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DIACYLGLYCEROLS ENHANCE HUMAN NEUTROPHIL DEGRANULATION RESPONSES: RELEVANCY TO A MULTIPLE MEDIATOR HYPOTHESIS OF CELL FUNCTION

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SUMMARY: At 10 μM, 1-O-oleoyl-, 1-O-palmitoyl-, and 1-O-myristoyl-2-O-acetylglycerol weakly stimulated neutrophils to release lysozyme, an enzyme in secondary granules, but had no such effect on the release of a primary granule enzyme, β-glucuronidase. The glycerides (1-10 μM) had a second effect on both granule populations: they enhanced the degranulating potencies of leukotriene B_4, platelet-activating factor, a formylated oligopeptide, and C5a by 10- to 30-fold. In contrast, they were much less effective in enhancing responses to ionophore A23187 and partially inhibited responses to phorbol myristate acetate. The diether analogue, 1-O-hexadecyl-2-O-ethylglycerol was inactive in these regards. We suggest that diacylglycerols are a novel class of bioactive products mobilized from phosphoglycerides in stimulated neutrophils; as co-products of this mobilization, platelet-activating factor and leukotriene B_4 may interact with diacylglycerols to promote cell function.

Stimulated PMN rapidly mobilize their phosphoglycerides (1-12) to form free arachidonic acid (1-2,5,6,8,10) and 1-O-alkyl-2-lyso-sn-glycero-3-phosphocholine (9,10). Arachidonate is then oxidized to LTB_4 and 5-HETE while the latter lipid is acetylated to PAF. These products may mediate PMN function: LTB_4 and PAF degranulate PMN; 5-HETE dramatically enhances their effects (13,14). Nevertheless, individually or in combination, the three lipids are required at high concentrations to achieve even moderate amounts of degranulation. Moreover, PMN made completely unresponsive to each or all of the lipids respond fully to FMLP or C5a (15). Thus, LTB_4, PAF and 5-HETE

ABBREVIATIONS:
OAG: rac-1-O-oleoyl-2-O-acetylglycerol; PAG: rac-1-O-palmitoyl-2-O-acetylglycerol; MAG: rac-1-O-myristoyl-2-O-acetylglycerol; EEG: 1-O-hexadecyl-2-O-ethylglycerol; LTB_4: leukotriene B_4; PAF: Platelet activating factor; FMLP: N-formyl-(S)-methionyl-(S)-leucyl-(S)-phenylalanine; C5a: the chemotactic fragment from human C5; PMA: phorbol-12-myristate-13-acetate; 5-HETE: 5(S)-hydroxy-cis-8,11,14-trans-6-eicosatetraenoate; PMN: polymorphonuclear neutrophil; TLC: thin layer chromatography

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do not successfully explain most PMN degranulation responses. Other mediators, perhaps acting in concert with these, appear required. Diacylglycerols are candidates for this. These lipids play a central role in the metabolic interconversions of phosphoglycerides. For example, stimulated platelets rapidly convert phosphoglyceride into diacylglycerols (16,17). Furthermore, diacylglycerols possess relevant bioactivity: they degranulate platelets (18) by directly activating protein kinase C, a protein-phosphorylating enzyme implicated in exocytotic reactions (19,20). A similar pathway linking phosphoglycerides, diacylglycerols, protein kinase C, protein phosphorylation, and cell function may exist in PMN (5,7,11,12,20,21). Here we present evidence supporting this and indicating that diacylglycerols can interact with other products of phosphoglyceride mobilization, e.g., LTB_4 and PAF, to elicit prominent degranulation responses in human PMN.

MATERIALS AND METHODS

Reagents and Buffers:

C5a was purified to apparent homogeneity from zymosan-activated, 6-aminohexanoic acid-pretreated human sera; LTB_4 was biosynthesized using rabbit peritoneal PMN, arachidonic acid, and ionophore A23187; PAF was prepared by catalytic hydrogenation, saponification, and acetylation of beef heart plasmalogens; PMA, A23187, FMLP, cytochalasin B, and bovine serum albumin were purchased (13,14). For all bioassays, modified Hanks' balanced salt solution (1.4 mM calcium chloride, 0.7 mM magnesium chloride) was used (13).

Preparation of Glycerols:

rac-1-O-Oleoylglycerol, rac-1-O-palmitoylglycerol and rac-1-O-myristoylglycerol (Serdary Research, London, Ontario, Canada) were protected at the 3-position with 4,4'-dimethoxytrityl chloride and acetylated at the 2-position with acetic anhydride in triethylamine. Acetylated product was then heated for 24 hr at 60°C with boric acid in trimethyl borate (22) to remove the protecting group. The respective products, i.e., OAG, PAG, and MAG, were purified as described below. Chimyl alcohol (i.e., 1-alkyl-sn-glycerol containing 82% hexadecyl and 15% octadecyl alkyl ether residues at sn-1) (23) was protected at sn-3 with trityl chloride in methylene chloride containing triethylamine and treated with KOH followed by ethyl methane sulfonate to form the corresponding sn-2-ethoxy derivative. Product was detritylated by suspending for 4 hr in ethanol:diethyl ether (3:1) made 3 M with hydrochloric acid. The resulting 1-O-alkyl-2-O-ethylglycerol (EEG), as well as OAG, PAG, and MAG were individually purified by TLC. Preparative TLC silicic acid plates were first developed in water:ethanol (1:1, v:v) containing 12.5% boric acid and then heat-activated (180°C, 3 hr). Products were spotted and developed in diethyl ether:hexane (90:10, v:v), a system which separates 1,2-diacyl isomers from their spontaneously forming 1,3-isomers (24). Without

delay, the 1,2-isomer zone was isolated and extracted with diethyl ether through a sintered glass filter. Subsequent TLC analyses of 1,2-isomer isolates indicated little (i.e., <5%) or no 1,3-isomer contamination. To confirm product structure, EEG and OAG were analysed by ammonia chemical ionization-mass spectroscopy. Results demonstrated M+1 ions with both glycerides and unambiguously confirmed product identity and (>95%) purity. All products were stored at -70°C in hexane. Before use, glycerides were freed of hexane under a stream of nitrogen and taken up in Hanks' buffer containing 2.5 mg/ml bovine serum albumin. All other stimuli were also dissolved in the same buffer. Stimuli and glycerides were diluted in the same buffer before being individually added to PMN suspensions. All final cell suspensions contained 250 µg of bovine serum albumin per ml and 0.1% dimethylsulfoxide (used with cytochalasin B).

Bioassay

Human leukocytes (2.6×10^6/ml) (>95% PMN, isolated from the blood of normal human donors who abstained from all medications for >7 days [13]) were treated with 5 µg/ml cytochalasin B for 2 min and then challenged for specified periods. Cells were exposed to a glyceride 15, 10, 8, 4, 2, or 0 min before challenge, as indicated. Following challenge, cell suspensions were placed on ice and centrifuged (200g x 4'; 4°C) to obtain supernatant fluid which was assayed for lysozyme (E.C. 3.2.1.17), β-glucuronidase (E.C. 3.2.1.21), and lactic dehydrogenase (E.C. 1.1.1.27). Results are reported as net enzyme release, which is the percentage of total cellular enzyme released by stimulated minus that found in identically (and simultaneously) treated but unstimulated cell suspensions. When used to modulate the actions of standard degranulating agents, the glycerides or PMA were not considered as stimuli; net enzyme release was therefore calculated by subtracting release induced by glyceride or PMA alone from that induced by enhancer plus standard degranulating agent.

RESULTS AND DISCUSSION

At 10 µM (lower dosages were inactive) MAG and, less effectively, OAG and PAG, weakly stimulated PMN to release lysozyme, an enzyme located principally in secondary granules (Figure 1). Their actions developed over 15-30 min and required cytochalasin B. Contrastingly, EEG, the diether analogue of PAG, was inactive. None of the glycerides detectably influenced the release of a primary granule constituent, β-glucuronidase, or a cytosolic enzyme, lactic acid dehydrogenase. Hence, degranulation was selective for diacylglycerols, involved only secondary granules, and occurred as a limited, slowly-developing response. This response resembles that elicited by PMA: in the presence of cytochalasin B, PMA stimulates principally secondary granule enzyme release over 15-30 min; in contrast, LTB_4, PAF, FMLP, C5a, and A23187 rapidly (<3-5 min) trigger release from both granule populations (13,14). PMA, also like diacylglycerols, possesses vicinal fatty acyl and acetate residues, and directly activates protein kinase C (26). These data, then, agree

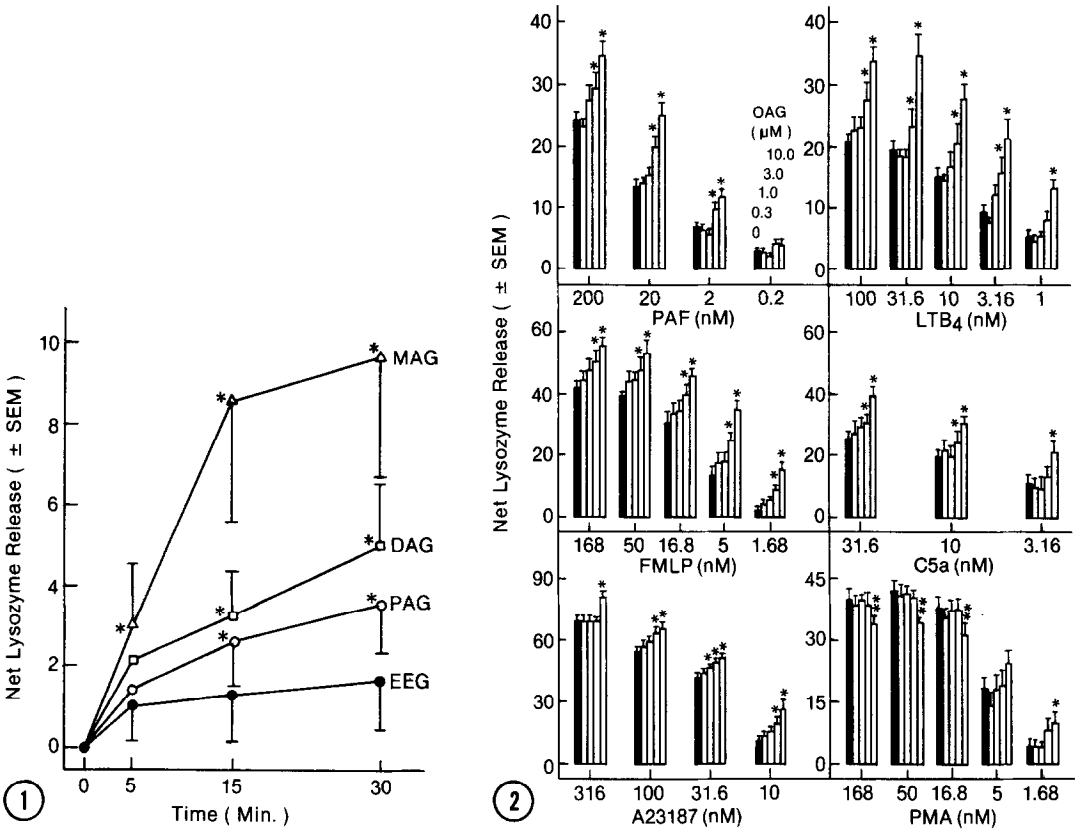

Figure 1: Net lysozyme released by neutrophils following exposure to various glycerides. Astericks indicate values significantly greater ($p < 0.05$, Student's paired t-test) than for cells treated with bovine serum albumin alone. Each point gives the mean for 11 separate experiments.

Figure 2: Net lysozyme released by neutrophils pretreated with 1-10 μM OAG for 2 min before challenged with the indicated dosage of PAF, LTB_4, FMLP, C5a, A23187, or PMA. Astericks indicate values significantly greater ($p < 0.05$) than the release produced by challenging agent alone (i.e., first shaded bar in each series). Double astericks indicate values significantly lower ($p < 0.05$) than the release produced by the challenging agent alone. Each bar gives the mean of more than 7 experiments. Similar results were found in cells pretreated with OAG for 4 or 0 min before challenge; little or no enhancement occurred in cells pretreated

with those of Fujita et al (21) in suggesting that OAG and PMA activate PMN via a common mechanism that involves protein kinase C.

In addition to their direct but feeble actions, diacylglycerols had a much more profound effect on degranulation. Figure 2 demonstrates that OAG enhanced the lysozyme-releasing actions of other stimuli. It was most effective on responses to LTB_4 and PAF, increasing their potencies by 10- to 30-fold (i.e., it shifted dose-response curves toward 10- to 30-fold lower stimulus concentrations) as well as their optimal degranulating effects by 50 to

TABLE 1

EFFECT OF VARIOUS GLYCERIDES OR PMA ON THE NEUTROPHIL DEGRANULATION RESPONSE TO FOUR STIMULI [1]

Glyceride or PMA (μM)	Stimulus (nM)			
	PAF (2)	LTB_4(10)	FMLP (5)	A23187 (31.6)
None	5.1 ± 1.1 [2]	10.7 ± 1.3	7.6 ± 1.9	22.2 ± 5.1
EEG (10)	5.4 ± 1.4	10.8 ± 1.8	7.4 ± 2.2	22.3 ± 7.4
EEG (3.16)	5.1 ± 1.3	9.6 ± 1.7	7.8 ± 2.4	23.5 ± 6.8
PAG (10)	10.3 ± 1.9*	16.5 ± 1.7*	18.5 ± 2.5*	37.4 ± 3.2*
PAG (3.16)	7.6 ± 1.4*	14.8 ± 1.8*	13.6 ± 2.7*	30.8 ± 5.0*
MAG (10)	8.9 ± 2.7*	16.2 ± 1.7*	16.4 ± 1.7*	30.1 ± 4.7*
MAG (3.16)	7.4 ± 1.5*	13.7 ± 1.7*	11.0 ± 1.7*	28.6 ± 5.7*
OAG (10)	8.0 ± 1.2*	17.6 ± 1.6*	16.6 ± 2.2*	32.2 ± 3.1*
OAG (3.16)	7.1 ± 1.0*	13.1 ± 1.4*	13.5 ± 2.8*	28.1 ± 3.0*
PMA (0.005)	8.9 ± 0.8*	15.5 ± 1.1*	16.1 ± 2.1	30.1 ± 2.7*
PMA (0.0005)	7.1 ± 0.4*	12.9 ± 0.7*	10.4 ± 1.9*	28.0 ± 2.7*

[1] Cells were preincubated with cytochalasin B plus glyceride or PMA for 2 min and challenged for 5 min.
[2] Net β-glucuronidase release, ± SEM, for eight experiments. Similar results were found for lysozyme release.
* Indicates values significantly ($p<.05$, Student's paired t-test) greater than those for cells not incubated with a glyceride or PMA (i.e., first value in each column).

150%. It somewhat less effectively influenced FMLP and C5a, was only moderately active in enhancing A23187-induced responses, and actually inhibited responses to higher concentrations of PMA (Figure 2).

In interpreting the above results, several points require emphasis. First, OAG identically influenced β-glucuronidase release. In particular, it inhibited responses to PMA (data not shown) while enhancing those to the other stimuli (Table 1). Second, PAG and MAG also enhanced lysozyme (data not shown) and β-glucuronidase release (Table 1); EEG, however, had no such activity (Table 1). Thus, enhancement was a diacyl-dependent, structurally specific effect rather than some nonspecific action of lipids in general. Relevant to this, PAF desensitizes (i.e., inhibits) responses to itself (13, 23) and does not augment responses to LTB_4 (14) or FMLP (data not shown); and 5-HETE, while potentiating PMN degranulation, enhances responses to PAF and LTB_4, without influencing responses to FMLP, C5a, or A23187 (13,14). Thus, in addition to structural specificity, diacylglycerols demonstrate a unique spectrum of potentiating actions. They do not act like PAF or 5-HETE. Third, PMA also enhanced degranulation (Table 1). This plus the inhibitory actions of OAG on PMA further indicate that phorbol diesters and diacylglycerols

represent a family of at least partially analogous stimuli. Fourth, diacylglycerols and PMA did not merely add their intrinsic agonistic actions to those of other stimuli. At the dosages and time periods employed, their independent degranulating effects were minimal (for lysozyme) or nil (for β-glucuronidase) and, in any case, were subtracted from the results reported.

It is therefore suggested that events (e.g., protein kinase C activation) triggered by the structurally analogous stimuli, PMA and diacylglycerols, are especially conducive to exocytotic reactions only when other events, such as those induced by LTB_4, PAF, FMLP, C5a, or A23187, are concurrently initiated. In this view, release of secondary and primary granule enzymes is seen as a response optimally mediated by the coordinated production of distinctly different mediators each of which govern different elements of the final exocytotic reaction. Two pathways seem already identified. The first involves the release and metabolism of arachidonate into agonists (e.g. LTB) and potentiators (e.g., 5-HETE) of function. The second involves deacylation of alkyl ether glycerolipids to form, ultimately, the potent agonist PAF. A third pathway, wherein diacylglycerols or metabolic derivatives thereof are produced, appears quite attractive based on the results reported here. We note that diacylglycerols are particularly effective in enhancing the actions of LTB_4 and PAF (Figure 2) and that glycerides, LTB_4, and PAF may be obligate co-products: all form as a consequence of phosphoglyceride mobilization (1-12). This mobilization, therefore, yields multiple products with different effects on degranulation. The combined interactions of these co-products and, in particular, the enhancing actions of diacylglycerols may successfully explain the ultimate cellular response.

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