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1-O-ALKYL-2-N-METHYLCARBAMYL-GLYCEROPHOSPHOCHOLINE: A BIOLOGICALLY POTENT, NON-METABOLIZABLE ANALOG OF PLATELET-ACTIVATING FACTOR

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SUMMARY: We prepared unlabeled and ^{3}H-labeled analogs of platelet-activating factor (PAF) containing a N-methylcarbamyl residue at the sn-2 position. PAF and its methylcarbamyl analog competed for binding to high affinity receptors on human polymorphonuclear neutrophils; their respective dissociation constants for these receptors were 0.2 and 1.1 nM. The binding affinities of the two analogs correlated precisely with their capacities to stimulate neutrophil degranulation responses. Unlike PAF, however, the methylcarbamyl analog completely resisted metabolic inactivation by neutrophils and by human sera. Thus, these compounds' biological potencies are determined predominately by receptor binding: cellular metabolism of the ligands neither contributes to nor appreciably limits their stimulating actions.

PAF (1-O-alkyl-2-acetyl-GPC) is a bioactive product of stimulated cells. The compound is rapidly lethal in several animal species and may mediate autotoxic reactions such as anaphylaxis and shock (1,2). Levels of PAF are regulated by serum and cytosolic acetylhydrolases which convert PAF to its essentially bioinactive and innocuous derivative, 1-O-alkyl-2-lyso-GPC (lysoPAF). Cellular lyso-

Abbreviations used: GPC, sn-glycero-3-phosphocholine; PAF, platelet-activating factor or 1-O-hexadecyl-2-acetyl-GPC; lyso-PAF, 1-O-alkyl-2-lyso-GPC; CPAF, rac-1-O-hexadecyl-2-N-methylcarbamyl-GPC; [^{3}H]PAF, 1-O-[^{3}H-9,10] hexadecyl--2-acetyl-GPC; [^{3}H]CPAF, 1-O-[^{3}H-9,10]hexadecyl-2-N-methylcarbamyl-GPC; LTB_4, leukotriene B_4; PC, 1,2-dioleoyl-GPC; PMN, polymorphonuclear neutrophil; K_d and K_{di}, dissociation constants for agonist and antagonist, respectively, binding to PMN; R_t, receptor number; TLC, thin-layer chromatography.

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PAF is then acylated, forming 1-O-alkyl-2-acyl-GPC (3-8). These metabolic steps proceed with remarkable rapidity: human PMN, for instance, inactivate PAF at rates as high as 10 pmol/min/10^7 cells (6). Serum has a similar capacity to metabolize PAF (7,8). Consequently, many studies on the binding, subcellular distribution, and in vivo actions of PAF have been restricted. Compounds resistant to acetylhydrolase would be particularly useful for examing these problems provided that they: a) approach PAF in potency; b) possess full agonistic action; c) interact specifically with PAF receptors; and d) can be prepared easily and inexpensively both as unlabeled and labeled analogs. Here, we report that 1-O-hexadecyl-2-N-methylcarbamyl-GPC (CPAF) (Fig. 1) has each of these properties. CPAF proved useful for examining the role of ligand metabolism in PMN responses to PAF.

MATERIALS AND METHODS

Reagents and Buffers:

[^{3}H]PAF (56 Ci/mmol), [^{3}H]lysoPAF (56 Ci/mmol), PAF, lysoPAF, and LTB_4 were prepared (6,9) and [^{3}H]LTB_4 (186 Ci/mmol) was purchased from Amersham, Arlington Heights, IL. Our buffer was a modified Hanks' balanced salt solution containing 1.4 mM $CaCl_2$ and 0.7 mM $MgCl_2$ (6). L652731 was a generous gift from Merck, Sharp, & Dohme, Rahway, NJ. We determined that the PAF antagonist inhibited [^{3}H]PAF specific binding to PMN (K_{di} ~ 35 nM) but had no such effect on [^{3}H]LTB_4 binding. Sources of all other reagents are indicated in (6,9).

Preparation of CPAF:

rac-1-O-Hexadecyl-2-lyso-GPC was converted to its hydrochloride salt by extraction with methanol/chloroform/concentrated HCl (2:1:0.1, v/v). Extracted material was dried, dissolved in dimethylformamide (4 ml), and treated x 5 hr at 50C with methyl isocyanate (0.5 ml). The dried residue of this reaction was purified by TLC on Silica Gel H layers developed in solvent system I (chloroform/methanol/ammonium hydroxide, 70:35:7, v/v). The reactant (R_f = 0.30) represented >70% of starting material. Its structure (see Fig. 1) and purity (>95%) were confirmed by: a) TLC in solvent system II (chloroform/methanol/glacial acetic acid/water, 50:25:8:4, v/v) (plates were developed to 14 cm twice); b) fast atom bombardment-mass spectroscopy (M+1 = 539 m/z); c) ^{1}H-nuclear magnetic resonance spectroscopy; and d) elemental analyses. [^{3}H]CPAF (56 Ci/mmol) was prepared from 1-O-[^{3}H-9,10]hexadecyl-2-lyso-GPC by identical procedures except that the reaction was conducted at 65C; radiolabeled product co-migrated with authentic CPAF on TLC, solvent systems I and II.

Assays

PMN (i.e., a leukocyte mixture containing >95% PMN, <5 platelets/100 PMN, and no erythrocytes) were isolated from normal human donor blood (6). Degranulation

was conducted as described (6) and reported as net enzyme release (i.e., percentage of total cellular enzyme activity released by stimulated cells minus that released by unstimulated but otherwise identically treated cells). Binding was conducted with 10^7 PMN suspended in 1 ml of buffer containing radiolabel, $\pm$ unlabeled ligand, and 125 μg of BSA. Suspensions were incubated x 60 min at 4C and suctioned through GF/C glass fiber filters. Filters were rinsed with 5 ml of buffer (4C), air dried, placed in 0.5 ml methanol for 5 min, overlaid with 7 ml of scintillation fluid, and counted for radioactivity (6,9). Results are reported in Scatchard plots of high affinity binding data (6) or as specific binding (i.e., fraction of added radioactivity bound to filters from cell suspensions incubated with radiolabel minus that bound to filters from suspensions incubated with radiolabel plus excess unlabeled ligand). For metabolic assays, 10^7 PMN in 1 ml of buffer containing radiolabel, $\pm$ unlabeled ligand, and 125 μg BSA were incubated 60 min at 37C. Alternatively, 100 μl of human sera was incubated 20 min at 37C with 100 μl of buffer containing radiolabel and 250 μg BSA. Reactions were stopped by the addition of equal volumes of methanol/chloroform (1:1), extracted (6), and analyzed on TLC (developed to 14 cm twice with solvent system II). TLC plates were scraped in 3 mm zones and each zone was counted for radioactivity (6).

RESULTS AND DISCUSSION

PAF and, at ~10-fold higher concentrations, CPAF stimulated PMN degranulation (Fig. 1, upper panel). CPAF is similarly potent in stimulating platelets (10). Since our PAF is the natural isomer and acts stereospecifically (9) whereas our CPAF is a racemic mixture, these data suggest that the two analogs have a relative potency ratio of 5:1, respectively. At 4C, the radiolabeled analog, [^{3}H]CPAF (which is 100% natural isomer), progressively bound to PMN over 20-40 min after which apparent equilibrium occurred (data not shown). We therefore conducted binding studies using 60 min incubation periods and ambient temperature of 4C to avoid any ligand metabolism (6). Scatchard plots of high affinity binding data indicated that [^{3}H]CPAF bound to 2500 receptors/PMN with a K_d of 1.1 nM; for [^{3}H]PAF, these respective values were 2000 receptors/PMN and 0.2 nM. Parameters for low affinity binding were: [^{3}H]PAF, 200,000 receptors/PMN, K_d of 0.25 μM; [^{3}H]CPAF, 240,000 receptors/PMN, K_d of 1.2 μM (Fig. 1). Thus, [^{3}H]CPAF had ~5-fold lower affinity than [^{3}H]PAF but each ligand bound to approximately the same number of receptors. The interrelationship of these receptors was examined in three types of studies. First, PMN were pretreated with PAF to produce a state of selective desensitization or down-regulation. The cells were hyporesponsive to a second challenge with either PAF or CPAF but

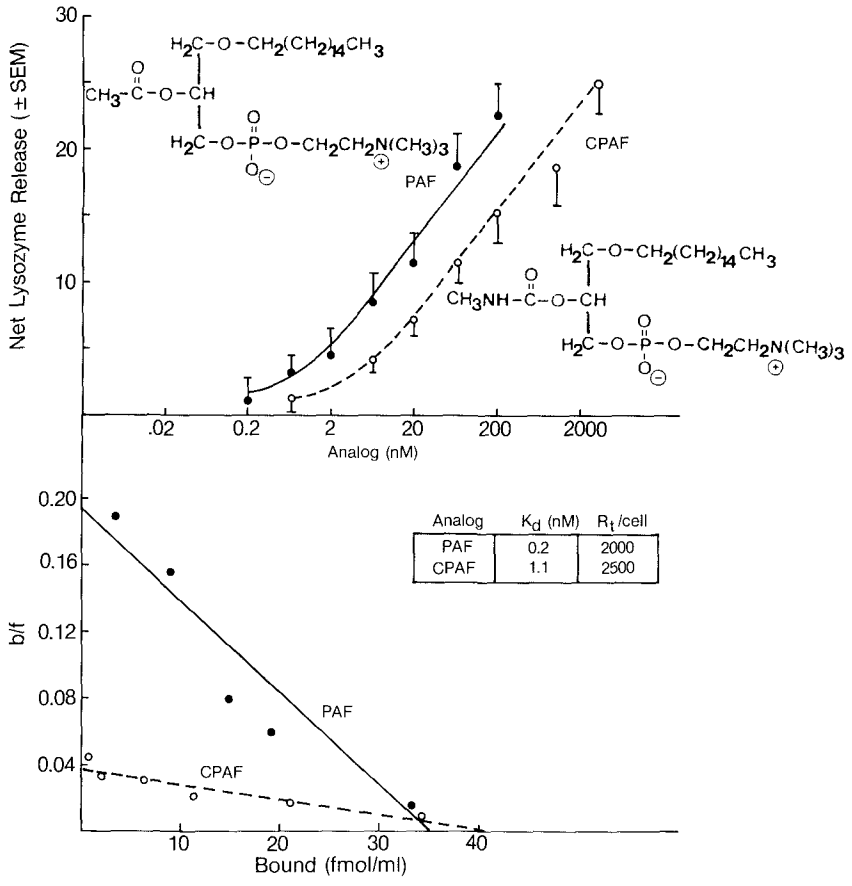

Fig. 1. Neutrophil degranulation responses to (upper panel) and binding of (lower panel) PAF and CPAF. Binding is given as Scatchard plots of high affinity binding data and reports on the natural isomers of [^{3}H]PAF and [^{3}H]CPAF; degranulation is given as concentration-response curves for the natural isomer of PAF and rac CPAF. Data points are the mean of >9 experiments. The structures of PAF (left) and CPAF (right) are given.

responded normally to LTB_4 (Table 1). Second, L652731, a competitive PAF antagonist (11), inhibited PMN degranulation responses to PAF and CPAF but not to LTB_4 (Table 1). Third, PAF and CPAF competed with [^{3}H]PAF as well as with [^{3}H]CPAF for binding sites but neither analog influenced [^{3}H]LTB_4 binding; conversely, LTB_4 did not interfere with the two phospholipids' binding (Table 2). These results indicate that PAF and CPAF bind with, and stimulate function through, common receptor sites.

PMN metabolize PAF at rates as great as 10 pmol/min/10^7 cells. Under these same conditions, however, the cells did not metabolize [^{3}H]CPAF over 60 min

Table 1
Effects of PAF Pretreatment and L652731 on
Neutrophil Degranulation Responses to PAF, CPAF, and LTB_4

	Cell Treatment			
Stimulus	None[1]	2nM PAF[1]	20 nM PAF[1]	5000nM L652731[2]
PAF, 200 nM	11.3 ± 1.3[3]	3.0 ± 1.0*	0.1 ± 0.1*	6.3 ± 0.9*
PAF, 20 nM	5.7 ± 0.9	0.4 ± 0.6*	0.0 ± 0.2*	1.9 ± 0.6*
CPAF, 2000 nM	12.2 ± 1.1	3.6 ± 1.0*	0.3 ± 0.3*	6.7 ± 0.9*
CPAF, 200 nM	8.0 ± 0.8	1.0 ± 0.5*	0.0 ± 0.0*	2.8 ± 0.4*
LTB_4, 100 nM	11.6 ± 1.2	12.0 ± 2.6	12.1 ± 2.1	10.9 ± 0.9
LTB_4, 10 nM	8.8 ± 0.7	9.1 ± 2.2	9.0 ± 1.9	8.4 ± 0.6

[1] Cells were pretreated with 0, 2, or 20 nM of PAF for 4 min, exposed to cytochalasin B for 2 min, and challenged with the indicated stimuli for 5 min.

[2] Cells were simultaneously exposed to L652731 and the indicated stimulus.

[3] Net β-glucuronidase release, ± SEM, for 6-8 experiments.

* indicates values significantly ($p < .05$, Student's unpaired t-test) below those for untreated cells.

Table 2

Specificity of Ligand Binding to Neutrophils[1]

	Radiolabel		
Competing ligand	[^{3}H]PAF	[^{3}H]CPAF	[^{3}H]LTB_4
none	13.0 ± 1.6[2]	5.2 ± 0.9	15.8 ± 2.6
PAF, 200 nM	3.3 ± 0.3*	2.5 ± 0.2*	14.7 ± 2.5
CPAF, 200 nM	4.9 ± 1.4*	3.2 ± 0.2*	15.3 ± 1.7
LTB_4, 100 nM	12.0 ± 1.7	6.0 ± 2.2	0.9 ± 0.3*

[1] PMN (1×10^7/ml) were incubated at 4C with 63.2 pM [^{3}H]PAF, 63.2 pM [^{3}H]CPAF, or 31.6 pM [^{3}H]LTB_4, ± a competing ligand. After 60 min, cell suspensions were filtered and filters were counted for radioactivity.

[2] Percentage of total radioactivity bound, ± SEM (N=5 experiments).

* indicates values significantly ($P < .01$, students paired t test) below those found in the absence of competing ligand.

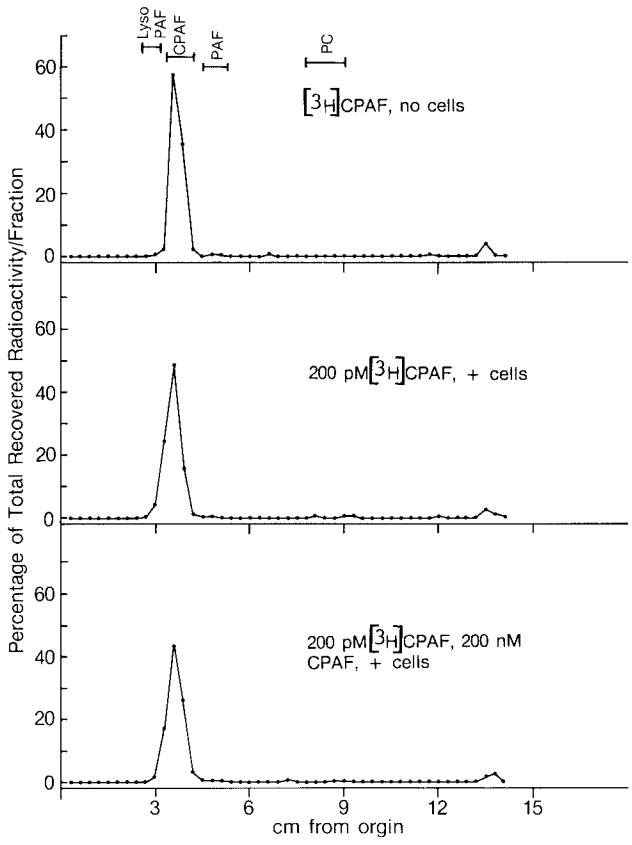

Fig. 2. TLC analysis of labeled products following 60 min incubations of [^{3}H]CPAF in buffer (upper panel), buffer containing 10^7 PMN (center panel), or buffer containing 10^7 PMN plus excess unlabelled CPAF (lower panel). Results are typical of 6 separate experiments.

Table 3

Metabolism of [^{3}H]PAF and [^{3}H]CPAF by Human Sera[1]

Condition	Standard		
	LysoPAF	CPAF	PAF
[^{3}H]PAF + serum	92.2 ± 1.1[2]	0.5 ± 0.1	4.6 ± 0.3
[^{3}H]CPAF + serum	0.2 ± 0.1	95.2 ± 0.3	1.0 ± 0.2
[^{3}H]PAF + buffer	0.3 ± 0.1	0.4 ± 0.1	98.0 ± 0.7
[^{3}H]CPAF + buffer	0.1 ± 0.1	95.2 ± 0.1	1.1 ± 0.2

[1] 2 pmol of radiolabel was incubated with 100 μl sera plus 100 μl buffer or with 200 μl buffer for 20 min at 37C. Extracts of reactions were analyzed on TLC developed twice with solvent system II.

[2] Percentage of recovered radioactivity migrating with the indicated standard, ± SEM (N=4). Greater than 92% of added radioactivity was recovered.

(Fig. 2). [^{3}H]CPAF was equally resistant to degradation by serum (Table 3).

Our results indicate that PAF induces 1/2 maximal degranulation responses at 16 nM and binds to high and low affinity receptors with K_d values of 0.2 nM and 200 nM. For CPAF (considering only the natural isomer), these respective values are 80, 1.1, and 1,200 nM. The two compounds, therefore, have almost identical stimulating potencies relative to their receptor affinities; their bioactions clearly reflect receptor occupancy rather than susceptibility to metabolism. This refutes previous suggestions that a) PAF stimulates cells by donating its acetate to regulatory transacetylating reactions (12) and b) PMN degranulation responses to PAF are appreciably reduced by the cell's inactivation of PAF (6). CPAF should be useful for examining these issues in other systems, for measuring receptor binding under conditions in which PAF is metabolized, and for in vivo studies.

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