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EVIDENCE FOR EXISTENCE OF VARIOUS HOMOLOGUES AND ANALOGUES OF PLATELET ACTIVATING FACTOR IN A LIPID EXTRACT OF BOVINE BRAIN

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Vasodepressor phospholipid with platelet-aggregating activity was highly purified from a lipid extract of bovine brain and subjected to field desorption-mass spectrometry. It was further analyzed by gas-liquid chromatography-mass spectrometry after hydrolysis with phospholipase C and conversion to tert-butyldimethylsilyl derivatives. Results indicated the presence of four species of platelet activating factor(1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, PAF) and ten acyl analogues of PAF. The acyl analogues of PAF included species having an sn-2-propionyl or sn-2-butyryl group, which have not been previously detected in natural sources. The total amount of acyl analogues of PAF was much higher than that of PAF.

Martini et al.(1, 2) reported the existence of an unknown vasodepressor substance in an acetone extract of nervous tissues from various animals. Its chemical nature long remained unknown, but in 1976 we confirmed and expanded their findings by demonstrating vasodepressor activity in a phospholipid fraction from a lipid extract of bovine brain(3). We found that the highly purified vasodepressor, which we tentatively named Depressor-I, resembled lysolecithin in chemical nature, judging from its chromatographic behaviors(3). Not one of the synthetic lysolecithins that we tested, however, was identical to Depressor-I with potent hypotensive activity(4). Thus we speculated the existence of a new type of biologically active phospholipid(5,6).

In the late of 1970's two groups(7, 8) identified PAF released from immunoglobulin E-sensitized rabbit basophils as a unique phospholipid, 1-O-alkyl-2-

Abbreviations: PAF, Platelet-activating factor(1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine; acyl PAF, acyl analogue of PAF(1-long-chain acyl-2-short-chain-acyl-sn-glycero-3-phosphocholine); 16:0, sn-1-O-hexadecyl or sn-1-palmitoyl; 18:0, sn-1-O-octadecyl or sn-1-stearoyl; 2:0, sn-2-acetyl; 3:0, sn-2-propionyl; 4:0, sn-2-butyryl; tBDMS, tert-butyldimethylsilyl.

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acetyl-sn-glycero-3-phosphocholine. Shortly afterwards, Blank et al.(9) reported that semi-synthetic 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine mimicked the biological activities of an unidentified antihypertensive phospholipid derived from the renomedullary polar lipid fraction after chemical treatment. PAF has now been shown to have diverse biological activities, and is considered to be an important chemical mediator in certain pathophysiological processes, such as acute inflammatory reactions(10, 11). We have been purifying hypotensive phospholipid from a bovine lipid extract, and very recently reported that this extract contains at least two types of active phospholipids, Depressor-IA and IB(12). The former has platelet-aggregating activity and is similar in chemical nature to PAF, whereas the latter differs in the following biological and chemical properties from PAF: 1) On treatment with lipase it is inactivated whereas PAF and Depressor-IA are not. 2) Treatment with diazomethane modifies its weak hypotensive and platelet-aggregating activities; the diazomethane-treated Depressor-IB induces a sharp drop in the blood pressure of rats and inhibits PAF-induced aggregation of rabbit platelets.

In this study, we achieved satisfactory separation of PAF-like Depressor-IA from the diazomethane-sensitive Depressor-IB, and so could analyze highly-purified Depressor-IA by MS. Results show that Depressor-IA consisted of various species of PAF and acyl PAF, including those with an sn-2-propionyl or sn-2-butyryl moiety, which have not been previously detected in natural materials.

MATERIALS AND METHODS

Extraction of complex lipids from bovine cerebra. Ox brain(400-450g) was promptly removed from animals in a slaughter-house and placed on ice. Cerebral blood vessels and mucus membranes were removed as completely as possible, and then the cerebra were homogenized with acetone(2 liters) in a blender. These processes were completed within 4 hours after death of the animals. The homogenate was filtered, and cerebral lipids were extracted from both the acetone-soluble fraction and acetone-insoluble fraction by the method of Folch et al.(13). The lipid extracts were combined and evaporated to dryness. The residue was mixed with 1 liter of ethanol, and ethanol-soluble material was partitioned between n-hexane and 80 % aqueous ethanol to separate complex lipids from neutral lipids.

Structural analysis of vasoactive phospholipids by FD-MS. Vasodepressor phospholipids in the complex lipid fraction recovered in aqueous ethanol from 40 bovine cerebra were purified as described previously(5, 12). Briefly, the complex lipids were roughly fractionated by silicic acid column chromatography(5). Fractions expressing hypotensive activity were pooled and subjected

to a second silicic acid column chromatography(5). Active fractions were again pooled and then purified by Sephadex LH-20 column chromatographies(5). Active fractions eluted from the second Sephadex LH-20 column contained Depressor-IA and IB. These were separated on a cellulose column as described previously(12). Active fractions containing Depressor-IA were finally purified by preparative TLC on silica gel 60(Merck) with a solvent system of chloroform-methanol-water(65:35:5, by vol.). The highly-purified Depressor-IA was analyzed by FD-MS in a Hitachi M-80 mass spectrometer under the following conditions: An emitter current of 20-30 mA, an emitter voltage of 3.0 kV, a cathod voltage of -3.2 kV and an accelerating voltage of 3.0 kV.

Identification of PAFs and acyl PAFs by GC-MS. Certain amounts of deuterated $PAF_{16:0-2:0}$ and acyl $PAF_{16:0-2:0}$ were added to complex lipids from bovine cerebrum, and partially purified by silicic acid column chromatographies as described above. The Depressor-IA fraction was further purified by reverse-phase TLC on Silica gel 60(Merck) with a solvent system of acetone-methanol-water(1:2:1, by vol.). The lipids were located by spraying the plates with a solution of 6-p-toluidino-2-naphthalene sulfonic acid, and the plates were divided into several zones with the aid of phospholipid standards ($PAF_{16:0-2:0}$, acyl $PAF_{16:0-2:0}$, phosphatidylcholine, sphingomyelin and lyso-phosphatidylcholine). The phospholipids to be analyzed were extracted from the silica by the method of Bligh and Dyer(14). Then the active phospholipids were hydrolyzed with phospholipase C(Bacillus cereus), and the resultant glycerides were converted to their tBDMS derivatives. The tBDMS derivatives were then purified by TLC as described by Oda et al.(15) except that the derivatization was done at 150°C for 30 min. For identification of PAFs, the purified Depressor-IA was hydrolyzed with lipase (Rhizopus arrihizus) at 37°C for 60 hours to decompose acyl PAFs in the preparation. The remaining PAFs that were resistant to lipase treatment were extracted from the incubation mixture, hydrolyzed with phospholipase C and derivatized as described above.

The tBDMS derivatives were analyzed in a JEOL JMS-D300 mass spectrometer coupled with a gas chromatograph with a glass column(1.5 m x 2 mm I.D.) packed with 1.5 % OV-1 on Gas Chrom Q(80-100 mesh). The column was maintained at 270°C and developed at a flow rate of 40 ml/min. The temperatures of the injection port and separator were 300°C and 290°C, respectively. Mass spectrometry was done under the following conditions: A 20 eV-ionizing voltage and an ion source temperature of 230°C. The amounts of PAFs and acyl PAFs in the bovine brain lipid extract were estimated by quantification of tBDMS derivatives from the corresponding parent phospholipids by selected ion monitoring of $[M-57]^+$ against internal standards as described elsewhere (15). Various authentic PAFs and acyl PAFs were prepared by treatment of the corresponding lyso-compounds(2-5 mg) with 1 ml of acetic anhydride, perdeuteroacetic anhydride, propionic anhydride or butyric anhydride at room temperature for 1-2 sec after addition of one drop of perchloric acid(60 %, w/v) essentially as described by Kumar et al.(16).

Biological activities of Phospholipids. The hypotensive activities of active fractions from the bovine brain lipid extract were measured as described previously(3). Their platelet-aggregating activities were also tested with both washed rabbit platelets and rabbit platelet-rich plasma as described elsewhere (12).

RESULTS AND DISCUSSION

Our first aim in this study was to obtain highly-purified Depressor-IA, a PAF-like material, in sufficient amount for analysis by FD-MS. For this purpose, phospholipids were extracted from 40 bovine cerebra, and purified by chromatographies monitoring with hypotensive and platelet-aggregating activi-

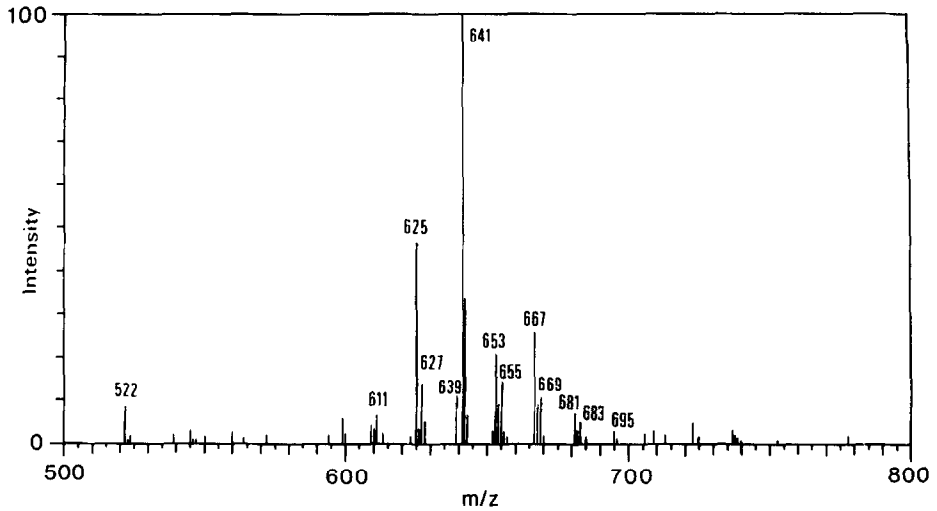


Figure 1. FD-MS of purified Depressor-IA.

ties. The final yield of Depressor-IA was about 15 μg as phosphorus. The FD-MS of the preparation is shown in Figure 1. As expectedly, the spectrum suggested the presence of PAFs and acyl PAFs, but the molecular composition appeared complicated. Thus, careful interpretation of the mass spectrometric data was necessary. Under our conditions, authentic $PAF_{16:0-2:0}$ and acyl $PAF_{16:0-2:0}$ gave base peaks assignable to $[M + \text{choline}(104)]^+$ together with very small ions such as $[M + H]^+$, $[M + H - CH_3CO]^+$, $[M + \text{phosphocholine}(183)]^+$ and $[\text{choline}]^+$. On the basis of $[M + \text{choline}]^+$, the observed ions in the spectrum were tentatively assigned as shown in Table 1. For example, the highest peak at m/z 641 can be regarded as $[M + 104]^+$ of $PAF_{17:0-2:0}$ or acyl $PAF_{16:0-2:0}$. Furthermore, it seemed possible that $PAF_{16:0-3:0}$, $PAF_{15:0-4:0}$, acyl $PAF_{15:0-3:0}$ or acyl $PAF_{14:0-4:0}$ might be responsible for the observed ion at m/z 641, although their existence in natural materials has not yet been reported. These results indicated that Depressor-IA consisted of various species of PAFs and acyl PAFs, but more direct evidence for this was required. Therefore, we prepared deuterated $PAF_{16:0-2:0}$ and acyl $PAF_{16:0-2:0}$ as internal standards, and mixed with the complex lipids from bovine cerebrum. Partially purified Depressor-IA was subjected to hydrolysis with phospholipase C, and the resultant glycerides were converted to tBDMS derivatives and analyzed by

Table 1
Possible assignments of $[M+104]^+$ in FD-MS of purified Depressor-IA and of $[M-57]^+$ in EI-MS of its tBDMS derivatives

Ions		1-O-alkyl species[a]			1-acyl species[b]		
$[M+104]^+$	$[M-57]^+$	acetyl[c]	propionyl	butyryl	acetyl	propionyl	butyryl
m/z							
611	399	15:1[d]	14:1	13:1	14:1	13:1	12:1
613	401	15:0	14:0	13:0	14:0	13:0	12:0
625	413	16:1	15:1	14:1	15:1	14:1	13:1
627	415	16:0	15:0	14:0	15:0	14:0	13:0
639	427	17:1	16:1	15:1	16:1	15:1	14:1
641	429	17:0	16:0	15:0	16:0	15:0	14:0
653	441	18:1	17:1	16:1	17:1	16:1	15:1
655	443	18:0	17:0	16:0	17:0	16:0	15:0
667	455	19:1	18:1	17:1	18:1	17:1	16:1
669	457	19:0	18:0	17:0	18:0	17:0	16:0
681	469	20:1	19:1	18:1	19:1	18:1	17:1
683	471	20:0	19:0	18:0	19:0	18:0	17:0
695	483	21:1	20:1	19:1	20:1	19:1	18:1
697	485	21:0	20:0	19:0	20:0	19:0	18:0

[a] 1-O-long-chain alkyl-2-short-chain acyl-sn-glycero-3-phosphocholines or their sn-3-tBDMS derivatives
[b] 1-long-chain acyl-2-short-chain acyl-sn-glycero-3-phosphocholines or their sn-3-tBDMS derivatives
[c] a short-chain acyl moiety at an sn-2-position
[d] Chain length:degree of unsaturation in an sn-1-long-chain alkyl or acyl moiety

GC-MS。 Figure 2-A shows tracings of the ion-current profiles at m/z 401, 427, 429, 443, 455, 457, 471, 483 and 485, which corresponded to $[M-57]^+$ of tBDMS derivatives of various PAF and acyl PAFs(see Table 1), respectively. The EI-MS at the points shown as a-h on these tracings are given in Figure 3. These spectra suggest that the major peak(s) on each tracing was due to the tBDMS derivative of acyl PAF shown on the right of Figure 2-A. Data on the retention times of authentic tBDMS derivatives from acyl PAFs(Figure 4) supported these assignments。 Figure 2-B shows the ion-current profiles at m/z 415 and 443 in the EI-MS of the tBDMS derivative of Depressor-IA pretreated with lipase to remove acyl PAFs. The mass spectra at i and j in Figure 2-B, which were due to $PAF_{16:0-2:0}$ and acyl $PAF_{18:0-2:0}$, respectively, are shown in Figure 3. Although tBDMS derivatives of glycerides with an sn-1-saturated long-chain moiety were poorly separated from the corresponding tBDMS derivative having an sn-1-monounsaturated long-chain moiety with the same chain length under our GC conditions, mass spectrometric data indicated that 10 acyl PAFs, including species having

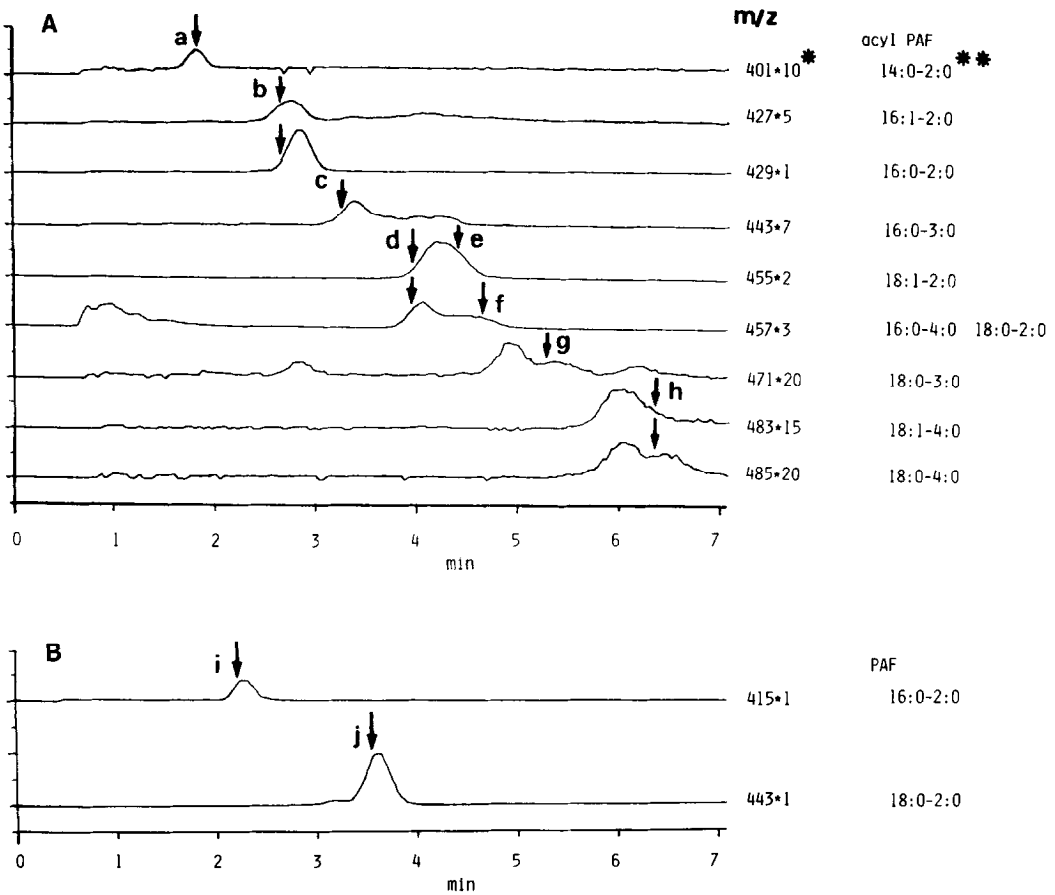


Figure 2. Ion-current profiles of diagnostic ions in EI-MS of tBDMS derivatives of purified Depressor-IA. A: Various ions that can be assigned to $[M-57]^+$ of tBDMS derivatives of acyl PAFs in purified Depressor-IA were selected, and their ion-current profiles are shown. B: Purified Depressor-IA was hydrolyzed with lipase to decompose various acyl PAFs, and the remaining PAFs were converted to tBDMS derivatives and subjected to GC-MS. * magnification for intensity of ion. ** assignment of tBDMS derivatives of acyl PAF and PAF.

an sn-2-propionyl or sn-2-butyryl group, were present in the lipid extract from bovine cerebra as well as much smaller amounts of PAFs. Many of these acyl PAFs and four species of PAFs could be quantified by the selected ion monitoring technique(15) for the respective $[M-57]^+$. Table 2 shows the results, expressed as μg per bovine cerebrum. Thus, the occurrence of μg levels of PAFs and much higher amounts of acyl PAFs from ox cerebrum 4 hours after death was shown, and the molecular species of PAFs and acyl PAFs were much more numerous than those found in extracts of other tissues or cells(17-20). Furthermore, we identified sn-2-propionyl or sn-2-butyryl homologues of acyl PAFs in bovine cerebrum which

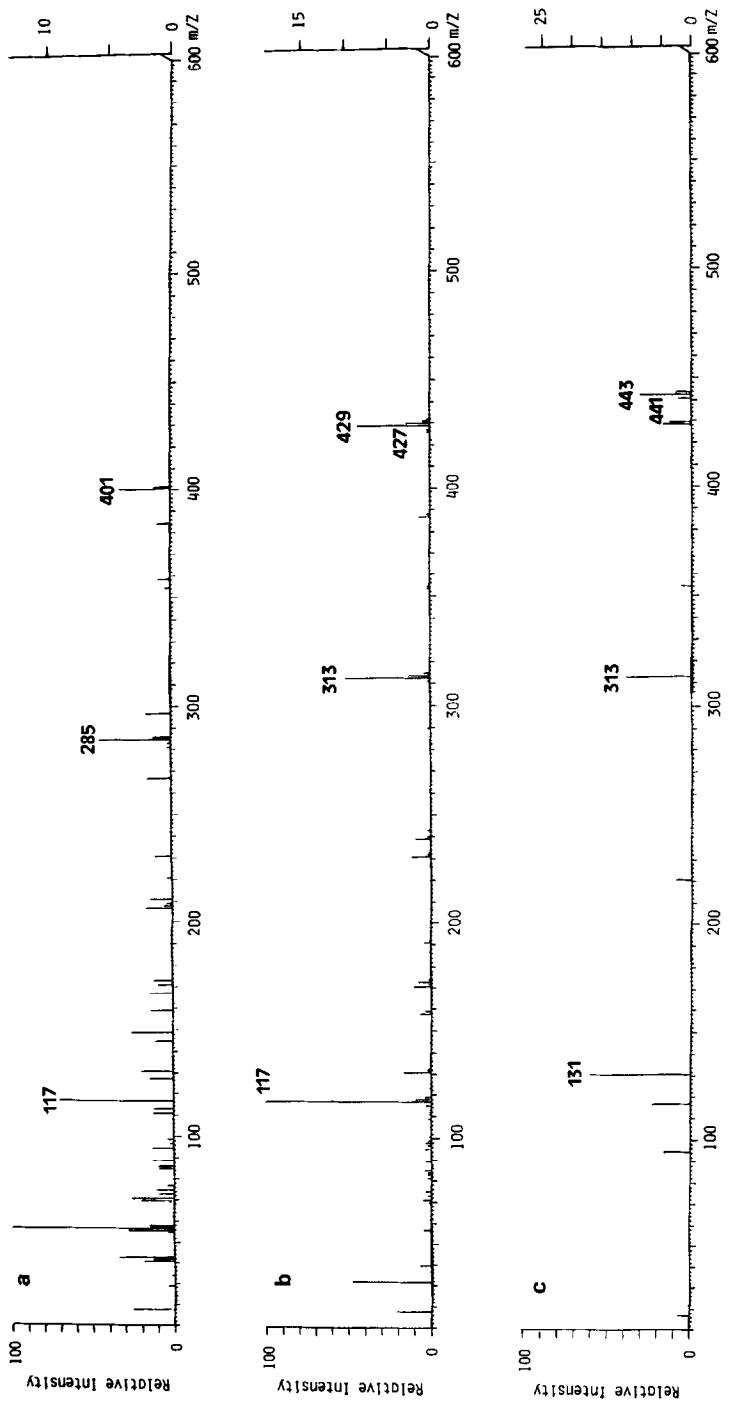
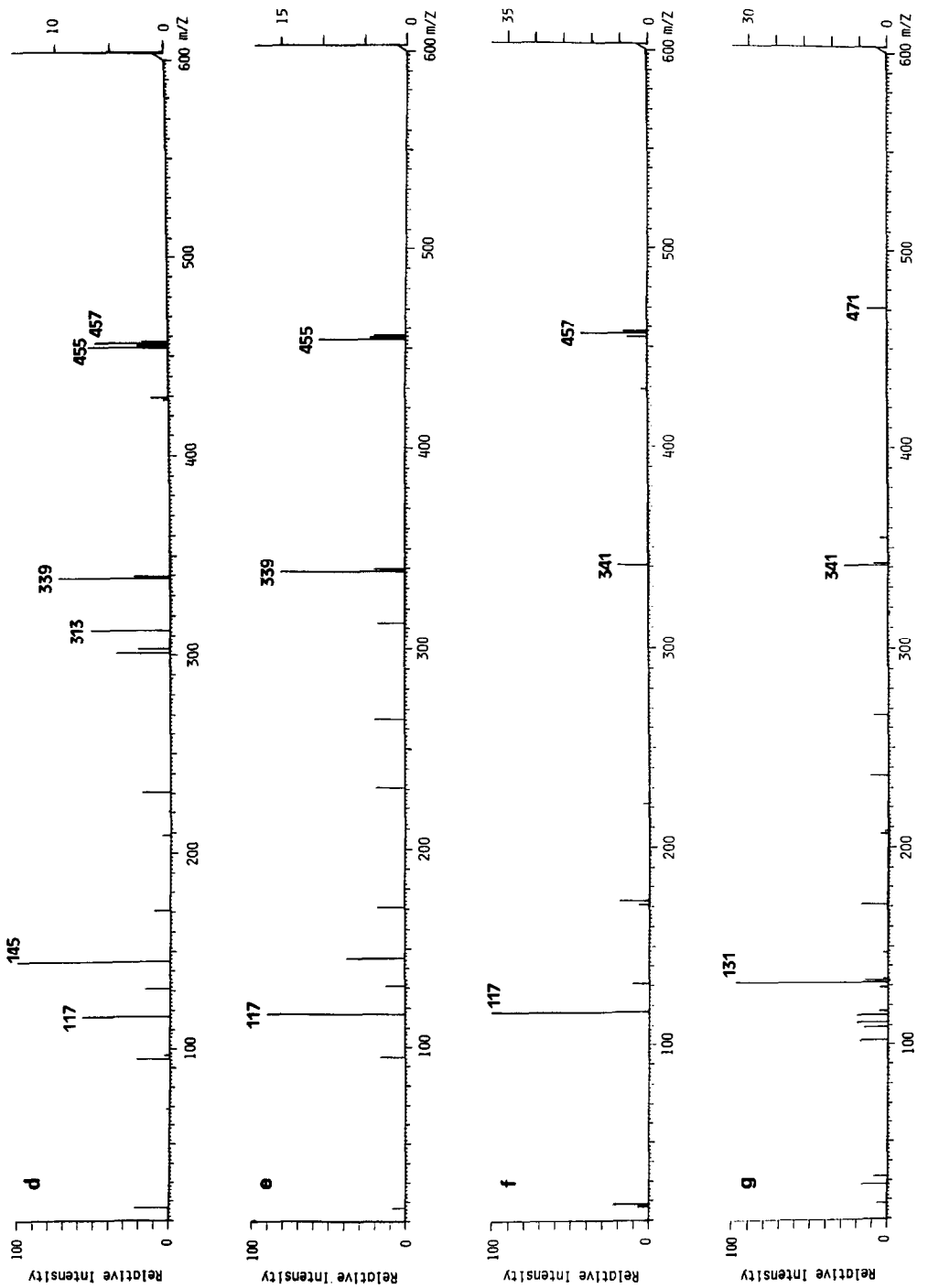


Figure 3. EI-MS of various components of the tBDMS derivatives of purified Depressor-IA. EI-MS measured at points a-j on Figure 2 are shown. These spectra were due to tBDMS derivatives of the following acyl PAFs and PAFs: a) acyl $PAF_{14:0-2:0}$ b) acyl $PAF_{16:0-2:0}$ and a trace amount of acyl $PAF_{16:1-2:0}$ c) acyl $PAF_{16:0-3:0}$ d) acyl $PAF_{16:0-4:0}$ and a trace amount of acyl $PAF_{18:1-2:0}$ e) acyl $PAF_{18:1-2:0}$ f) acyl $PAF_{18:0-2:0}$ g) acyl $PAF_{18:0-3:0}$ h) acyl $PAF_{18:0-4:0}$ and acyl PAF 18:1-4:0 i) $PAF_{16:0-2:0}$ and j) $PAF_{18:0-2:0}$ and a trace amount of $PAF_{18:1-2:0}$.


d
e
f
g
Relative Intensity
100
0
m/z
600
500
400
300
200
100
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10
15
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30
145
117
339
313
455
457
341
471
131

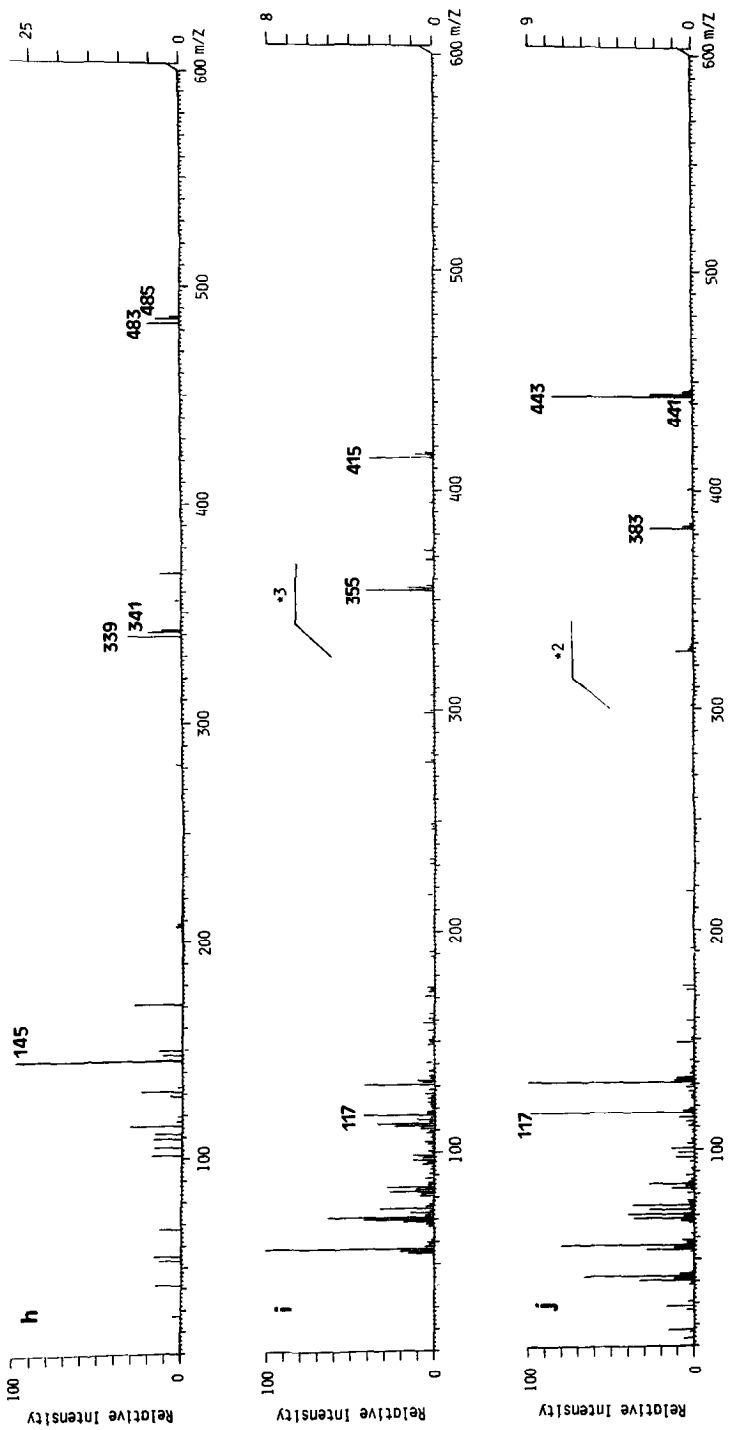

Figure 3. — Continued.

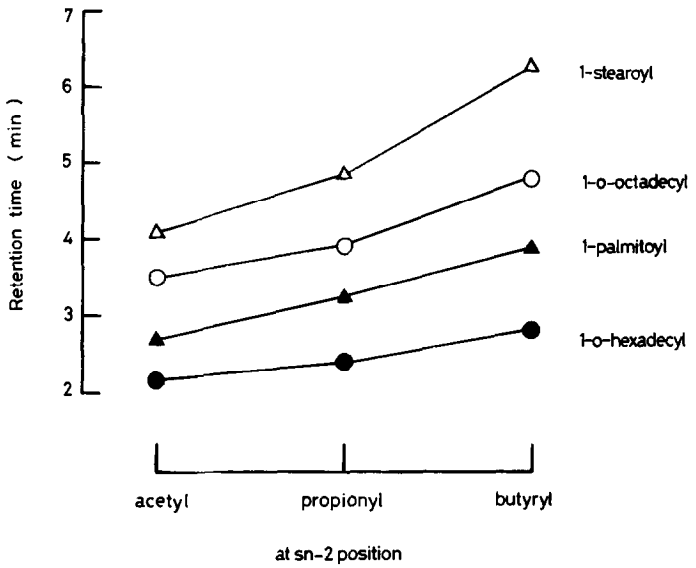

Figure 4. Retention times of tBDMS derivatives of authentic 1-O-long-chain alkyl(or acyl)-2-short-chain acyl-sn-glycero-3-phosphocholines.

have not been previously detected in animal tissues, body fluids or cells. However, there was no evidence for the presence of corresponding species of PAF homologues. The question on the types of cells and stimuli that were responsible for the production of PAF and, or acyl PAF in bovine cerebra remains yet undissolved. Attempts to clarify the problem are in progress.

Table 2
Molecular species of PAF and acyl PAF in lipid extract from bovine cerebra

	Species									
sn-1	14:0	16:1	16:0	16:0	16:0	18:1	18:0	18:0	18:1	18:0
sn-2	2:0	2:0	2:0	3:0	4:0	2:0	2:0	3:0	4:0	4:0
	μg/cerebrum									
PAF[a]	-[c]	0.1[d]	2.5	-	-	0.16[d]	7.5	-	-	-
acyl PAF[b]	5.5	5.2[d]	236	26	52	174	46	4.7	6.3	7.5

[a]Amounts of various PAFs were determined by selected ion monitoring of $[M-57]^+$ of tBDMS derivatives from lipase-treated purified Depressor-IA.
[b]Amounts of various acyl PAFs were determined by selected ion monitoring of $[M-57]^+$ of tBDMS derivatives from purified Depressor-IA without treatment with lipase.
[c]not detected
[d]Because authentic samples of these species were not available, values were calculated tentatively based on the ratio of the peak area of the species to the peak area of tBDMS derivatives of the internal standards, deuterated $PAF_{16:0-2:0}$ and acyl $PAF_{16:0-2:0}$.

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