PLATELET ACTIVATING FACTOR (1-*O*-ALKYL-2-*O*-ACETYL-*sn*-GLYCERO-3-PHOSPHOCHOLINE)

Activity of analogs lacking oxygen at the 2-position

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1. Introduction

1-*O*-Alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (AAGPC or 1-*O*-alkyl-2-*O*-acetyl-GPC), platelet-activating factor (PAF) [1–3], activates platelets and neutrophils (PMN) of rabbits and humans [1,3–8] and is antihypertensive in rats [9]. Analogs of AAGPC in which the acetyl group is replaced by an *O*-ethyl or *O*-methyl group are biologically active [4,10–11]. Since the 2-*O*-ethyl analog (1-*O*-alkyl-2-*O*-ethyl-GPC) is more active, we selected it earlier to probe the mechanism of action of AAGPC. Although the 2-*O*-ethyl analog is somewhat less active (20–40-fold) than AAGPC, it elicits identical responses [4,5] and selectively cross-desensitizes PMN to the action of AAGPC [5] and thus appears to act through the same mechanism. These studies provide evidence that AAGPC does not require transfer of its acetyl group to a protein or other cellular component for the expression of activity. However, as has been pointed out [11], some cells contain an oxidative alkyl cleavage system [12] that possibly could remove the 2-*O*-ethyl group as an aldehyde and form 1-*O*-alkyl-2-lyso-GPC, which might then be acetylated to form AAGPC. Here, to further investigate the mechanism of action of AAGPC, we have prepared and tested analogs that contain nonhydrolyzable alkyl groups at the 2-position. The most active of these is the *n*-propyl derivative, which aggregates platelets at 6 nM.

2. Materials and methods

2.1. *Synthesis of the 2-alkyl analogs of AAGPC (rac-1-O-hexadecyl-2-alkyl-3-O-phosphorylcholine–propane)*

The appropriate alkyl bromide (*n*-propyl, isopropyl, or isobutyl) was reacted with diethylmalonate in a standard malonic ester synthesis to produce predominately mono-alkylation. The product was reduced with lithium aluminum hydride to give the corresponding 2-alkyl-1,3-propanediol, which was reacted as in [10] with hexadecyl bromide to give predominately the monoether, 1-*O*-hexadecyl-2-alkyl-3-propanol. After purification by column chromatography on Silica Gel 60 (E.M. Merk), the 3-propanol derivatives were converted to their corresponding phosphorylcholines as in [13]. The structure of each of the products in the sequence leading up to the introduction of the phosphocholine group was confirmed by infrared and nuclear magnetic resonance spectroscopy. The isobutyl and isopropyl compounds were monitored for the presence, and the *n*-propyl compounds for the absence of the *gem*-dimethyl groups. The final products were purified by thin-layer chromatography and had R_F-values the same or slightly higher than AAGPC (Silica gel H; chloroform:methanol:acetic acid:water, 50:25:8:4, by vol.). In addition there was a 1:1 ratio of P/N in each of the analogs. Each of the analogs gave molecular (M) and ($M + 1$) ions by desorptive chemical ionization mass spectrometry using methane as the ionizing gas. In addition there were characteristic fragments for each of the

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compounds. Details of the synthesis of these analogs, confirmation of their structure and their complete spectral characteristics will be published later. AAGPC was prepared from choline-linked plasmalogens of beef heart [4]. Concentrations of all analogs were determined by measuring lipid phosphorus [14].

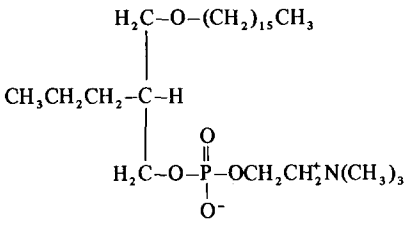
2.2. *Preparation of cells and bioassays*

Rabbit platelets and peritoneal PMN were isolated and their aggregation response assayed as in [4]. The PMN contained <5 platelets/100 PMN. Human PMN were isolated and their degranulation measured as in [5]; these leukocyte preparations were 98% PMN and contained <2 platelets/100 cells. AAGPC and analogs were added to cells in small volumes (5–50 μl/ml) of bovine serum albumin-containing buffer as in [4].

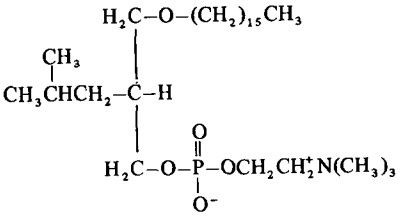
AAGPC and the analogs were assayed in vivo by intravenous infusion over 20 s into a femoral vein of adult New Zealand rabbits. Blood samples were taken from a femoral artery at selected times and platelets and PMN counted.

3. Results

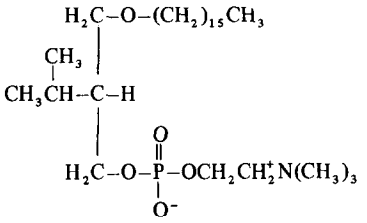
The following analogs of PAF were prepared and tested:



2-*n*-Propyl-PAF



2-Isobutyl-PAF



2-Isopropyl-PAF

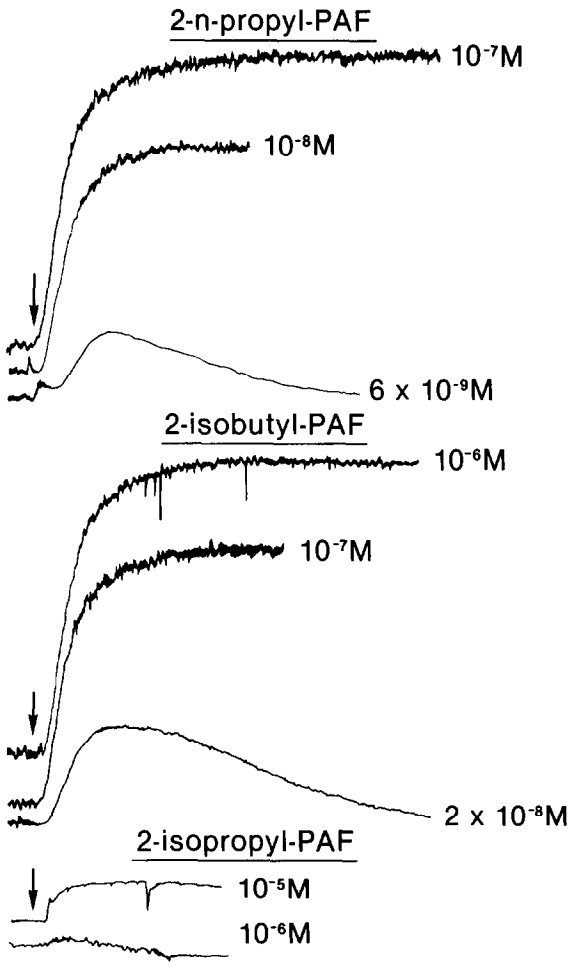


Fig.1. Aggregation of rabbit platelets in response to analogs of PAF. Rabbit platelets were suspended at 2.5 × 10^8/ml in Tyrode's-gelatin containing Ca^{2+} and were incubated with stirring at 1000 rev./min. Aggregation was followed by measuring increased light transmission; the upper tracings represent a maximum increase of 60% in transmission and 2 min from the point of addition of the analogs, indicated by the arrows. Final concentrations of the analogs are indicated for each tracing.

The aggregation of rabbit platelets in response to the analogs is shown in fig.1. The 2-*n*-propyl analog was most active whereas the 2-isopropyl analog showed little activity even at 10^{-5} M.

The in vitro aggregation of rabbit PMN in response to 2-*n*-propyl-PAF is shown in fig.2C; PMN responded to levels of $<10^{-7}$ M. The effects of 2-*n*-propyl-PAF administered intravenously to rabbits are also shown in fig.2. The disappearance of platelets and PMN from circulation following administration of the analog is similar to that elicited by AAGPC.

Activities of the analogs and AAGPC are compared in table 1. As with all responses, the 2-*n*-propyl-PAF was more active than the 2-isobutyl-PAF in the degranulation of human PMN; 2-isopropyl-PAF elicited little response in any instance at ≤ 1 μM.

4. Discussion

The most active of the analogs tested was the 2-*n*-propyl-PAF. All the analog preparations were racemic, whereas the 1-*O*-alkyl-2-acetyl-GPC tested was the pure *sn*-glycero-3-phosphocholine isomer. Based on [3,10,15] only one of the enantiomers is presumed to be active; if the potencies observed are adjusted accordingly, the activity of the 2-*n*-propyl analog is similar to the activity of the 2-*O*-ethyl

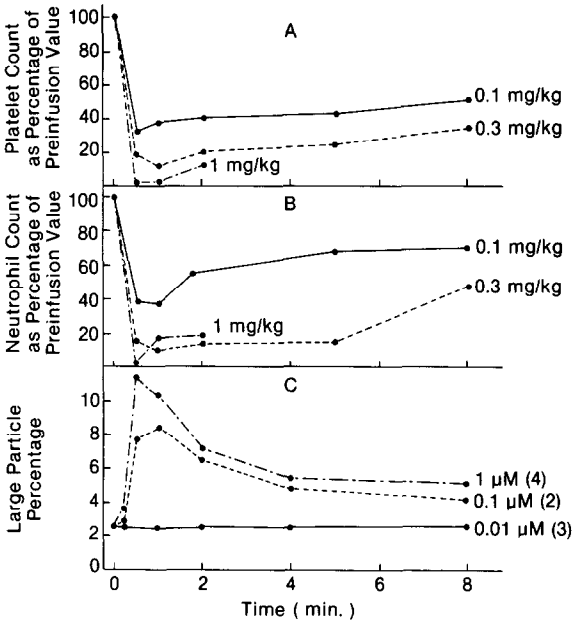


Fig.2. Effect of *n*-propyl-PAF on platelet and neutrophil counts after injection into rabbits and the in vitro aggregation of rabbit neutrophils. For the in vivo studies, analogs were administered at the doses shown and cells counted as in section 2. The aggregation of PMN is shown as the large particle percentage of PMN suspensions after addition of *n*-propyl PAF. Final concentrations of the analog are given. PMN were incubated in Hank's buffer containing 1.4 mM Ca^{2+} and 0.7 mM Mg^{2+} for 5 min (37°C) before challenge.

Table 1
Relative potencies of PAF and its analogs in various assays

Assay	Analog (molarity or dose/kg)				
	1-*O*-Alkyl-2-acetyl-GPC	*n*-Propyl-PAF	Isobutyl-PAF	Isopropyl-PAF	1-*O*-Alkyl-2-lyso-GPC
Platelet aggregation (rabbit)	5×10^{-11} M	4×10^{-9} M	6×10^{-8} M	$>10^{-6}$ M	$>10^{-6}$ M
Neutrophil aggregation (rabbit)	4×10^{-9} M	4×10^{-7} M	4×10^{-6} M	$>10^{-6}$ M	$>10^{-6}$ M
Neutrophil degranulation (human)	3×10^{-9} M	4×10^{-7} M	2×10^{-6} M	$>10^{-6}$ M	$>10^{-6}$ M
In vivo neutropenia (rabbit)	0.8 μg/kg	100 μg/kg	n.d.	>1 mg/kg	>1 mg/kg
In vivo thrombocytopenia (rabbit)	0.3 μg/kg	50 μg/kg	n.d.	>1 mg/kg	>1 mg/kg

For in vitro assays, cells were suspended with varying doses of stimulus (as described in fig.1,2 and section 2) and the ensuing response measured. For in vivo assays, rabbits were infused with varying doses of stimulus. For all assays, responses were plotted against the Log of dose. The concentration (M) or dose of agent that caused 50% of the maximal platelet or neutrophil aggregation response, net release of 5% lysozyme (for degranulation), or depression of the number of circulating cells by 50% of preinfusion values (for neutropenia and thrombocytopenia) were extrapolated; n.d., not determined

analog in [4] and much more active than the 1-*O*-alkyl-2-lyso-GPC (table 1). The 2-*n*-propyl-PAF has very low antihypertensive activity in rats (in preparation) suggesting it may be possible to separate the anaphylactic and antihypertensive effects of AAGPC.

The spatial dimensions of the 2-alkyl group in the analogs appear critical for activity since the 2-*n*-propyl analog is active whereas the 2-isopropyl analog is not. Similar propanediol analogs containing either no alkyl group at the 2-position [11] or *gem*-dimethyl groups [11] are inactive, and that the substitution of the acetyl group of 1-*O*-alkyl-2-acetyl-GPC with chains longer than propionyl groups leads to loss of activity [1,16]. Furthermore, the 2-*O*-ethyl derivative is more active than the 2-*O*-methyl derivative [10]; thus there appear to be strict steric constraints on groups at the 2-position. This sharply restricted range of size fits a model in which activity is mediated by a receptor requiring a specific steric fit for activation.

The activities of the 2-*n*-propyl and 2-isobutyl analogs cannot be attributed to activation by the *O*-alkyl cleavage system. It was warned that contamination by AAGPC can be a serious source of error [16]. This was ruled out here and in [4] (on 1-*O*-alkyl-2-*O*-ethyl-GPC) by mild saponification [17], which removes the acetyl group of AAGPC but not the phosphocholine group; the treatment reduced the activity of AAGPC by >1000-fold but had virtually no effect on the activity of the 2-*n*-propyl analog. In contrast to [16], we conclude that appropriate non-ester groups can replace the short-chain of AAGPC with retention of activity. These findings strengthen our evidence [3,4] that in platelets and PMN transfer of the acetyl group is not required for activity of AAGPC.

Acknowledgements

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References

[1] Demopoulos, C. A., Pinckard, R. N. and Hanahan, D. J. (1979) J. Biol. Chem. 254, 9355–9358.
[2] Hanahan, D. J., Demopoulos, C. A., Liehr, J. and Pinckard, R. N. (1980) J. Biol. Chem. 255, 5514–5516.
[3] Vargaftig, B. B., Chignard, M., Benveniste, J., Lefort, J. and Wal, F. (1981) Ann. NY Acad. Sci. 370, 119–137.
[4] O'Flaherty, J. T., Wykle, R. L., Miller, C. H., Lewis, J. C., Waite, M., Bass, D. A., McCall, C. E. and DeChatelet, L. R. (1981) Am. J. Pathol. 103, 70–78.
[5] O'Flaherty, J. T., Lees, C. J., Miller, C. H., McCall, C. E., Lewis, J. C., Love, S. H. and Wykle, R. L. (1981) J. Immunol. 127, 731–737.
[6] Pinckard, R. N., McManus, L. M., Demopoulos, C. A., Halonen, M., Clark, P. O., Shaw, J. O., Kniker, W. T. and Hanahan, D. J. (1980) J. Reticuloendothel. Soc. 28 suppl., 95S–103S.
[7] Goetzl, E. J., Derian, C. K., Tauber, A. I. and Valone, F. H. (1980) Biochem. Biophys. Res. Commun. 94, 881–888.
[8] McManus, L. M., Hanahan, D. J., Demopoulos, C. A. and Pinckard, R. N. (1980) J. Immunol. 124, 2919–2924.
[9] Blank, M. L., Snyder, F., Byers, L. W., Brooks, B. and Muirhead, E. E. (1979) Biochem. Biophys. Res. Commun. 90, 1194–1200.
[10] Wykle, R. L., Miller, C. H., Lewis, J. C., Schmitt, J. D., Smith, J. A., Surles, J. R., Piantadosi, C. and O'Flaherty, J. T. (1981) Biochem. Biophys. Res. Commun. 100, 1651–1658.
[11] Hanahan, D. J., Munder, P. G., Satouchi, K., McManus, L. and Pinckard, R. N. (1981) Biochem. Biophys. Res. Commun. 99, 183–188.
[12] Wykle, R. L. and Snyder, F. (1976) in: The Enzymes of Biological Membranes (Martonosi, A. ed) vol. 2, pp. 87–117, Plenum, New York.
[13] Brockerhoff, H. and Ayengar, N. K. N. (1979) Lipids 14, 88–89.
[14] Rouser, G., Siakotos, A. N. and Fleischer, S. (1966) Lipids 1, 85–86.
[15] Heymans, F., Michel, E., Borrel, M.-C., Wichrowski, B., Godfroid, J.-J., Convert, O., Coeffier, E., Tence, M. and Benveniste, J. (1981) Biochim. Biophys. Acta 666, 230–237.
[16] Tence, M., Coeffier, E., Heymans, F., Polonsky, J., Godfroid, J.-J. and Benveniste, J. (1981) Biochimie 63, 723–727.
[17] Wykle, R. L., Kraemer, W. F. and Schremmer, J. M. (1980) Biochim. Biophys. Acta 619, 58–67.