Evidence that biosynthesis of platelet-activating factor (paf-acether) by human neutrophils occurs in an intracellular membrane

Gérard Ribbes, Ewa Ninio+, Pascal Fontan, Michel Record, Hugues Chap, Jacques Benveniste+ and Louis Douste-Blazy*

INSERM Unité 101, Biochimie des Lipides, Hôpital Purpan, 31059 Toulouse and *+INSERM Unité 200, Université Paris-Sud, 32 rue des Carnets, 92140 Clamart, France*

Received 13 July 1985

Human polymorphonuclear leukocytes (PMN) were incubated in the absence or presence of the calcium ionophore A23187 (6 μM) for 10 min at 37°C. They were then lysed by nitrogen cavitation and fractionated using Percoll gradients. Three major fractions of increasing density corresponding to plasma membrane, intracellular membranes and secretory granules were detected using [^{3}H]concanavalin A, NADH-dehydrogenase and β-D-glucuronidase as respective markers. In both cases, the acetyltransferase activity responsible for biosynthesis of paf-acether (platelet-activating factor of 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) was detected in the intermediary fraction, the enzyme activity being increased 3–4-fold in stimulated cells. From the comparison with the distribution of various markers, it is concluded that in human PMN the final step of paf-acether assembly occurs in an intracellular membrane, possibly the endoplasmic reticulum.

Platelet-activating factor *Polymorphonuclear leukocyte* *Acetyltransferase* *Subcellular fractionation*

1. INTRODUCTION

The biosynthetic pathways of formation of paf-acether (platelet-activating factor of 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) were extensively studied in various cells or tissues (reviews [1–3]). It is now well accepted that paf-acether is synthesized by remodelling of membrane phospholipids, i.e. deacylation of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine by a phospholipase A_2 activity yielding the inactive precursor, lyso-paf-acether (1-*O*-alkyl-*sn*-glycero-3-phosphocholine) [4–6] and its subsequent acetylation by an acetyl-CoA: 1-alkyl-*sn*-glycero-3-phosphocholine acetyltransferase (EC 2.3.1.67) [7,8]. This enzyme can be stimulated several times by specific secretagogues in human neutrophils [9,10], peritoneal [11] and alveolar macrophages [6] and eosinophils from patients with eosinophilia [12]. Kinetic data suggested that an increase in the number of active enzyme molecules rather than a change in kinetic parameters was responsible for the enhanced acetyltransferase activity [11]. Recently, some evidence of a phosphorylation/dephosphorylation mechanism in acetyltransferase activation was shown using rat spleen microsomes [13].

The aim of this study was to localize precisely the subcellular structure(s) supporting the acetyltransferase, in stimulated and unstimulated human polymorphonuclear leukocytes (PMN). Among the various human and animal cells so far studied, the PMN are those which generate the highest amount of paf-acether [14].

* To whom correspondence should be addressed

Part of this work has been presented as a preliminary report at the 69th Annual Meeting of the Federation of American Societies for Experimental Biology, Anaheim, CA, USA, April 22–27, 1985 [(1985) Fed. Proc. 44, 781]

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

2. MATERIALS AND METHODS

[^{3}H]Concanavalin A (60 Ci/mmol) and [*N-methyl*-^{14}C]platelet-activating factor (50 mCi/mmol) were purchased from Amersham, England; [^{3}H]acetyl-CoA (4.8 Ci/mmol) and uridine diphosphate [^{14}C]galactose (250–360 mCi/mmol) from New England Nuclear, Dreieich; Plasmagel® from Laboratoires Roger Bellon, Neuilly, France; Percoll® from Pharmacia, Uppsala. 1-*O*-Hexadecyl-*sn*-glycero-3-phosphocholine was obtained from Tebu S.A., Le Perray and Yvelines, France. Acetyl-CoA, fatty acid-free bovine serum albumin, calcium ionophore A23187 and all other chemicals were obtained from Sigma, St Louis, MO.

2.1. *Isolation and activation of PMN*

PMN isolation on a Percoll gradient is detailed in [15]. Briefly, the leucocyte-rich supernatants of human blood treated with Plasmagel were fractionated on Percoll (15.4 ml Percoll, buffered with 6.6 ml of 490 mM NaCl, 33 mM Tris-HCl, pH 7.4). Centrifugation was performed at 48000 × *g* for 10 min. Neutrophils concentrated in a lower band were collected and washed twice with buffer. After cell surface labelling with [^{3}H]concanavalin A, PMN were washed twice in a solution containing (mM): Hepes, 4.2, pH 7.4; NaCl, 137; KCl, 75.6; glucose, 5.5. Finally, cells (10^6) were suspended in 1 ml washing buffer supplemented with 1.3 mM $CaCl_2$ and 1 mM $MgCl_2$ and incubated with 6 μM A23187 for 10 min at 37°C). The stimulation was stopped by adding 40 ml cold isotonic washing buffer.

2.2. *Subcellular fractionation of PMN*

As in [15], cells were resuspended in an isotonic lysis buffer consisting of 100 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 25 mM Tris-HCl (pH 9.6). PMN were lysed by nitrogen cavitation using a pressure of 40 bar and an equilibration time of 20 min. After centrifugation at 1000 × *g* for 15 min, the supernatant was mixed with Percoll (4 ml supernatant, 11 ml Percoll and 2.2 ml distilled water, buffered with 4.8 ml of 400 mM KCl, 20 mM $MgCl_2$, 400 mM Tris-HCl, pH 9.05). Centrifugation was performed at 160000 × *g* for 15 min and 11 fractions of 2 ml were collected from the top of the tube. Each fraction was then centrifuged at 200000 × *g* for 45 min in 8 ml buffer and each pellet was used for further determinations.

2.3. *Assay for acetyltransferase*

Acetyltransferase activity was determined as in [11] by measuring the transfer of [^{3}H]acetate from [^{3}H]acetyl-CoA (200 μM, 0.5 μCi/100 nmol) to lyso-paf-acether (40 μM) used as an acceptor in the reaction mixture (0.5 ml, pH 7.0) containing (mM): Hepes, 4.2; NaCl, 137; KCl, 2.6; $CaCl_2$, 0.65; $MgCl_2$, 0.5 and 0.25% bovine serum albumin. Incubations were performed for 10 min at 37°C under conditions where paf-acether production remained linear with time and protein concentration. They were stopped by addition of 1 ml methanol containing 1-[^{14}C]paf-acether (3000 dpm) as an internal standard. After extraction, the amounts of paf-acether found in the assay are expressed in nmol/min per mg protein.

2.4. *Other methods*

Enzyme markers were determined as reported [15,16], except for succinate-cytochrome *c* reductase which was tested by the method of Tisdale [17]. Protein was measured by the method of Lowry et al. [18] in the presence of SDS (0.07%, w/v). Radioactivity was evaluated with a Packard Tri-carb 4530 spectrometer equipped for automatic quenching correction.

3. RESULTS

As shown in fig.1 and in agreement with our previous results [15], fractionation of PMN lysates on Percoll gradients allowed a clear separation between a low-density protein peak corresponding to [^{3}H]concanavalin A, used as a plasma membrane marker and a high-density protein peak coinciding with β-D-glucuronidase, a secretory granule marker. Results were essentially the same in A23187-stimulated and in resting PMN, except for a lower recovery of material from stimulated cells in both fractions. Indeed, A23187 treatment decreased the protein content of the lysate by 25 ± 7% (mean ± SD, n = 5) and that of the granule peak by 42 ± 3% (n = 5). This probably reflects some loss of adherent cells as well as granule discharge.

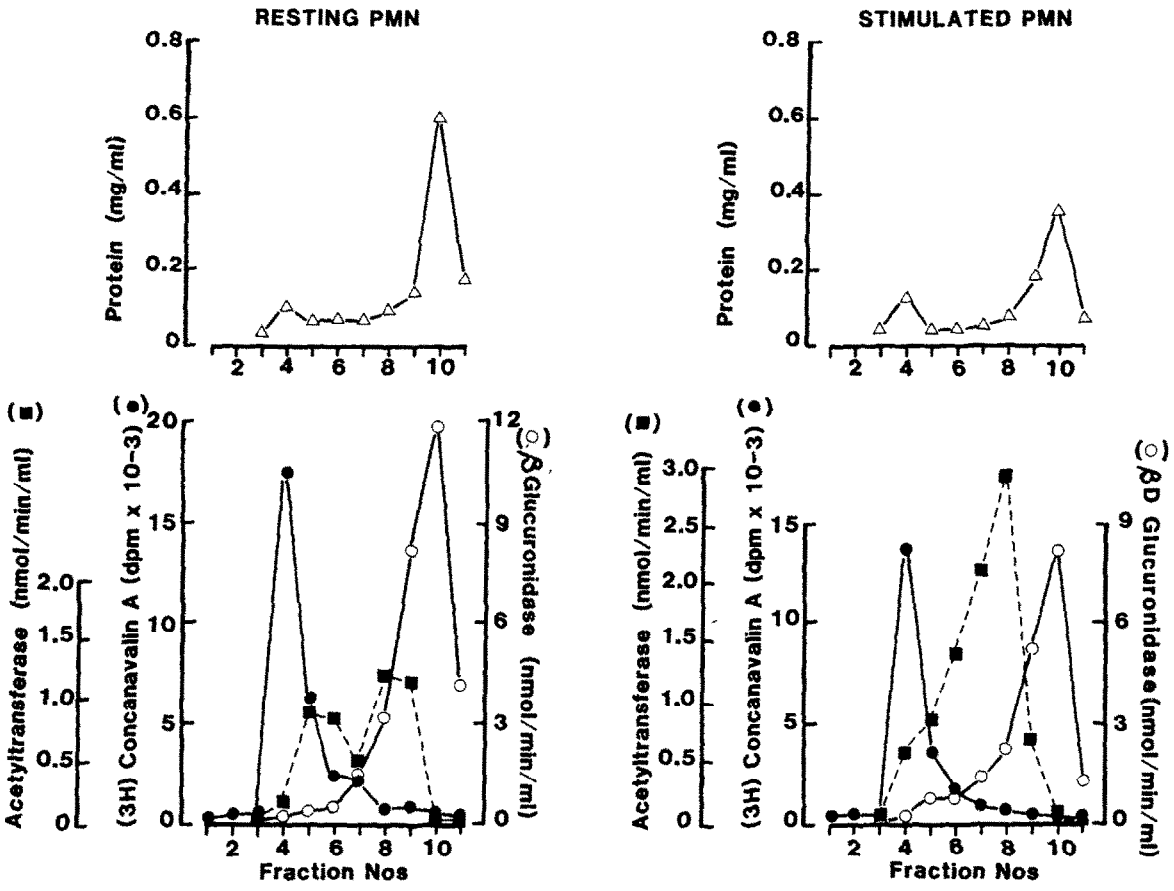

Fig.1. Subcellular fractionation of resting and A23187-stimulated PMN on Percoll gradients. This represents a typical profile obtained from the same pool of PMN and is representative of 5 experiments with similar results.

However, acetyltransferase activity did not coincide with any of the 2 markers previously mentioned. In resting PMN, it was detected as a broad intermediary peak with the appearance of a shoulder, as depicted in fig.1. Confirming previous results [10,14], A23187 treatment resulted in a dramatic increase in acetyltransferase activity, which was then recovered as a unique peak focusing at an intermediary density (fig.1). These data were further confirmed by determination of the specific activity of the enzyme in the 3 fractions. It was always the highest in the intermediary fraction for both resting and activated PMN (table 1). To identify the membrane supporting acetyltransferase activity more clearly, stimulated PMN were fractionated on Percoll gradients and the distribution of acetyltransferase was compared to that of various markers. Acetyltransferase profile was clearly different from those of galactosyltransferase (a marker for Golgi membranes) and succinate-

Table 1

Specific activity of acetyltransferase in subcellular fractions obtained from resting and A23187-stimulated PMN

	Resting PMN	Stimulated PMN
Plasma membrane fraction	0.3 ± 0.1	2.7 ± 2.0
Intermediary fraction	2.8 ± 1.3	10.6 ± 2.3
Granule fraction	1.4 ± 1.0	0.4 ± 0.4

Results (mean ± SD, 6 experiments) are given in nmol/min per mg protein

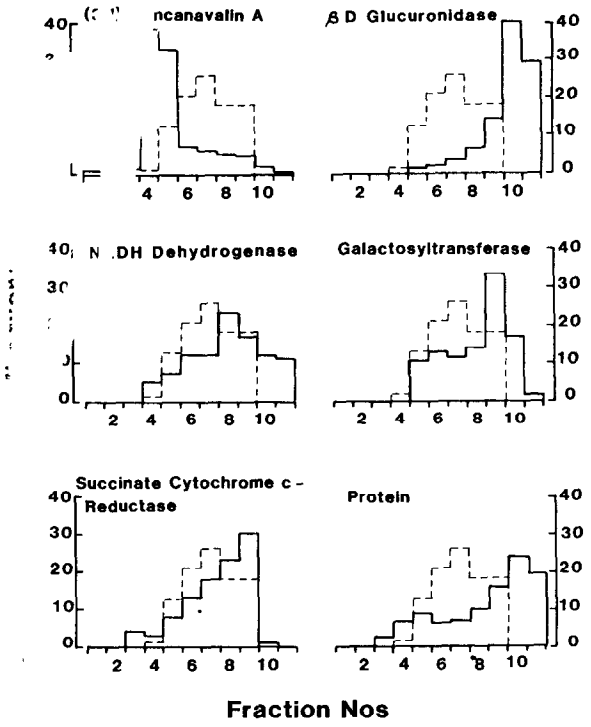

Fig.2. Subcellular distribution of acetyl transferase (---) and of various markers (——) in A23187-stimulated PMN. Data are representative of 5 experiments (except for galactosyltransferase, 2 experiments), with similar results.

cytochrome *c* reductase, a mitochondrial marker, besides those of [^{3}H]concanavalin A and β-D-glucuronidase (fig.2). However, although not absolute, some coincidence appeared with the peak of NADH dehydrogenase, used as a marker for endoplasmic reticulum [15,16].

4. DISCUSSION

Taken together, our data clearly indicate that PMN acetyltransferase is localized in a membrane fraction different from plasma membrane, granule membrane, mitochondria and Golgi membranes. To the best of our knowledge, this is the first time that the final step of paf-acether biosynthesis has been shown to occur in an intracellular membrane, possibly the endoplasmic reticulum. It is interesting to compare such a finding with a similar observation dealing with the localization of cyclooxygenase and thromboxane synthetase in the dense tubular system from human platelets [19,20]. Therefore, this is a further example of a lipid mediator released from cells, the synthesis of which does not occur in the plasma membrane or in a secretory organelle. This immediately raises the question as to the mechanism allowing paf-acether transfer from its site of synthesis to the plasma membrane and about the process of its transport through the membrane. Such a role could be partially devoted to a transfer protein specific for paf-acether [21]. Indeed, preliminary studies revealed that paf-acether synthesized in response to A23187 treatment and not yet released from PMN was mostly found in plasma membrane and granules, indicating that some redistribution from the site of synthesis did occur.

Our data may also help in understanding the mechanism of acetyltransferase activation. It is now generally accepted that specific stimulation of various cells triggers 2 synergistic pathways involving diacylglycerol and protein kinase C, on the one hand, and inositol triphosphate, Ca^{2+} and Ca^{2+}-(calmodulin)-dependent protein kinases on the other [22]. Since some preliminary evidence exists that activation of acetyltransferase might involve a phosphorylation step [13], one can speculate that the putative protein kinase responsible for this would have to reach the intracellular membrane containing the acetyltransferase. Such a point is certainly important to consider in the light of the properties of the various protein kinases so far described.

In conclusion, as outlined above, this study opens a series of important questions relevant to the biology of paf-acether. However, although acetyltransferase represents the final step of paf-acether assembly, other studies are still needed to determine the subcellular localization of phospholipid precursors as well as of phospholipase A_2. Moreover, whether such a localization of acetyltransferase in an intracellular membrane is a general feature will require further investigations dealing with other cell types capable of synthesizing paf-acether. In this respect, preliminary data seem to indicate that this could be the case for 2 tumor cell lines, Krebs ascites cell (P. Fontan et al., in preparation) and HL60 (M. Record et al., in preparation).

ACKNOWLEDGEMENTS

This study is part of a program supported by the Association pour la Recherche contre le Cancer and grant no.837 from UER Kremlin-Bicêtre.

REFERENCES

[1] Roubin, R., Tencé, M., Mencia-Huerta, J.M., Arnoux, B., Ninio, E. and Benveniste, J. (1983) in: Lymphokines (Pick, E. ed.) vol.8, pp.249–276, Academic Press, New York.
[2] Benveniste, J. and Vargaftig, B.B. (1983) in: Ether Lipids: Biomedical Aspects (Mangold, H.K. and Paltauf, F. eds) pp.355–376, Academic Press, New York.
[3] Snyder, F. (1985) Med. Res. Rev. 5, 107–140.
[4] Polonsky, J., Tencé, M., Varenne, P., Das, B.C., Lunel, J. and Benveniste, J. (1980) Proc. Natl. Acad. Sci. USA 77, 7019–7023.
[5] Mencia-Huerta, J.M., Ninio, E., Roubin, R. and Benveniste, J. (1981) Agents Action 11, 536–568.
[6] Albert, D.H. and Snyder, F. (1983) J. Biol. Chem. 258, 97–102.
[7] Wykle, R.L., Malone, B. and Snyder, F. (1980) J. Biol. Chem. 255, 10256–10260.
[8] Ninio, E., Mencia-Huerta, J.M., Heymans, F. and Benveniste, J. (1982) Biochim. Biophys. Acta 710, 23–31.
[9] Alonso, F.M., Gill, G., Sanchez-Crespo, M. and Mato, J.M. (1982) J. Biol. Chem. 257, 3376–3378.
[10] Lee, T.C., Malone, B., Wasserman, S.I., Fitzgerald, V. and Snyder, F. (1982) Biochem. Biophys. Res. Commun. 105, 1303–1308.
[11] Ninio, E., Mencia-Huerta, J.M. and Benveniste, J. (1983) Biochim. Biophys. Acta 751, 298–304.
[12] Lee, T.C., Lenihan, D.J., Malone, B., Roddy, L.L. and Wasserman, S.I. (1984) J. Biol. Chem. 259, 5526–5530.
[13] Lenihan, T.J. and Lee, T.C. (1984) Biochem. Biophys. Res. Commun. 120, 834–839.
[14] Jouvin-Marche, E., Ninio, E., Beaurain, G., Tencé, M., Niaudet, P. and Benveniste, J. (1984) J. Immunol. 133, 892–898.
[15] Record, M., Laharrague, P., Fillola, G., Thomas, J., Ribbes, G., Fontan, P., Chap, H., Corberand, J. and Douste-Blazy, L. (1985) Biochim. Biophys. Acta, in press.
[16] Record, M., Bès, J.C., Chap, H. and Douste-Blazy, L. (1982) Biochim. Biophys. Acta 688, 57–65.
[17] Tisdale, T.H. (1967) Methods Enzymol. 10, 213–215.
[18] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[19] Gerrard, J.M., White, J.G., Rao, G.H.R. and Townsend, B.W. (1976) Am. J. Pathol. 83, 283–287.
[20] Carey, F., Menashi, S. and Crawford, N. (1982) Biochem. J. 204, 847–851.
[21] Lumb, R.H., Pook, G.L., Dubacz, D.G., Blank, M.L. and Snyder, F. (1983) Biochim. Biophys. Acta 750, 217–222.
[22] Berridge, M.J. (1984) Biochem. J. 220, 345–360.