Journal of Cellular Biochemistry 40:353–359 (1989)
Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production 199–205

Subcellular Localization of Phospholipids and Enzymes Involved in PAF-Acether Metabolism

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The biosynthesis of platelet-activating factor (PAF-acether or 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) through the remodeling pathway was investigated at the subcellular level in two different cell lines. In human neutrophils, plasma membrane was isolated not only from granules, but also from internal membranes related to endoplasmic reticulum. Interestingly, the latter exhibited enhanced acetyltransferase upon neutrophil stimulation with ionophore A_{23187}. A similar study was undertaken on the tumor strain Krebs-II cells. The enzyme acetyltransferase was found to be located only on an endoplasmic reticulum subfraction, whereas most alkylacyl-GPC, the source of PAF-precursor alkyl-lyso-GPC, was located in the plasma membrane inner leaflet. The topographical separation of enzyme and precursor emphasizes the central role of the intracellular phospholipase A_2 in providing lyso-PAF to the acetyltransferase to form PAF-acether.

Key words: platelet activating factor (PAF, PAF-acether), neutrophils, Krebs II cells, phospholipid asymmetry, phospholipase A_2, acetyltransferase

The biosynthesis of platelet activating factor (PAF, PAF-acether or 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is known to proceed via two main routes: acetylation of 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (lyso-PAF) by a specific membranous acetyltransferase [1,2], and de novo biosynthesis involving cytidine diphosphate (CDP)-choline and 1-*O*-alkyl-2-acetyl-*sn*-glycerol as substrates of the final step [3,4]. The first one is responsible for rapid PAF production in stimulated cells, whereas the de novo pathway seems to support a continuous production of the phospholipid mediator.

The first pathway of PAF biosynthesis involves the sequential action of two key enzymes: phospholipase A_2 and acetyltransferase. The first provides the second with lyso-PAF formed upon a specific hydrolysis of membrane precursors, i.e., 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (alkylacyl-GPC). Some evidence has been obtained indicating that acetyltransferase could be activated in some stimulated cells through a biochemical mechanism involving protein phosphorylation by serine/threonine kinases [5–7].

Received April 17, 1988; accepted December 1, 1988.

Phospholipase A_2 is an ubiquitous enzyme whose activation is mainly dependent on cytoplasmic-free calcium concentration, although other regulation mechanisms might be involved. These include lipocortins and other related calcium/phospholipid binding proteins [8–11], alkalinization of cytoplasm through the action of Na^+/H^+ exchanger [12–13], as well as direct stimulation by G-proteins coupled to membrane receptors [14–15]. These various data have led to the concept that phospholipase A_2 activation should occur at a membrane side facing the cytoplasm, most probably in the inner leaflet of the plasma membrane. Indeed, such a localization might explain the possible direct coupling of the enzyme with G-proteins and specific membrane receptors.

The present paper reviews data concerning the subcellular localization of alkylacyl-GPC, the membrane phospholipid precursor of PAF, and that of acetyltransferase.

METHODS

Subcellular Fractionation Procedure

Two kinds of cells have been the object of investigation: human neutrophils and Krebs-II cells. In both cases, a convenient procedure of subcellular fractionation on Percoll gradients was adapted to both kinds of cells [16–17]. This method consists of preparing a 1,000 × g supernatant from cell homogenates obtained by nitrogen cavitation that is mixed with a suspension of Percoll and centrifuged at high speed in a fixed-angle rotor, allowing generation of a density gradient. As the latter centrifugation does not take longer than about 20 min, separation of different kinds of membranes can be done quite rapidly. A very critical point of the procedure is to adjust the pH of the gradient to an alkaline value (9.0 to 9.6), which allows shifting of the intracellular membranes to a higher density as compared with plasma membrane. Indeed, whereas both plasma membrane and endoplasmic reticulum markers comigrate at the top of the gradient at a neutral pH, endoplasmic reticulum is selectively recovered at the bottom of the gradient under alkaline conditions without any change in the apparent density of plasma membrane. Actually, the same procedure has been used successfully with other kinds of cells such as platelets [18,19] or the human epidermic cell line A_{431} [20].

RESULTS

Biosynthesis of PAF-Acether in Human Neutrophils

Acetyltransferase in human neutrophils. When neutrophils are challenged with various stimuli, PAF-acether is rapidly synthesized and released. Because, in addition, synthesis of the mediator has been achieved by incubating whole cells with acetyl-CoA (a nonpermeant substrate) [21], it was tempting to assume that the acetyltransferase was located on the plasma membrane.

In this study, both resting and A_{23187}-stimulated cells were submitted to subcellular fractionation [22]. Using a pH of 9.05 allowed two major fractions to be obtained within one step: plasma membrane and granules at low and high density, respectively, and an intermediary fraction that was shown to contain heterogenous membrane vesicles with some evidence for the presence of endoplasmic reticulum [17].

A main peak of acetyltransferase activity was detected in this intermediary fraction isolated from A_{23187}-stimulated neutrophils (in which case the activity was increased fourfold over control). Therefore, acetyltransferase is present neither in the

plasma membrane nor in the granules of neutrophils, as its activity over the Percoll gradient did not coincide with the position of their respective markers ([^{3}H]concanavalin A and β-D-glucuronidase). Moreover, using marker profiles of the various membranes present in neutrophils (NADH dehydrogenase for endoplasmic reticulum, galactosyltransferase for Golgi membrane, succinate cytochrome-c reductase for mitochondria) did not reveal a perfect coincidence with that of acetyltransferase.

It was thus concluded that the acetyltransferase responsible for PAF biosynthesis in neutrophils is in no way located in the plasma membrane. A recent paper demonstrated that the enzyme is located partly in the tertiary granules [23].

For a better efficiency in PAF synthesis, it is possible that the phospholipase A2 acting on alkylacyl-GPC might be colocated with the acetyltransferase. Both intracellular localization and characterization of this phospholipase A2 are currently under investigation, using two successive Percoll gradients to improve the separation of internal membranes.

Subcellular Distribution of Acetyltransferase and Alkylacyl-GPC in a Tumor Cell Line

Acetyltransferase in Krebs-II cells. Because the ascitic strain Krebs-II cells contains high amounts of alkylacyl-GPC [24] and because PAF released from tumor cells could trigger cross-aggregation with circulating platelet ("tumor emboli" involved in metastatic dissemination), the presence of an acetyltransferase in these cells was investigated. Enzyme activity was detected in microsomes, although PAF production in total cell could not be obtained with various stimuli such as A_{23187} or phorbol esters. When different cell membranes were separated across the Percoll gradient, various possible localizations could be eliminated for acetyltransferase, whose activity did not coincide with the following markers: [^{3}H]concanavalin A and 5′-nucleotidase (plasma membrane), galactosyltransferase (Golgi membranes), succinate dehydrogenase (mitochondria), catalase and dihydroxyacetone-phosphate acyltransferase (peroxisomes), N-acetyl-β-D-glucosaminidase (lysosomes), NADH dehydrogenase of NADH cytochrome c reductase (endoplasmic reticulum).

In all experiments, acetyltransferase activity was detected in a membrane recovered in a high-density range at the bottom of the gradient. The only marker found to coincide with acetyltransferase was the radioactivity of [^{3}H]uridine previously incorporated into intact cells and precipitable with trichloroacetic acid. As discussed in a very recent work [25], this corresponds to RNA and allows identification of a specific subfraction of endoplasmic reticulum that is particularly rich in ribosomes ("heavy-rough" endoplasmic reticulum). A similar subfraction was previously observed in the same cell by Pryme et al. [26], who found it to be specific for tumor cells. The significance and the origin of this very specific subfraction is presently unknown, especially when considering the metabolism of PAF. These data are interesting to discuss in relation to those concerning the localisation of alkylacyl-GPC in the plasma membrane [24].

Alkylacyl-GPC distribution in the plasma membrane of Krebs-II cells (Fig. 1). The specific localization of a given phospholipid within a cell might be the result of a difference in the lipid composition between various membranes (for instance, plasma membrane, endoplasmic reticulum, Golgi complex, mitochondria), but also of an asymmetric transverse distribution within a membrane. The concept of membrane

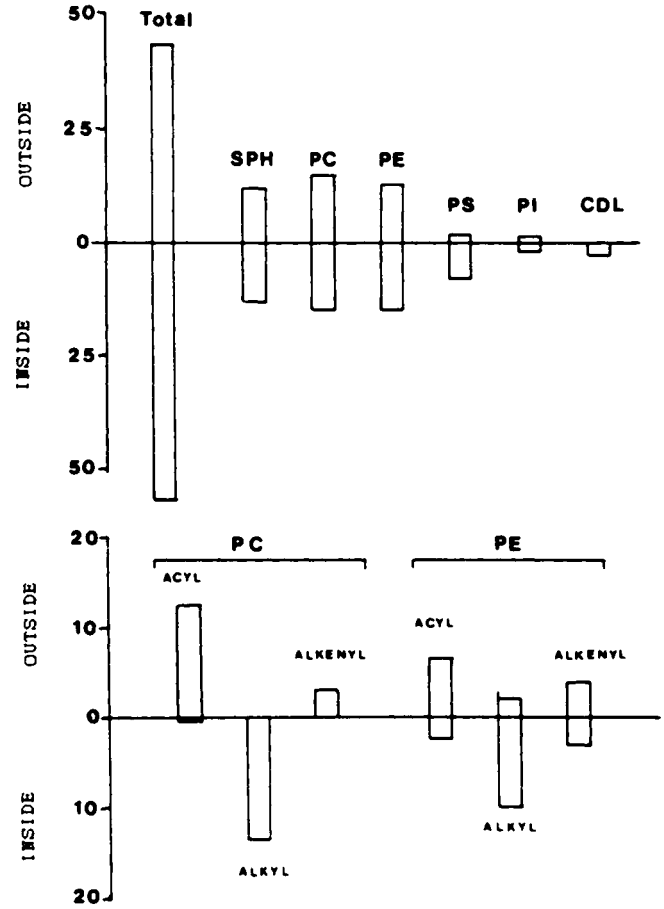

Fig. 1. Asymmetric distribution of phospholipid classes and subclasses in Krebs-II cell plasma membrane [24]. Total, total phospholipids; SPH, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; CDL, cardiolipin.

phospholipid asymmetry evolved in 1972–1973 from studies dealing with the red cell membrane [27,28]. These studies then were extended to other cells, e.g., platelets [29–31]. A general concept has emerged about eucaryotic cell membranes to indicate that the inner leaflet facing the cytoplasm contains almost the totality of anionic phospholipids, i.e., phosphatidylserines and phosphoinositides, and the majority of phosphatidylethanolamines. In contrast, the external leaflet appears to contain mainly choline phospholipids, i.e., phosphatidylcholine and sphingomyelins [for review, see 32,33]. In turn, a previous study revealed a more symmetrical distribution of phospholipids in the plasma membrane of Krebs-II cells, except phosphatidylserines, which again were detected in the cytoplasmic leaflet of the membrane [24].

A simple procedure for measuring phosphatidylcholine subclasses [34] showed enrichment of the plasma membrane in alkylacyl-GPC as compared with total cell. From this data, it can be calculated that 84% of total cell alkylacyl-GPC is present in the plasma membrane, which actually contains 53% of total cell phospholipids.

Further analysis based on selective degradation by purified phospholipases acting on intact cells under nonlytic conditions revealed that no alkylacyl-GPC molecules are directly accessible to phospholipase A_2 attack, indicating that they present a specific localization in the cytoplasmic face of the membrane. In contrast, more than 90% of diacyl-GPC was accessible to phospholipase A_2 hydrolysis. In other words, the apparent symmetrical distribution mentioned above for the total choline phospholipids actually masked a very sharp separation between diacyl- and alkylacyl-GPC. Therefore, the present results demonstrate an almost complete topographical separation between the location of acetyltransferase and alkylacyl-GPC, the reservoir for PAF-acether direct precursor alkyl-lyso-GPC. The bulk of internal membranes contains only 16% of total cell alkylacyl-GPC, this proportion being probably less in the membrane domain bearing the acetyltransferase.

Therefore, it is possible that the internal half of the plasma membrane might represent a privileged site where lyso-PAF could be produced by the action of phospholipase A_2. The authors previously arrived at a similar conclusion for platelets, in which arachidonic acid, the precursor of various eicosanoids, also was found to be most abundant in plasma membrane inner leaflet [30]. In this respect, it is interesting to recall that alkylacyl-GPC contains rather high amounts of arachidonic acid, leading to the view that the two precursors of lipid mediators (lyso-PAF and arachidonic acid) could be liberated from the membrane phospholipids through the same reaction [35].

DISCUSSION

The possible significance of the present data is summarized in Figure 2.There is no doubt that the final step of PAF biosynthesis occurs in intracellular membranes. Under these conditions, the most efficient coupling between lyso-PAF production and lyso-PAF acetylation would occur if a phospholipase A_2 were activated in the same membrane. Although no detailed study was performed in that sense, such a possibility cannot be excluded, because phospholipase A_2 is a ubiquitous enzyme. It must be borne in mind that stimulated cells usually release lyso-PAF in great excess compared with PAF [5,36,37], up to 1,000-fold in alveolar macrophages [38].

In addition, the plasma membrane as the privileged site of lyso-PAF biosynthesis is consistent with several aspects of phospholipase A_2 regulation, including a direct coupling with membrane receptors through G-proteins [14,15], regulation by calpactins/lipocortins, and other related calcium/phospholipid binding proteins, which will preferentially interact with a membrane that is particularly rich in phosphatidylserines [8–11]. In both cases also, a major regulation of phospholipase A_2 by cytoplasmic-free calcium [39] or cytoplasmic pH [12,13] remains possible. Phospholipase A_2 activity and its subsequent lyso-PAF production could be the link between the topographical separation of PAF-precursor, alkylacyl-GPC, and the PAF-synthesizing enzyme, acetyltransferase. In this respect, some deficiency in this phospholipase A_2 activity in Krebs-II cells could explain the lack of PAF production in total cells, although they contain a high amount of alkylacyl-GPC and an active acetyltransferase. Evidently, further studies are needed on the activity and intracellular localization of the phospholipase A_2 acting on alkylacyl-GPC.

Another issue raised by our data concerns the cellular processing of PAF following its biosynthesis. Indeed, considering its exclusive synthesis in an intracellular membrane, emphasis must be given to the mechanisms that might allow PAF to reach the plasma

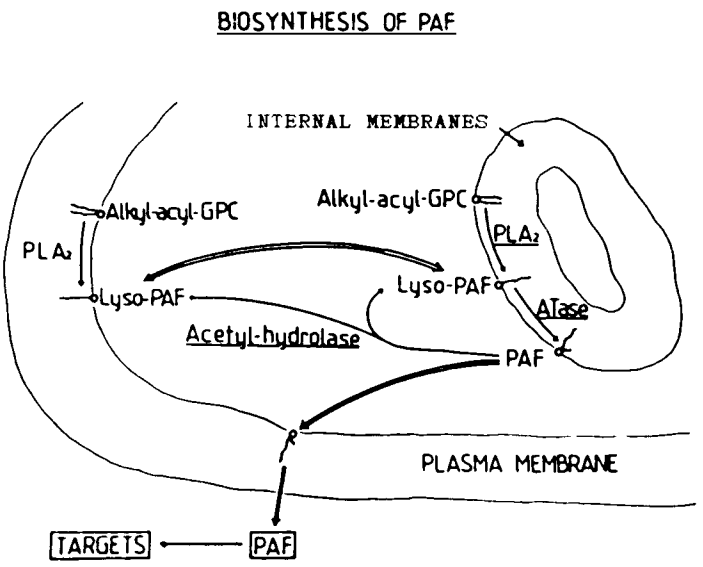

Fig. 2. Proposed intracellular pathway for PAF metabolism. The topographical separation of alkylacyl-GPC (inner plasma membrane) and acetyltransferase (endoplasmic reticulum) in Krebs-II cells might be overcome by an ubiquitous phospholipase A_2 providing the lyso-PAF to the acetyltransferase. To date a similar scheme cannot be drawn for human PMN, because the subcellular distribution of alkylacyl-GPC is not known. However, the acetyltransferase in PMN, is also located intracellularly and, a transport mechanism for PAF-acether is necessary to allow its release out of the cell. Thin arrows indicate enzymatic reactions; broad arrows correspond to putative transfer of PAF and lyso-PAF within the cell. PLA_2, phospholipase A_2; ATase, acetyltransferase.

membrane and farther on its target cells in the external medium. This might occur either through traffic along the various membrane systems present in each cell or with the help of specific transfer proteins, which have been detected, for instance, in lung cytosol [40]. Whatever the mechanism, this might also help to protect PAF from excessive degradation in the cytosol, where cellular acetylhydrolase activity is currently detected [41].

Together the present data demonstrate that the synthesis of PAF-acether, the only phospholipidic mediator known to date, takes place in the same membrane as that of constitutive cell phospholipids, i.e., the endoplasmic reticulum. As a consequence, an intracellular processing of newly synthesized PAF, and perhaps lyso-PAF, is necessary to achieve PAF release in the outer medium to elicit its biological activity.

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