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Isolation, Chemical Characterization, and Subcellular Distribution of 1-*O*-Alkyl-2-Acetyl-*sn*-Glycerol in *Tetrahymena pyriformis* Cells[1]

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1-*O*-Hexadecyl-2-acetyl-*sn*-glycerol, the immediate precursor of platelet-activating factor (PAF) in its *de novo* formation, was detected in the protozoon *Tetrahymena pyriformis*. It was purified from the total lipid extract by TLC, after successive developments in two different solvent systems. Characterization was assessed by (a) gas-liquid chromatography with electron capture detection, and (b) gas chromatography combined with mass spectrometry in selected ion monitoring mode, after derivatization with heptafluorobutyric acid anhydride and *tert*-butyldimethylchlorosilane/imidazole, respectively. Its quantity was found to be 0.1 nmol/10^7 cells from the GC-MS, using authentic alkylacetylglycerol as external standard. Cell fractionation revealed that alkylacetylglycerol is located exclusively in the microsomal fraction of the protozoon. Previously, we have reported the occurrence of PAF in the microsomal fraction, as well as a dithiothreitol-insensitive CDP-choline: cholinephosphotransferase activity that utilizes exogenous alkylacetylglycerol as substrate in the mitochondrial and microsomal fractions. The above findings indicate that PAF can be formed in the cell by the *de novo* pathway.

Key words: alkylacetylglycerol, diacylglycerols, GC-MS, platelet-activating factor, *Tetrahymena pyriformis*.

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Alkylacetylglycerols are known as the immediate precursors in the *de novo* biosynthesis of 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) (*1*). Platelet-activating factor, a phospholipid with a wide spectrum of biological activities (*2*), is biosynthesized by two distinct pathways: (a) "remodelling," which is activated by inflammatory stimuli; and (b) *de novo*, considered responsible for the maintenance of endogenous levels of PAF under physiological conditions (*3*). In its last part, the *de novo* pathway involves a DTT-insensitive CDP-choline:cholinephosphotransferase activity that catalyzes the addition of phosphocholine to the third position of 1,2-alkylacetylglycerols. Although activity of this enzyme has been detected in various rat tissues (*4-6*), in rabbit platelets (*7*), HL-60 cells (*8*), and in *Tetrahymena pyriformis* (*9*), endogenous levels of alkylacetylglycerols have not been identified yet in the above systems.

Alkylacetylglycerols themselves are a class of biologically active lipids, since they are known to reduce blood pressure and to aggregate platelets (*1*). The latter response is probably due to conversion to PAF by a PAF-specific CDP-choline:cholinephosphotransferase (*10*), and anticipates AAG internalization. It has a different duration, a different biological half time, and for comparable results, the required concentration of AAG is 10^3 times higher than that of PAF. The presence of Ca^{2+} in the incubation medium is not an absolute requirement, but it accelerates the manifestation of the biological effect. Alkylacetylglycerols stimulate differentiation of HL-60 to macrophage-like cells (*11*) and affect vascular smooth muscle cell proliferation in a similar way to PAF (*12*). They also activate protein kinase C in vascular smooth muscle cells and in NB41A3 neuroblastoma cells and compete for phorbol ester binding sites (*13*). Various 1,2-diacylglycerols, structural analogs of alkylacetylglycerols, have been shown to regulate protein kinase C in human platelets (*14*). Interestingly, incubation of the synthetic, membrane-permeable 1-oleyl-2-acetyl-*sn*-glycerol *in vitro* with platelets, in concentrations above 50 μg/ml, results in serotonin release without ionophore-mediated Ca^{2+} fluxes (*15, 16*), unlike PAF.

Alkylacetylglycerols are not susceptible to serum acetylhydrolase that utilizes PAF as substrate. A different, membrane-bound acetylhydrolase activity related to the *de novo* pathway, acting on alkylacetylglycerols, has been characterized in Erlich ascites cells (*17*).

We have previously reported the occurrence of PAF in the microsomal fraction of *T. pyriformis* under physiological conditions (*18*), as well as the presence of a CDP-choline:cholinephosphotransferase that mediates PAF formation (*9*). This enzymic activity is predominantly distributed in the mitochondrial and microsomal fractions of the protozoan. The aim of the present work was to detect alkylacetylglycerol in *T. pyriformis* and to investigate its

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Abbreviations: AAG, 1-*O*-alkyl-2-acetyl-*sn*-glycerol, alkylacetylglycerol; PAF, platelet-activating factor; HFBA, heptafluorobutyric acid anhydride; TBDMS, *tert*-butyldimethylsilyl; TBDMCS/Im, *tert*-butyldimethylsilylchloride/imidazole; DTT, dithiothreitol; TNS, 6-*p*-toluidine-2-naphthalene sulfonic acid; CDP-, cytidylyldiphospho-; R_f, relative to the front; EI, electron impact; ECD, electron capture detector; MSD, mass selective detector; MS, mass spectrometry; SIM, selected ion monitoring.

subcellular distribution, in order to provide direct evidence for the *de novo* formation of PAF in the cell.

AAG was purified from lipid extracts by TLC, using two different solvent systems, and analyzed by GLC with ECD, and MSD, after derivatization with heptafluorobutyric acid anhydride and TBDMCS/Im, respectively. Quantitative estimation was made by using authentic AAG as external standard in GC-MS.

EXPERIMENTAL PROCEDURES

Materials—1-*O*-Hexadecyl-2-acetyl-*sn*-glycerol was from NovaBiochem (Switzerland); TBDMCS/Im reagent was obtained from Alltech (Switzerland); TNS was from Sigma (St. Louis, USA). Heptafluorobutyric acid anhydride was from Pierce-Warringer (UK); solvents were of analytical or HPLC grade, LabScan (Ireland); Silica Gel G precoated TLC plates No. 5721, without fluorescence indicator, from Merck (Germany), were pre-washed in the most polar solvent system and activated before use.

Purification and Quantitative Determination of Commercial AAG—Commercial AAG was purified by TLC with chloroform-methanol-acetic acid (95 : 5 : 1, v/v/v). The relevant R_f values of authentic neutral lipid classes were as follows: triglycerides, 0.90; 1,3-diglycerides, 0.83; 1,2-diglyceride, 0.76; 1,3-alkylacetylglycerol, 0.74; 1,2-alkylacetylglycerol, 0.64; cetyl alcohol, 0.61; fatty acids, 0.55; batyl alcohol, 0.38; and monoglycerides, 0.34. Both 1,2- and 1,3-isomers of alkylacetylglycerol were extracted together for derivatization, in order to avoid further isomerization and subsequent loss of the 1,2-isomer, which is the "active" form involved in PAF biosynthesis. Quantitative determination was performed according to Ref. *19*, using batyl alcohol as reference compound. In this method, sodium periodate reacts with the vicinal hydroxyl groups of glyceryl ethers after alkaline hydrolysis, to produce formaldehyde, which is determined colorimetrically after it is complexed with chromotropic acid.

Purification of Lipids from Tetrahymena pyriformis—*T. pyriformis* cells were grown axenically and harvested at the late log phase according to Ref. *18*. Cells were fractionated into membrane, mitochondrial, and microsomal fractions and cytosol as described in detail elsewhere (*9*). Total lipids were extracted according to Ref. *20* and lipid phosphorus was determined according to Ref. *21*. Separation of lipid classes was performed by two successive TLC systems: the first is as described above. The region comigrating with authentic AAG was scraped off the plate and extracted. The lipid residue was re-chromatographed on TLC using *n*-hexane-diethylether-methanol-acetic acid (80 : 20 : 5 : 1, v/v/v/v). The relevant R_f values of authentic neutral lipid classes were: triglycerides, 0.74; fatty acids, 0.52; 1,3-diglycerides, 0.29; 1,2-diglyceride, 0.26; cetyl alcohol, 0.20; 1,3-alkylacetylglycerol, 0.15; 1,2-alkylacetylglycerol, 0.12; batyl alcohol, 0.06 and monoglycerides, 0.03.

Preparation of Heptafluorobutyroyl Derivatives—Lipid residue, purified by TLC as described above, was taken to dryness in a screw-capped tube and recovered in 50 μl of toluene-5% triethylamine. Then, 10 μl of HFBA was added, the atmosphere was purged with N_2, and the tube, tightly closed, was placed in a 70°C waterbath for 10 min. The reaction was stopped with 1 ml of 2.5% ammonia and the products were extracted twice with 0.5 ml of toluene. The organic phase was dried down and the residue was recovered in 0.5 ml of *n*-hexane. A blank reference sample without AAG was prepared by the same procedure.

Gas Chromatographic Analysis of 1-O-Hexadecyl-2-Acetyl-3-Heptafluorobutyroyl-sn-Glycerol—Analysis was performed on a Shimadzu 14A GC equipped with electron capture detector (ECD). Column type: methylsilicone fused silica capillary column, 30 m × 0.32 mm i.d. Film thickness: 0.25 μm. Column temperature: 240°C. Injector temperature: 260°C. Detector temperature: 270°C. Carrier gas: helium, at a flow rate of 8 ml/min.

Preparation of TBDMS Derivatives—Lipid residue, purified by TLC as described above, was taken to dryness in a screw-capped tube and 100 μl of TBDMCS/Im was added to each sample. The tube was sealed and heated at 70°C for 3 h, under continuous stirring. The excess of the reagent was removed under nitrogen with the aid of small quantities of methanol, and the residue was partitioned in chloroform-water (1 : 1, v/v). The chloroform phase was washed twice with water and dried down. The residue was recovered in 0.5 ml of *n*-hexane. A blank reference sample without AAG was prepared by the same procedure.

Gas Chromatographic Analysis of TBDMS Derivatives—Analysis was performed on a Hewlett-Packard model 5890B equipped with mass selective detector 5971A, interfaced to a model 59970C data system (Palo Alto, CA). Column type: HP-5 fused silica capillary column, coated with 5% phenyl-95% methyl polysiloxane, 25 m × 0.25 mm i.d. (Hewlett-Packard, Palo Alto). Carrier gas: helium, at 83 kPa. An automatic injection system was used. Column temperature: 250°C. Injection temperature: 260°C. The ion source was maintained at 200°C and EI mass spectra were obtained at 70 eV.

RESULTS

AAG was measured in *T. pyriformis* cells. Total lipids, corresponding to 1 mg of lipid phosphorus, were subjected to TLC, using chloroform-methanol-acetic acid (95 : 5 : 1, v/v/v). The lipid residue migrating to R_f from 0.63 to 0.75, corresponding to authentic 1,2- and 1,3-alkylacetylglycerol, was scraped off the plate and extracted according to Bligh and Dyer (*20*). Being efficiently separated from fatty acids, fatty alcohols, and 1-*O*-alkyl-glycerols (for R_f values see "EXPERIMENTAL PROCEDURES"), it was further purified after a second TLC development with *n*-hexane-diethylether-methanol-acetic acid (80 : 20 : 5 : 1, v/v/v/v) (for R_f values see "EXPERIMENTAL PROCEDURES"). The R_f of authentic AAG in that case was 0.12. The relevant region from the lipids of the protozoan was extracted and used for chemical characterization. A first approach was to derivatize the purified lipid residue with HFBA and analyze it by GLC-ECD. Figure 1 shows a typical gas chromatogram of the molecule (B) with a peak at 40 min, at the same retention time as authentic AAG (A). A separate lipid preparation was derivatized with TBDMCS/Im. This reagent can also be applied directly to the hepta-fluorobutyroyl derivatives (*22*). The product of the reaction was analyzed by GC-MS. Authentic AAG was eluted at 13.139 min (Fig. 2A). Its scan mass spectrum (Fig. 2B) revealed the following main fragments under our experimental conditions: *m/z* 415, $[M-(tert\text{-butyl})]^+$; *m/z* 355, $[M-(tert\text{-butyl}+\text{acetic acid})]^+$; *m/z* 217, $[M-(CH_2\text{-}O\text{-}C_{16}\text{-}$

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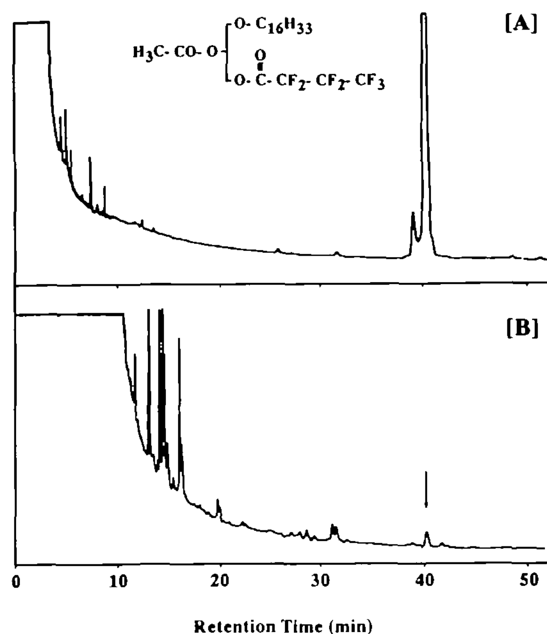

Fig. 1. **Gas-liquid chromatograms of heptafluorobutyroyl derivatives.** A: Authentic alkylacetylglycerol from NovaBiochem after TLC purification. B: The lipid fraction from *Tetrahymena pyriformis* co-migrating on TLC with authentic AAG. Gas chromatograph, Shimadzu 14A. Column type: fused silica capillary column, methyl silicone, 30 m×0.32 mm i.d., 0.25 μm film thickness. Detector: ECD. Injection temperature: 200°C. Column temperature: 240°C. Detector temperature: 270°C. Carrier gas: helium, at a flow rate of 8 ml/min.

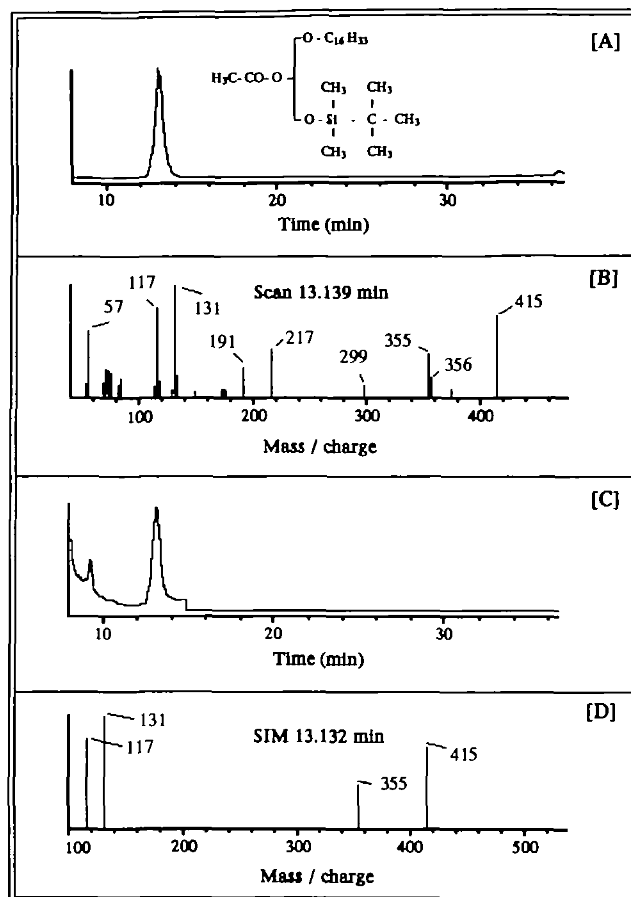

Fig. 2. **GC-MS of TBDMS derivatives.** A: Total ion chromatogram of authentic AAG from Nova Biochem, obtained by full scan. B: The relevant mass spectrum at 13.139 min. C: Total ion chromatogram of the lipid residue from *T. pyriformis* comigrating on TLC with authentic AAG. D: The relevant mass spectrum obtained by SIM mode, at 13.132 min. Gas chromatograph: Hewlett-Packard Mo. 5890B equipped with mass selective detector 5971A, interfaced to a Mo. 59970C data system (Palo Alto, CA). Column type: HP-5 fused silica capillary column, coated with 5% phenyl-95% methyl polysiloxane, 25 m×0.25 mm i.d. (Hewlett-Packard, Palo Alto). Carrier gas: helium, at 83 kPa. An automatic injection system was used. Column temperature: 250°C. Injection temperature: 260°C. The ion source was maintained at 200°C and EI mass spectra were obtained at 70 eV.

$H_{33})]^+$; *m/z* 131, $[TBDMSO]^+$; *m/z* 117, $[O\text{-}CH\text{-}CH_2O\text{-}Si(CH_3)_2]^+$; *m/z* 74, $[O\text{-}Si(CH_3)_2]^+$, and *m/z* 57, [*tert*-butyl]$^+$. The molecular ion corresponding to *m/z* 472 does not appear. For *T. pyriformis* lipid extract, selected ion monitoring mode (SIM) was applied in order to avoid background interference due to the low quantity of our material. Characteristic ion fragments were considered to be the following: *m/z* 415, 355, 131, and 117. Figure 2C shows the total ion chromatogram, and Fig. 2D a typical SIM profile for the lipid residue from *T. pyriformis*, with retention time 13.132 min, corresponding to that of authentic AAG. Comparison of the integrated areas of the characteristic ions at the same retention time shows that the two compounds are identical.

Quantitative determination was performed by using authentic alkylacetylglycerol as external standard in GC-MS, and comparing the peak intensities of the selected ions. The procedure revealed that *T. pyriformis* contains 0.1±0.05 nmol AAG per 10^7 cells, this value representing the mean±SD for three separate preparations. The same approach was followed in investigating the subcellular distribution of AAG, which was found to be located exclusively in the microsomal fraction of the protozoon. In the other fractions, namely pellicles, mitochondrial fraction and cytoplasm, AAG was not detectable under our experimental conditions. Detection limit was 0.001 nmol per 10^7 cells.

DISCUSSION

T. pyriformis is especially rich in alkylglycerolipids of the phosphocholine and aminoethylphosphonate types. These lipids are biosynthesized *de novo*, starting from acyl dihydroxyacetone phosphate (for review see Ref. *23*). Extensive research has been conducted on the biosynthesis, regulation and uptake of these compounds within the protozoon (*24*). Diacylglycerols and 1-*O*-alkyl-2-acyl (long chain)-*sn*-glycerols, known to be involved in cell signalling processes, occur only transiently, as intermediates in lipid biosynthesis. Alkylacetylglycerols have not been detected under physiological conditions in cells.

To investigate whether AAG is present in the lipid extract from *T. pyriformis*, two different gas-liquid chromatographic techniques were used. The first was electron

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capture detection of the heptafluorobutyroyl derivatives, which shows high sensitivity for halogenated compounds. Under our experimental conditions, AAG was efficiently separated from other peaks in the chromatogram, appearing at a retention time of 40 min. Quantitative determination in this case presented a problem due to the relatively poor linearity of the detector. To overcome this analytical problem and to reconfirm our results, a combination of selected ion monitoring-gas liquid chromatography coupled with mass spectrometry was applied. Similar methods have been used for the characterization of TBDMS derivatives of 1,2-diacylglycerols and alkylacetylglycerols derived from phosphatidylcholine (*25*) and PAF, respectively, after phospholipase C cleavage of the phosphocholine moiety. The ions selected have been reported to be characteristic of 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol (*22*). At higher column temperatures, different fragmentation of the molecule was observed.

No detectable amounts of 1-*O*-octadecyl-2-acetyl-*sn*-glycerol were found under our experimental conditions. This finding is consistent with our previous results that PAF identified in the protozoon is of the 1-*O*-hexadecyl type (*18*). Our method permits detection of very low amounts of alkylacetylglycerols, regardless of high background levels in the chromatograms. Both chromatographic methods revealed identical chromatograms for authentic 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol and for the lipid residue isolated from *T. pyriformis*, showing that the protozoon contains AAG with a hexadecyl carbon alkyl chain in the first position.

The quantity of AAG was assessed as 0.1 nmol AAG/10^7 cells, by comparing the peak of SIM from *T. pyriformis* with authentic AAG as external standard. This estimation, however, does not take into account the quantity of 1,2-AAG that may have been converted to the 1,3-isomer during the extensive purification procedure. This value is 15 times higher than the endogenous levels of PAF that we previously reported (*18*). Therefore, to comprehend the biological significance of AAG and to have a complete justification for the levels of the compounds detected, we need to elucidate all the enzymic activities that may be involved in the regulation of the levels of alkylacetylglycerol within the protozoon.

In the present work, alkylacetylglycerols were encountered as the immediate precursors of PAF biosynthesis *via* the *de novo* pathway (*4*). It is suggested that they are generated after phosphate hydrolysis from alkylacetylglycerophosphate, a reaction catalyzed by alkylacetylglycerophosphate phosphohydrolase. The levels of alkylacetylglycerols might conceivably be regulated by the following enzymes: a specific acetylhydrolase that does not utilize PAF or long chain diradylglycerols as substrate (*17*); an acylCoA:1-alkyl-2-acetyl-*sn*-glycerol acyltransferase, producing alkylacetylacyl glycerols, structural analogs of triacylglycerols (*26*); and a CDP-choline:cholinephosphotransferase, that leads to PAF formation (*4*).

We have previously reported the presence of a DTT-insensitive CDP-choline:cholinephosphotransferase, utilizing exogenous alkylacetylglycerol as substrate to form PAF. This activity was distributed predominantly between the mitochondrial and microsomal fractions of the protozoon (*9*). In previous work we have also detected endogenous levels of PAF that are not influenced by inflammatory stimuli, corresponding approximately to 4 ng/10^7 cells (*18*). PAF was located mainly in the microsomal fraction. This is consistent with the fact that the majority of phospholipid biosynthesis occurs on the membranes of endoplasmic reticulum. A question arises from the lack of AAG from the mitochondrial fraction. This could be the result of mechanisms that regulate PAF biosynthesis. Different possibilities are currently under investigation in our laboratory, concerning the occurence of other enzymic activities utilizing AAG as substrate in the mitochondrial fraction. In addition, a different compartmentalization of the enzymic activity and the relevant substrate cannot be excluded.

Our method is appropriate for the detection of the low, endogenous levels of alkylacetylglycerols and provides a tool for the complete study of the last step of the biosynthesis of PAF *via* the *de novo* pathway.

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