# Transbilayer reorientation of platelet-activating factor in the erythrocyte membrane

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Platelet-activating factor (PAF) is a phospholipid, intermediate in its structure between diacyl- and lysophospholipids. The reorientation of this highly bioactive compound to the inner membrane layer of human erythrocytes after its primary incorporation into the outer membrane layer has been studied. Reorientation was shown to be a slow process with an initial rate of only 0.012 $h^{-1}$. It does not depend on energy supply. From the steady-state distribution of PAF between inner and outer membrane layers, after very long incubation times (40–50 h), a preference of PAF for the outer membrane layer analogous to that of endogenous lecithin is derived. Our data indicate that the process of simple transbilayer reorientation of PAF is probably too slow to account for very fast transmembrane signal transmission or for the fast uptake and metabolism observed in certain cell types.

*Platelet-activating factor* *Erythrocyte membrane* *Flip-Flop*

## 1. INTRODUCTION

1-Alkyl-2-acetyl-*sn*-glycerol-3-phosphocholine is known to induce rapid aggregation of blood platelets and secretion of granular constituents from the cells [1,2]. It owes the name platelet-activating factor (PAF) to this property. PAF is released from platelets, basophilic and neutrophilic leucocytes and macrophages upon stimulation [3]. On the other hand, PAF causes chemotaxis, enhanced adhesiveness, aggregation and degranulation [4,5] of polymorphonuclear leucocytes, induces the oxidative burst in macrophages [6], produces contraction of ileum smooth muscle, diminishes coronary blood flow and induces anaphylaxis [3]. The platelet-activating effect of PAF is highly specific and depends on: (i) the ether-bonded 1-alkyl chain; (ii) a short fatty acid (acetyl or propionyl) or alkyl chain at the 2 position; and (iii) its phosphorylcholine head group [7–10].

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Recently, specific high-affinity binding sites for PAF have been demonstrated on platelet and smooth muscle membranes [11–14]. Receptor binding of PAF is thought to induce phosphoinositide breakdown by phospholipase C and thereby to activate $Ca^{2+}$-calmodulin-dependent kinase and protein kinase C [15–17].

Investigations of a direct action of PAF on intracellular processes, of the rate of its uptake by cells and its subsequent metabolization, as well as of the rate of its release from activated cells are hampered by the lack of information on the rate of transmembrane movement of PAF (either by simple transbilayer reorientation or protein-mediated). One aim of this work was therefore to measure the rate of transmembrane reorientation of PAF using the erythrocyte as a model system. In addition it was intended to check whether PAF might serve as an analogue of diacylphosphatidylcholines for the quantiification of transbilayer mobility of phospholipids using a procedure previously elaborated for lysophospholipids [18,19]. PAF has the advantage over lyso-

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phospholipids that it is less amenable to enzymatic and nonenzymatic conversions. First, it has an ether-bonded alkyl chain instead of an ester at position 1 of the glycerol backbone, which is therefore not susceptable to hydrolysis [18]. Second, it has an acetyl group in the 2-position of glycerol, which still enables the quantification of transmembrane reorientation by its extraction from the membrane with albumin, but should prevent conversion of PAF to long-chain diacyllecithins by acylation.

## 2. MATERIALS AND METHODS

Heparinized human blood was obtained from the local blood bank. Erythrocytes were isolated by centrifugation, the buffy coat removed and erythrocytes washed three times with saline. PAF, 1-*O*-[$^3$H]octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (spec. act. 90 Ci/mmol or 3.33 TBq/mmol), was obtained from Amersham, Braunschweig. 50 $\mu$l packed erythrocytes were suspended in 950 $\mu$l of a medium containing (mmol·l$^{-1}$): 1 KCl (90), NaCl (45), $Na_2HPO_4$ /$NaH_2PO_4$ (12.5) and sucrose (44) (medium A, pH 7.4). Subsequently, 10 $\mu$l labelled PAF (50 nCi) from a stock solution in methanol were added and the suspension incubated for 5 min at 22°C to incorporate about 400 molecules of PAF per cell into the outer membrane layer. Following centrifugation (5000 × *g*, 10 min), cells were suspended in 10 vols medium A containing gentamycin (0.1 mg/ml medium) to prevent bacterial growth. Essentially no PAF (<2%) was contained in the medium. A parallel suspension was incubated in the presence of glucose (5 mmol·l$^{-1}$). The phosphate content of this medium was twice that of medium A. After various periods of incubation (up to 50 h), 50 $\mu$l samples were centrifuged, the erythrocytes washed once with medium A and the fraction of PAF in the outer layer quantified by 3 sequential albumin extractions at 22°C as described [18,19]. To detect metabolic conversions of PAF, 100 $\mu$l of the cell suspension were centrifuged, the erythrocytes hemolysed with 2 vols $H_2O$ and lipids extracted and separated by thin-layer chromatography on silica plates using chloroform-methanol-acetic acid-water (60:30:12:2) as described in [18]. Spots containing PAF ($R_f$ = 0.19) and lecithin ($R_f$ = 0.37) were scraped from the plates and their radioactivity quantified by liquid scintillation counting.

## 3. RESULTS AND DISCUSSION

The transbilayer reorientation of PAF in the erythrocyte membrane can be quantified by its loss of extractability by albumin, analogous to the method previously elaborated for phospholipids. PAF, however, could be extracted less readily than lysolecithin at 0°C. Two sequential treatments of cells with 1.5% albumin at 22°C for 2 min, immediately after incorporation of PAF into the membrane, resulted in extraction of 98% of PAF from the cells. Specific binding of PAF to certain membrane constituents seems rather unlikely, since the same small inextractable fraction was found when 1000-times higher amounts of PAF were incorporated. The remaining 2% could not be extracted by increasing the albumin concentration, raising the extraction temperature or extending the extraction period. There fore the reorientation kinetics were corrected for this very small inextractable fraction. As shown in fig.1A, the rate of reorientation of PAF from the outer to inner membrane layer of the erythrocyte is slow. From the initial rate of increase of the inextractable fraction of PAF (at a fractional reorientation of < 5%) one can calculate a rate constant for the reorientation process of 0.012 h$^{-1}$. This rate is somewhat lower than that of palmitoyllysolecithin (0.019 h$^{-1}$ [18]).

In the presence of glucose the nonextractable fractions of PAF after 25 h of incubation were consistently higher than in the absence of glucose. The further analysis of this observation led to the finding that PAF-derived radioactivity is incorporated into the lecithin fraction and that this incorporation is dependent on the availability of energy supply (fig.1B). These findings make it likely that the acetyl group of PAF is slowly removed by hydrolysis and that the resulting free -OH group is re-esterified by long-chain fatty acids, particularly in the presence of energy supply. Such an enzymatic system was recently described for platelets. In these cells, PAF is rapidly converted into long-chain ether analogues of lecithins by means of the combined action of a cytoplasmic hydrolase and an inner membrane surface bound acyltransferase system [21-23]. Since phospho-

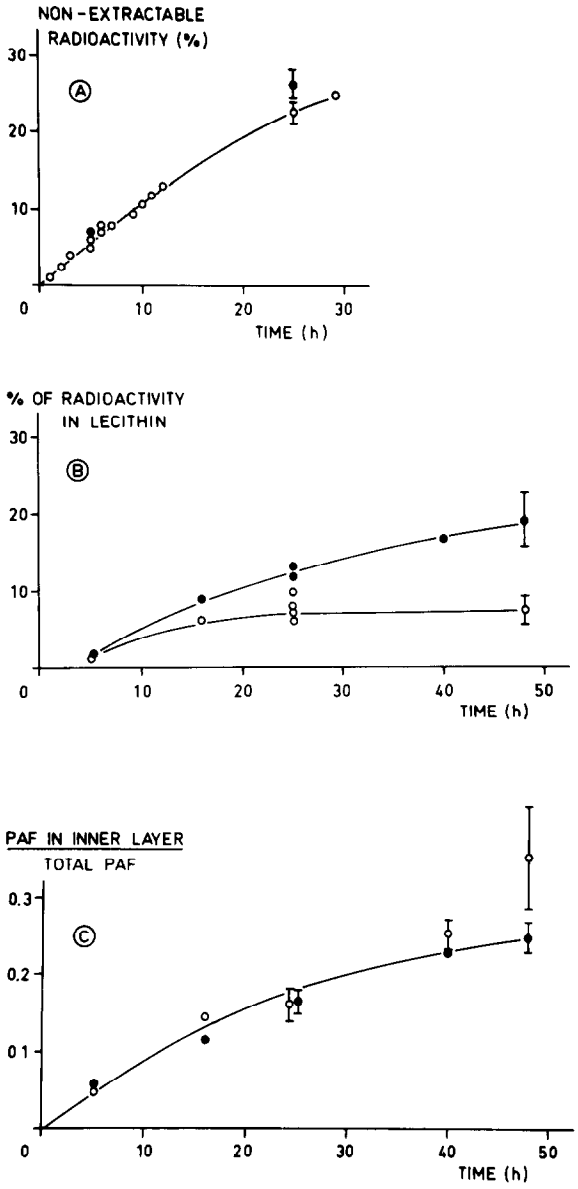


Fig.1. (A) Time-dependent increase of the fraction of PAF nonextractable by albumin. $^3$H-labelled PAF was incorporated into the outer membrane layer of human erythrocytes. Reorientation to the inner membrane layer in the absence or presence of glucose was followed by measuring the time-dependent decrease of extractability of PAF by albumin. (B) Time-dependent incorporation of PAF-derived radioactivity into lecithin. Incorporation of radioactivity into the membrane lecithin fraction was quantified after lipid extraction and separation of lecithin from PAF by thin-layer chromatography. (C) Time-dependent increase of the fraction of PAF in the inner membrane layer. The ratio $q$ of PAF in the inner membrane layer to total membrane bound PAF was calculated from the fraction of nonextractable PAF (cf. A) after correction for acylation (cf. B) by the following equation:

$q$ = (nonextractable radioactivity - radioactivity in lecithin)/(total radioactivity - radioactivity in lecithin)

Data of experiments in the absence (○) and presence (●) of glucose. The data represent mean values ± SD for 3–5 experiments.

lipids with two long chains are not extractable by albumin, higher acylation rates in the presence of glucose cause a higher fraction of nonextractable radioactivity. After correction for acylation no significant difference between incubations with and without glucose is obtained (fig.1C). The only deviation was observed after very long incubations (50 h) in the absence of glucose, where the variation of the data was rather high and nonextractable fractions were higher than those in the presence of glucose. This observation may be explained by hemolysis of the cells in the absence of glucose after these long incubation periods in salt medium.

Using the formula $t_{1/2} = 0.693/k \times q$ [18], where $q$ is the inner-leaf fraction of the total radioactivity, and a value of 0.012 $h^{-1}$ (see above) as a first approximation for the initial rate constant ($k$), one can calculate half-time ($t_{1/2}$) of reorientation assuming various steady-state distributions of PAF between the inner and outer membrane layers [9]. The best fit to the data could be obtained from a $q$ value of 0.3 resulting in a half-time (for a 2-compartment system) of 17 h. After this time period the fraction of PAF in the inner layer (relative to total membrane-bound PAF) should be 0.15 which fits the curve of fig.1C very well. A $q$ value of 0.5 would give an inextractable fraction of 0.25 after 29 h (the $t_{1/2}$ for this ratio), which is significantly higher than the value corresponding to the curve after this time period. Our data therefore indicate a preference of PAF for the outer membrane layer similar to that of exogenous lysolecithin [18] and endogenous lecithin [20].

Our present data and previous results demonstrate, that the transbilayer reorientation of PAF and that of palmitoyllysolecithin in the

erythrocyte membrane are both slow processes with half-times of 17 and 11 h, respectively [18]. If these slow rates are taken to reflect the 'simple', nonmediated movement across the lipid bilayer of nonspecialized biological membranes, it is immediately evident that the transmembrane signal transduction in cells stimulated by this transmitter requires an exofacial receptor. Moreover, our data suggest that in cells which take up PAF rapidly a selective transfer system, presumably involving proteins, must be operative. This is presumably true, e.g. for platelets. They take up PAF at a high rate ($t_{1/2} < 1$ h), much faster than lyso-PAF and convert it into long-chain ether lecithin [21–23].

Irrespective of these physiological aspects, PAF might become a promising tool for studies on the simple, nonmediated flip-flop of phospholipids in biological membranes. The use of lysophospholipids for this purpose is hampered to some extent by their acylation to diacylphospholipids, which as yet has proved to be unsuppressible in all erythrocytes. In the case of PAF, prevention of the hydrolytic cleavage of the acetyl residue in position 2 would forestall the transformation of the molecule. The blockage of hydrolysis of PAF by phenylmethylsulfonyl fluoride was reported [22] while this manuscript was in preparation.

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