-2-perdeuterated acetyl-GPC. These phospholipids were converted to the tBDMS derivatives after their hydrolysis with phospholipase C (6).

Analysis of PAF and acyl PAF in a lipid extract from bovine brain

Lipids were extracted from bovine cerebrum, and Depressor-IA in the extract was purified as described previously (14). The partially purified Depressor-IA was then hydrolyzed with phospholipase C, and the resultant glycer-

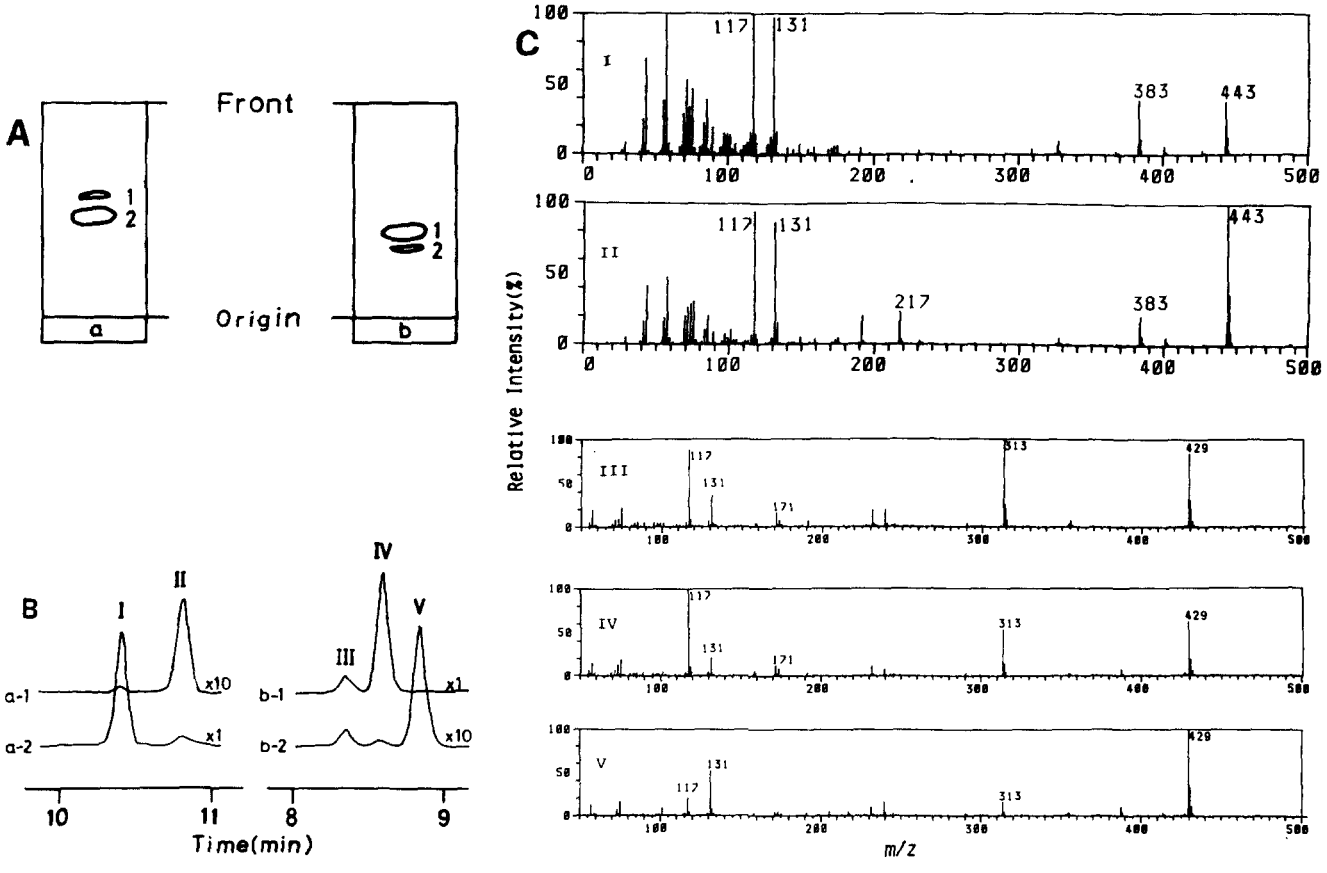


Fig. 1. Analysis of positional isomers of tBDMS derivatives of glycerides obtained by hydrolysis of authentic PAF and acyl PAF with phospholipase C. A: Thin-layer chromatograms of tBDMS derivatives of authentic 1-O-octadecyl-2-acetyl-GPC(a) and 1-palmitoyl-2-acetyl-GPC(b) in a solvent system of *n*-hexane–diethylether 9:1(v/v) on Merck Silica gel 60 plates. B: Gas chromatograms of compounds a-1, a-2, b-1, and b-2 recovered from the TLC plates. C: EI-MS of components I, II, III, IV, and V in gas chromatograms.

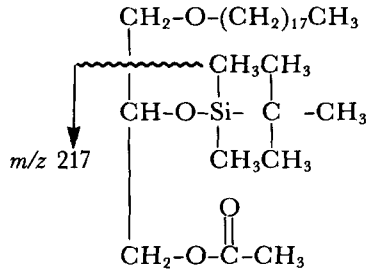
ides were converted to tBDMS derivatives (6). The tBDMS derivatives were purified by TLC (6), and analyzed in a Hitachi M-80B mass spectrometer coupled with a gas chromatograph with an ultraperformance capillary column (cross-linked methyl silicone, 50 m × 0.32 mm I.D.; 0.17 μm thin film). The column was maintained at 280°C and developed at a flow rate of 0.3 ml/min (He). The temperatures of the injection port and interface were 300°C. Mass spectrometry was done under the following conditions: 70 eV-ionizing voltage, 300 μA ionizing current, 3.5 kV accelerating voltage and 200°C ion source temperature.

Various PAFs and acyl PAFs were quantified by measurement of $[M-C(CH_3)_3]^+$ of their tBDMS derivatives against an internal standard, 1-O-hexadecyl-2-perdeuterated acetyl-GPC and 1-palmitoyl-2-perdeuterated acetyl-GPC, respectively.

RESULTS

Capillary GLC-MS of tBDMS derivatives of standard PAF and acyl PAF

Standard PAF having an *sn*-1-O-hexadecyl, octadecyl, or octadecenyl group was hydrolyzed with phospholipase C, and the resultant glyceride was converted to the tBDMS derivative and analyzed on a silica gel plate 60 (Merck) in a solvent system of *n*-hexane–diethylether 9:1 (v/v). **Fig. 1-A-a** shows a typical result for PAF($C_{18:0}$). The spots detected were extracted from the silica gel and analyzed by capillary GLC-MS. The slower-moving material (compound a-2) was eluted at 10.3 min from a column under our GLC conditions (Fig. 1-B-a, peak I), and its mass spectrum is shown in Fig. 1-C. The peak should be due to 1-O-octadecyl-2-acetyl-3-tBDMS-*sn*-glycerol, because the mass spectrum was essentially the same as that reported previously (17). The faster-moving material on the TLC plate (compound a-1) eluted slower than compound a-2 in the gas chromatogram. This peak should be due to a positional isomer, probably 1-O-octadecyl-2-tBDMS-3-acetyl-*sn*-glycerol, because its mass spectrum showed intense peaks at *m/z* 117, 383 and 443, all of which were also observed in the mass spectrum of peak I, and which could be assigned to $[CH_3CO + OSi(CH_3)_2]^+$, $[M-C(CH_3)_3-CH_3COOH]^+$ and $[M-C(CH_3)_3]^+$, respectively. However, an intense ion peak at *m/z* 217 was seen in the mass spectrum of peak II, but not in that of peak I. High resolution mass spectrometric analysis revealed that this ion had the elemental composition $C_{10}H_{21}O_3Si$(observed mass, 217.1257; calculated mass, 217.1260). An intense ion at *m/z* 217 was also seen in the mass spectra of the faster-moving tBDMS derivatives of PAF($C_{16:0}$) and PAF($C_{18:1}$), on TLC plates, indicating that this ion does not include a long-chain alkoxy moiety. Rather, the ion seemed to retain an acetyl group, because the faster-moving tBDMS derivatives on TLC of standard 1-O-octadecyl-2-propionyl-GPC and 1-O-octadecyl-2-butyryl-GPC showed the corresponding ion peaks in their mass spectra at *m/z* 231 and 245, respectively. Therefore, we suggest that the structure of the ion at *m/z* 217 may be as follows:



Next, the acetylated product of authentic 1-palmitoyl-2-lyso-GPC was analyzed by TLC. The faster-moving materials on TLC (Fig. 1-A, b-1) gave a major peak (Fig. 1-B, peak IV) and a minor peak (peak III) on the gas chromatogram. When the slower-moving material on TLC (Fig. 1-A, b-2) was analyzed by capillaryl GLC-MS, it gave a major peak (Fig. 1-B, peak V) eluted behind the minor peak III. Fig. 1-C shows the mass spectra of peaks III, IV, and V, in which $[M-C(CH_3)_3]^+$ (*m/z* 429), $[CH_3(CH_2)_{14}CO + OSi(CH_3)_2]^+$ (*m/z* 313), $[CH_3(CH_2)_{14}CO]^+$ (*m/z* 239), and $[CH_3CO + OSi(CH_3)_2]^+$ (*m/z* 117) were observed. Peaks IV and V would be 1-palmitoyl-2-acetyl-3-tBDMS-*sn*-glycerol and 1-palmitoyl-2-tBDMS-3-acetyl-*sn*-glycerol, respectively, because a base peak was seen at *m/z* 429 in the mass spectrum of peak V, but at *m/z* 117 in that of peak IV, and because a small, but significant ion peak at *m/z* 217 was observed in the mass spectrum of peak V, but not in that of peak IV.

The mass spectrum of peak III was very similar to that of peak IV. The former seemed to be due to 1-acetyl-2-palmitoyl-3-tBDMS-*sn*-glycerol, since it was the predominant peak (about 80%) in the tBDMS derivative of glyceride derived from the acetylated product of authentic 1-lyso-2-palmitoyl-GPC. A minor peak (corresponding to peak IV) amounted to about 20% of the tBDMS derivative of acetylated authentic 1-lyso-2-palmitoyl-GPC. This would be due to 1-palmitoyl-2-lyso-GPC produced by acyl migration of 1-lyso-2-palmitoyl-GPC which had been formed during the lipase treatment of 1,2-dipalmitoyl-GPC.

The parent glycerides of the *sn*-2-tBDMS derivatives described above seemed to be formed by isomerization of diglycerides, hydrolytic products of PAF($C_{18:0}$) and 1-palmitoyl-2-acetyl-GPC during the hydrolysis with phospholipase C. A small amount of the *sn*-2-tBDMS derivative was also detected in the case of authentic PAF($C_{16:0}$), PAF($C_{18:1}$), 1-myristoyl-2-acetyl-GPC, 1-stearoyl-2-acetyl-GPC, 1-oleoyl-2-acetyl-GPC, 1-stearoyl-2-propionyl-GPC, and 1-oleoyl-2-butyryl-GPC, under our conditions. There was, however, no significant production of the *sn*-2-tBDMS derivative in the case of 1-palmitoyl-2-butyryl-GPC, 1-stearoyl-2-butyryl-GPC, 1-palmitoyl-2-valeryl-GPC, 1-palmitoyl-2-caproyl-GPC, or 1-palmitoyl-2-heptanoyl-GPC.

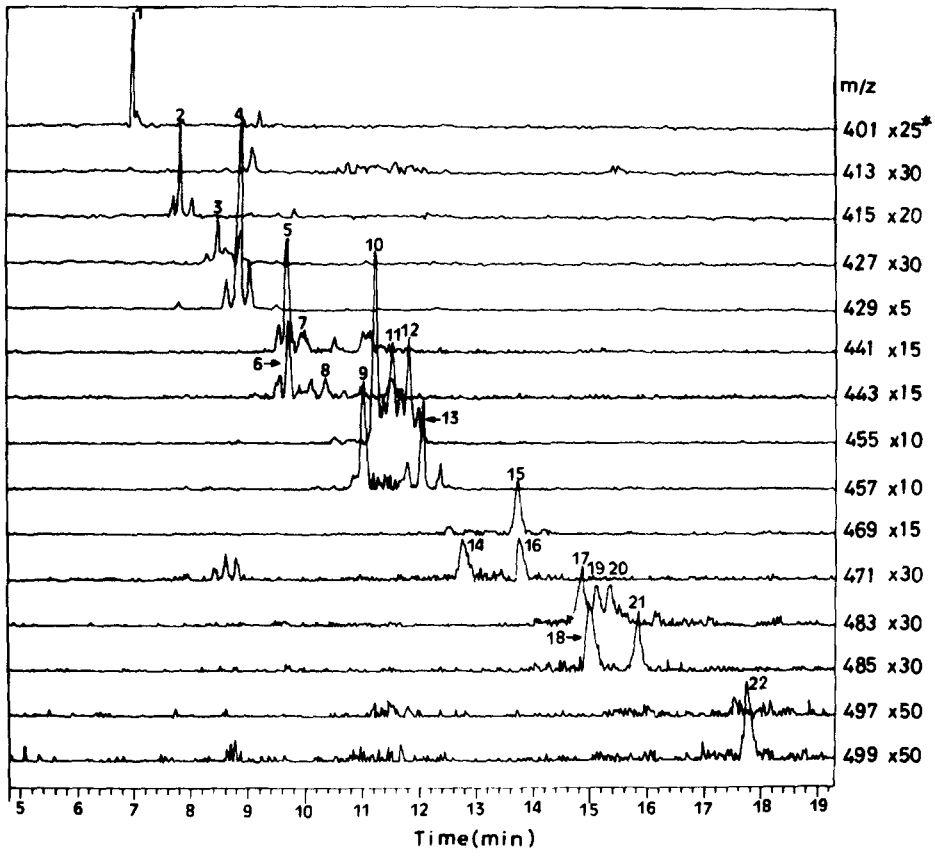


Fig. 2. Ion-current profiles of diagnostic ions in EI-MS of tBDMS derivatives of PAFs and acyl PAFs in purified Depressor-IA, obtained after their hydrolysis with phospholipase C. *Magnification for intensity of ion.

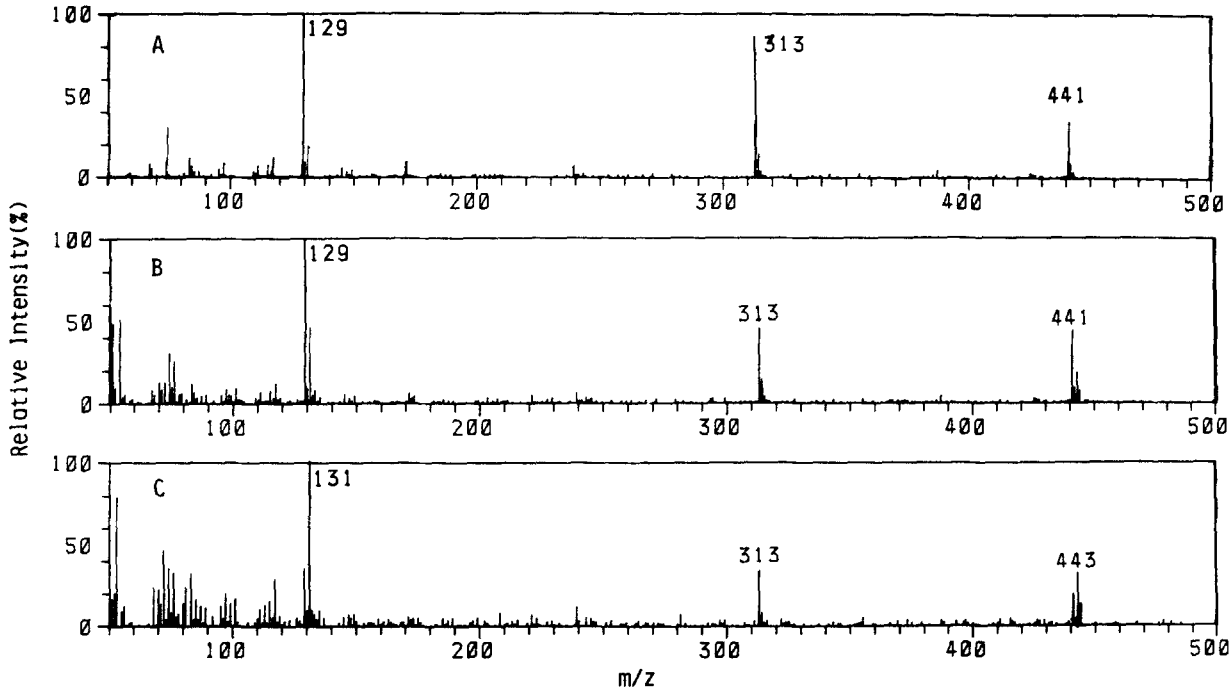


Fig. 3. EI-MS of two components of the tBDMS derivative of purified Depressor-IA, obtained after their hydrolysis with phospholipase C. A: EI-MS of tBDMS derivative of authentic 1-palmitoyl-2-acryloyl-GPC. B: EI-MS measured at the top of peak 5 in Fig. 2. C: EI-MS measured at the top of peak 6 in Fig. 2.

PAF and acyl PAF in Depressor-IA from bovine brain

Fig. 2 shows the ion-current profiles of tBDMS derivatives of glycerides derived from Depressor-IA at *m/z* 401, 413, 415, 427, 429, 441, 443, 455, 457, 469, 471, 483, 485, 497, and 499. Peaks 2, 7, and 8 were identified as due to $[M-57]^+$ of tBDMS derivatives of PAF($C_{16:0}$), PAF($C_{18:1}$), and PAF($C_{18:0}$), respectively. The assignments were based on the following findings. First, the mass spectrum measured at the top of peak 2, 7, or 8 coincided to that of the tBDMS derivative of the respective standard PAF. Second, the retention times of the tBDMS derivatives of peaks 2, 7, and 8 were the same as those of the tBDMS derivatives of the corresponding standard PAFs.

The other major peaks were all ascribed to tBDMS derivatives of glyceride having both a long-chain acyl group and a short-chain acyl group by comparison of their mass spectra and retention times with those of tBDMS derivatives of authentic acyl PAFs. These tBDMS derivatives could all be separated from each other by our capillary GLC system except for the combinations of peaks 5 and 6, and peaks 15 and 16. The mass spectra measured at the top of peaks 5 and 6 are shown in **Fig. 3** together with that of authentic 1-palmitoyl-2-acryloyl-3-tBDMS-*sn*-glycerol. Peaks 6 and 16 were found to be mainly due to 1-palmitoyl-2-propionyl-GPC and 1-stearoyl-2-propionyl-GPC, respectively. Judging from the major ions at *m/z* 129, 313, and 441, which can be assigned to $[CH_2=CHCO + OSi(CH_3)_2]^+$, $[CH_3(CH_2)_{14}CO + OSi(CH_3)_2]^+$ and $[M-C(CH_3)_3]^+$, peak 5 was ascribed mainly to the tBDMS derivative of 1-palmitoyl-2-acryloyl-GPC. Because intense ions were seen at *m/z* 129, 341, and 469 in the mass spectrum measured at the top of peak 15, this peak should be largely due to the tBDMS derivative of 1-stearoyl-2-acryloyl-GPC. The results with tBDMS derivatives of authentic 1-palmitoyl-2-acryloyl-GPC (Fig. 3-A) and 1-stearoyl-2-acryloyl-GPC (data not shown) support the assignments for peaks 5 and 15.

Similarly, the other peaks were assigned to the tBDMS derivatives of glycerides having a short-chain acyl group (**Table 1**). The amounts of various species of PAF and acyl PAF identified in this study were quantified as listed in Table 1.

TABLE 1. Molecular composition of PAF and acyl PAF in a lipid extract from bovine brain

Molecular Species	Peak Number on GLC (Fig. 2)	Retention Time	$[M-C(CH_3)_3]^+$	Amount
sn-1/*sn*-2		*min*	*m/z*	*μg/cerebrum*
[A] PAF				
16:0/2:0	2	7.6	415	2.6
18:1/2:0	7	9.9	441	2.1
18:0/2:0	8	10.3	443	2.9
[B] acyl PAF				
14:0/2:0	1	6.7	401	2.8
16:1/2:0	3	8.2	427	1.6
16:0/2:0	4	8.6	429	81
16:0/3:1	5	9.5	441	7.5
16:0/3:0	6	9.6	443	5.4
16:0/4:0	9	11.0	457	11
2:0/18:1	10	11.2	455	45
18:1/2:0	11	11.5	455	
18:0/2:0	13	12.0	457	13
16:0/5:0	14	12.8	471	2.3
18:0/3:1	15	13.7	469	3.8
18:0/3:0	16	13.8	471	1.4
4:0/18:1	17	14.8	483	6.8
18:1/4:0	19	15.1	483	
16:0/6:0	18	14.9	485	3.2
18:0/4:0	21	15.8	485	2.3
16:0/7:0	22	17.7	499	1.7

DISCUSSION

Our results demonstrated that Depressor-IA is a mixture of PAF and related compounds. Almost all these phospholipids have some vasodepressor activity in rats, but the vasodepressor activity of Depressor-IA can mainly be ascribed to three species of PAF identified in this study, because the activity of PAF has been reported to be 100 times or more that of the corresponding 1-long-chain acyl-2-acetyl-GPC in rats (1), and because the total amount of the latter in the lipid extract from bovine brain was about 20 times that of the former. In addition, it has been reported that an increase in the chain length of 1-palmitoyl-2-short-chain acyl-GPC resulted in progressive reduction in its hypotensive activity (18). Thus, 1-long-chain acyl-2-short-chain acyl-GPCs other than ones having an *sn*-2-acetyl group may contribute little to the vasodepressor activity of Depressor-IA. These results are consistent with our previous findings of no significant reduction in the depressor activity of Depressor-IA on its treatment with lipase from *Rhizopus arrhizus*, which hydrolyzes 1-acyl type of choline phospholipids, but not PAF (19).

Satouchi et al. (12, 13) previously detected three 1-long-chain acyl-2-acetyl-GPCs together with the corresponding PAFs in rabbit and human neutrophils by GLC-MS. By high-performance liquid chromatographic analysis of phospholipids having a [^{3}H]acetyl group, Mueller, O'Flaherty, and Wykle (20) also obtained evidence that several 1-long-chain acyl-2-acetyl-GPCs were synthesized concomitantly with various molecular compounds of PAF in human and rabbit neutrophils. Both research groups reported that the total amounts of 1-acyl-2-acetyl-GPC were less than that of PAF in rabbit and human neutrophils. However, the total amount of 1-long-chain acyl-2-acetyl-GPC was about 20 times more than that of PAF in the bovine brain lipid extract. Furthermore, novel molecular analogues having an *sn*-2-short-chain acyl group other than an acetyl group coexisted with 1-long-chain acyl-2-acetyl-GPC. This characteristic molecular heterogeneity of vasodepressor phospholipid in bovine brain lipid extract might be, in part, due to the

heterogeneous cells in brain tissue, which are able to synthesize PAF in response to unknown stimuli. Further studies are needed on this point.

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