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Selective Priming of Rate and Duration of the Respiratory Burst of Neutrophils by 1,2-Diacyl and 1-*O*-Alkyl-2-acyl Diglycerides

POSSIBLE RELATION TO EFFECTS ON PROTEIN KINASE C*

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Both 1,2-diacyl- and 1-*O*-alkyl-2-acyl-*sn*-glycerols are released during stimulation of human polymorphonuclear leukocytes (PMNL). 1,2-Diacylglycerols have received intense interest as intracellular "second messengers" due to their ability to activate protein kinase C (Ca^{2+} phospholipid-dependent enzyme). However, little is known about bioactivities of the alkylacylglycerols. This study compared the ability of 1,2-diacyl- and 1-*O*-alkyl-2-acylglycerols to modulate the respiratory burst of stimulated PMNL, a response which depends on the activation of an NADPH oxidase to generate bactericidal species of reduced oxygen. Direct stimulation by *N*-formyl-Met-Leu-Phe caused an abrupt release of H_2O_2 which ceased within 2.5 min. Preincubation with diacylglycerols (1-oleoyl-2-acetylglycerol, 5–30 μM, and 1,2-dioctanoylglycerol, 2–5 μM) caused a decrease in lag time, 3-fold increase in initial rate of H_2O_2 release, and marked prolongation of the response to *N*-formyl-Met-Leu-Phe (features characteristic of a priming effect). Preincubation with alkylacylglycerols (1-*O*-Δ9-octadecenyl-2-acetylglycerol, 5–30 μM, and 1-*O*-octyl-2-octanoylglycerol, 20–50 μM) primed initiation (shortened lag time and increased velocity) but, in contrast to diacylglycerols, did not alter duration of H_2O_2 release.

While low concentrations of diacylglycerols (5–30 μM) primed PMNL, higher concentrations (≥70 μM) stimulated the cells directly. In contrast, higher (70–100 μM) concentrations of alkylacylglycerols did not prime the responses but, in fact, inhibited priming (especially of duration) induced by diacylglycerol. The high concentrations of alkylacylglycerol also inhibited direct stimulation induced by high concentrations of diacylglycerol. Direct stimulation by high concentrations of diacylglycerol probably involves activation of protein kinase C, whereas alkylacylglycerol was found to inhibit activation of protein kinase C by diacylglycerol *in vitro*. Thus, diacylglycerols are complete priming agonists, altering both rate and duration of the response. In contrast, alkylacylglycerols may have biphasic, concentration-related effects in modulation of functions of PMNL. At low concentrations, they may facilitate initiation of functional events; however, as their concentration increases, they may serve to terminate responses.

The distinct priming effects of these diglycerides also reveal that priming can involve at least two distinct events: 1) initiation and 2) prolongation. The separate modulation of these events indicates that initial activation of the NADPH oxidase and maintenance of subsequent oxidase activity require different intracellular mechanisms.

Normal bactericidal function of human polymorphonuclear leukocytes (PMNL)[1] depends upon stimulation of a respiratory burst (1, 2). This involves activation of the NADPH oxidase, a multicomponent electron transport chain which transfers electrons from intracellular NADPH to extracellular (or phagosomal) oxygen, with the net production of superoxide and H_2O_2. Enhanced oxidative responses may be observed if normal PMNL are preincubated with one agonist and then stimulated with a different agonist (3–14). This increased responsiveness has been referred to as "priming" (5, 15), and as employed previously (14), the following terminology will be used in this report:

$$\text{Rest} \xrightarrow{\text{stimulant}} \text{direct stimulation}$$

$$\text{Rest} \xrightarrow[\text{(priming agent)}]{\text{1st agonist}} \text{priming} \xrightarrow[\text{(stimulant)}]{\text{2nd agonist}} \text{primed stimulation}$$

The primed response is greater than would be predicted by an additive effect of the two agonists alone, and maximal priming involves all the following kinetic aspects of the respiratory burst: shortened lag time, increased velocity, and prolongation. Priming is not a mere *in vitro* phenomenon;

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[1] The abbreviations used are: PMNL, polymorphonuclear leukocytes; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; alkyl-PC, 1-O-alkyl-2-acyl-*sn*-glycero-3-phosphocholine; HBSSg, Hanks' balanced salt solution with addition of 5 mM glucose and 0.1% gelatin; PAF, platelet activating factor (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine); PC, choline-containing phosphoglycerides; PKC, calcium and phospholipid-dependent protein kinase (protein kinase C); PMA, phorbol-12-myristate-13-acetate; TLC, thin layer chromatography; V_{init}, initial (maximal) velocity; $V_{3\,min}$, velocity 3 min after stimulation. Diglycerides are abbreviated according to side chains linked to the glycerol linkage, with "A" for acyl and "E" for ether (alkyl); chains at *sn*-1 and *sn*-2 positions are given in parentheses (number of carbons:number of double bonds). Thus, AAG, 1,2-diacylglycerol; EAG, 1-*O*-alkyl-2-acylglycerol; EEG, 1,2-dialkylglycerol.

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similar, greater-than-normal respiratory burst responses have been reported with granulocytes from patients having illnesses characterized by myeloid stimulation (16–19). Although both *in vivo* and *in vitro* priming were observed more than a decade ago (3, 16), the mechanism(s) of priming remain largely unknown.

Optimal priming is observed with agonists which activate different intracellular stimulus-response coupling mechanisms (6). Diacylglycerols (AAGs)[1] have been proposed as intracellular second messengers which activate protein kinase C (PKC) in human PMNL (14, 20, 21), as well as other cells (22–24). AAGs are released by a phospholipase C which cleaves phosphatidylinositol-4,5-bisphosphate; this pathway is activated by binding of certain membrane receptors (*e.g.* receptors for chemotactic peptides) but is inhibited by phorbol myristate acetate (25, 26). Recently, Daniel *et al.* (27) described a distinct pathway, activated by phorbol myristate acetate, which cleaves 1-*O*-alkyl-2-acyl-*sn*-glycero-phosphocholine (alkyl-PC) to release 1-*O*-alkyl-2-acyl-*sn*-glycerols (ether acyl glycerides, EAGs) in the Madin Darby kidney cell line; this pathway appeared to be catalyzed by a phospholipase C specific for PC. 1-*O*-alkyl-linked species constitute 46 mol % of the PC fraction of human PMNL (28) which provides a source of EAGs. Stimulation with the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), phorbol myristate acetate, AAG, or Ca^{2+} ionophores have been shown to cause EAG release in human PMNL (29–31). Thus, both 1,2-diacyl- and 1-*O*-alkyl-2-acyl-glycerols are released during responses of PMNL.

Biological activities of AAGs have been generally assumed to be due to activation of PKC (7, 8, 20, 22–24, 32). The respiratory burst of PMNL is directly stimulated by a cell permeant AAGs, 1-oleoyl-2-acetylglycerol (AAG(18:1–2:0)) (7, 8, 14, 20) and 1,2-dioctanoylglycerol (AAG(8:0–8:0)) (32). Direct stimulation by AAG(18:1–2:0) occurs at concentrations which cause translocation of PKC from the cytosol to the plasma membrane (14). Lower concentrations of AAG(18:1–2:0) also prime PMNL to produce more reduced oxygen species (*e.g.* H_2O_2) in response to fMLP (7, 8, 14), although such priming may involve mechanisms distinct from classical translocation or activation of PKC (14). The biological activities of EAGs are largely unknown. EAGs have little or no ability to activate PKC *in vitro* (33, 34) and may actually inhibit PKC activity ((35) and this study) but could modulate cellular function by other mechanisms. EAGs cause differentiation of the human myeloid leukemia cell line, HL-60, by unknown mechanisms (36). Effects of EAGs on functional responses by PMNL have not been reported.

We are investigating the ability of AAGs and EAGs to modulate cellular function using synthetic analogs of the natural diglyceride species. AAG(18:1–2:0) has been used previously since it is cell permeant (20, 32). We have synthesized the EAG species, 1-*O*-Δ9-octadecenyl-2-acetylglycerol (EAG (18:1–2:0)) and several similar EAG analogs for comparison. The AAG, 1,2-dioctanoylglycerol and its EAG analog, 1-*O*-octyl-2-octanoylglycerol, have also been synthesized and their priming and stimulatory effects examined. Since activation of PKC might have a central role in stimulation of PMNL, the effects of AAG(18:1–2:0) and EAG(18:1–2:0) on activity of PKC(s) from human PMNL were also studied.

EXPERIMENTAL PROCEDURES

Reagents—PMA, cytochalasin B, and azide were obtained from Sigma. fMLP was from Penninsula Laboratories, Belmont, CA. 1,2-Dioctanoylglycerol (AAG(8:0–8:0)) was obtained from Molecular Probes (Eugene, OR). Octyl bromide, *rac*-isopropylidene glycerol (Soketal), (4,4′-dimethoxy) trityl chloride, and trimethylborate were from Aldrich. Octanoyl chloride was from NuChek Prep (Elysian, MN). [γ-^{32}P]ATP was from DuPont-New England Nuclear. Hanks' balanced salt solution (HBSS) was from GIBCO, and Isolymph from Gallard Schlessinger (Carle Place, NY). Gelatin was purchased from Difco Laboratories (Detroit, MI).

Synthesis of Diglycerides and Platelet Activating Factor—1-Oleoyl-2-acetyl-*rac*-glycerol (AAG(18:1–2:0)), 1-*O*-Δ9-octadecenyl-2-acetyl-*sn*-glycerol (EAG(18:1–2:0)), 1-*O*-Δ9-octadecenyl-2-propionyl-*rac*-glycerol (EAG(18:1–3:0)), 1-*O*-hexadecyl-2-hexanoyl-*sn*-glycerol (EAG(16:0–6:0)), 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol (EAG(16:0–2:0)), and 1-*O*-hexadecyl-2-*O*-ethyl-*sn*-glycerol (EEG(16:0–2:0)) were synthesized by minor modifications of previously published techniques (37). Platelet activating factor (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, PAF) was synthesized according to the methods of Surles *et al.* (38). Ammonia chemical ionization mass spectrometry yielded characteristic fragmentation and molecular ions for the above compounds. TLC was routinely employed to further verify chemical and isomeric purities. The TLC was carried out on Silica gel G layers developed in ethyl ether/hexane 60:40 (v/v) and visualized by H_2SO_4:chromate charring. The 1,2- and 1,3-diglycerides are readily resolved in this system. All compounds demonstrated >95% chemical and isomeric purity. To minimize acyl migration (verified by TLC), the 1,2-diglycerides were stored in hexane at −70 °C.

1-*O*-Octyl-2-octanoyl-*rac*-glycerol (EAG(8:0–8:0)) was synthesized as follows: *rac*-isopropylidene glycerol was converted to the octyl ether by refluxing with sodium hydride followed by 1-octyl bromide in tetrahydrofuran. After cooling, an equal volume of water was carefully added. The mixture was extracted with an equal volume of ethyl ether and the ether was collected, washed twice with water, and then dried over sodium sulfate. After solvent removal, the resulting oil was vacuum distilled (100–110 °C at 2 mm Hg) with a yield of 85%. Acidic hydrolysis of the ketal was carried out by refluxing in 0.5 N H_2SO_4 for several hours, followed by stirring at room temperature overnight. The solution was extracted twice with equal volumes of ethyl ether followed by equal volumes of chloroform. The organic phases were combined, dried over sodium sulfate, and freed of solvent. The resulting monoglyceride was vacuum distilled (130–140 °C at 1 mm Hg), yield 80%. 1-*O*-octyl-*rac*-glycerol was protected at the primary hydroxyl position by reacting it with 4,4′-dimethoxytrityl chloride using pyridine as the solvent. The reaction mixture was poured into an equal volume of water and extracted twice with equal volumes of ether. After pooling the ether phases, they were washed with water and dried over sodium sulfate. The protected monoglyceride was acylated without further purification using octanoyl chloride in pyridine. The primary product, 1-*O*-octyl-2-octanoyl-3-(4,4′-dimethoxytrityl-*rac*-glycerol was purified after extracting from ethyl ether/water, as above (yield 55%). The crude material was freed of solvent and loaded onto a silica column (Merck Grade 60 Silica Gel, 230–400 mesh) and eluted with chloroform. Column fractions were monitored by TLC. The most pure fractions were pooled, the solvent evaporated, and applied to another Silica Gel 60 column. The lipid was eluted with hexane/ethyl acetate (4:1, v/v). The most pure fractions were again collected, evaporated, and loaded onto a third Silica Gel 60 column employing toluene eluant, giving a >98% pure product. Deprotection of the primary hydroxyl was carried out by refluxing 100 mg of lipid and 100 mg of boric acid in 1 ml of trimethyl borate overnight. The desired product, 1-*O*-octyl-2-octanoyl-*rac*-glycerol (EAG(8:0–8:0)), migrated on TLC with an R_F = 0.39 (ethyl ether/hexane, (60:40, v/v)). Purification was carried out on preparative TLC on borate impregnated plates to minimize acyl migration, using the same solvent system. Impregnation was achieved by prerunning 1-mm thick Silica Gel G plates in water/ethanol (1:1, v/v) saturated with boric acid, then activating overnight at 100 °C. Purity of the EAG(8:0–8:0) was >95% and was routinely examined for acyl migration by TLC. With this purification method, no contamination by the diacyl analog, AAG(8:0–8:0) should occur. This was confirmed by mass spectrometry.

For each experiment, aliquots of diglyceride or PAF solutions were dried under N_2 and suspended by sonication in HBSS containing 2.5 mg/ml bovine serum albumin (final bovine serum albumin concentrations in the assays was less than 0.25 mg/ml).

Preparation of PMNL—PMNL were isolated by a modification of the technique of Boyum (39) as described previously (40). Granulocytes were washed by centrifugation and resuspended in HBSS containing 5 mM glucose and 0.1% gelatin, without added Ca^{2+} or Mg^{2+} (HBSSg). PMNL concentration was determined by standard hemocytometer techniques. PMNL were kept at 5 × 10^7/ml at 4 °C until 5

min before study, at which time they were warmed to 37 °C and 1 mM Ca^{2+} (final concentration) was added to the buffer. Viability, assessed by trypan blue exclusion, was >95% and was not changed by preincubation with the diglycerides employed.

Priming and Stimulation Protocols—If the time of stimulant addition is considered time 0, at $t = -151$ min, PMNL were diluted to 5×10^6/ml in prewarmed, air-saturated HBSSg with 1 mM Ca^{2+}. At $t = -10$ min, the priming agent (or buffer) was added as appropriate to the experiment. At $t = -2$ min, 5 μg/ml cytochalasin B (unless stated otherwise) and 2 mM azide were added, followed by stimulant addition at $t = 0$. Azide was included to inhibit catalase and myeloperoxidase which would cause catabolism of H_2O_2 to water (peroxidase) or to regeneration of O_2 (catalase) and thereby cause unpredictable changes in net O_2 consumption and H_2O_2 release.

In certain experiments, the effect of removal of extracellular diglyceride was examined as follows: PMNL were prewarmed and diglyceride added, as above. After 2.5 min, the cells were washed by brief (5 min) centrifugation and resuspended in the same buffer, but lacking diglyceride. They were then treated with cytochalasin B and stimulated with fMLP, as in the standard priming protocol.

Measurement of Hydrogen Peroxide Release and Oxygen Consumption—Hydrogen peroxide release was measured polarographically using an oxidase probe, and oxygen consumption was measured using a Clark oxygen electrode (both from Yellow Springs Instruments, Yellow Springs, OH) in a 37 °C water-jacketed, dual electrode, 2-ml cuvette (Gilson Medical Electronics, Middleton, WI). Both H_2O_2 release and O_2 consumption were recorded simultaneously using a homemade nanoammeter. H_2O_2 was calibrated by addition of 10 μM H_2O_2 to the cuvette. The relative sensitivity of the H_2O_2 and O_2 electrodes was also calibrated by addition of glucose oxidase plus glucose to the cuvette; a stoichiometry of O_2 consumption to H_2O_2 generation of 1:1 was assumed. The two methods of H_2O_2 calibration agreed within 15%. Zero oxygen concentration was calibrated by addition of sodium dithionite, and the O_2 concentration of the air-saturated buffer was assumed to be 200 μM. The measurement of H_2O_2 generation was roughly 10-fold more sensitive than that of O_2 consumption and was considered the more precise measure of the responses. Therefore, the H_2O_2 data are given in this report. In all instances, oxygen consumption paralleled H_2O_2 release, except that slight O_2 consumption continued after cessation of detectable release of H_2O_2. The "initial velocity" (V_{init}) is the maximal rate of response. Since the response of fMLP normally terminated in less than 2.5 min, the rate of response occurring 3 min after stimulation ($V_{3\,min}$) was used as an indicator of prolongation of the respiratory burst.

Protein Kinase C Assays—Previously described techniques (21, 41) were employed. Briefly, PMNL, 5×10^7/ml, were suspended in extraction buffer containing 50 mM Tris-HCl, 2 mM EGTA, 50 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4. The PMNL were sonicated and fractionated as described (21). After a 500 × *g* centrifugation to remove nuclei and unbroken cells, the supernatant was centrifuged at 150,000 × *g* for 90 min; the resultant supernatant, referred to as "cytosol," was used to assay for PKC activity in PMNL (with resting PMNL, under these conditions, virtually all PKC activity is in this soluble fraction). The PKC assay (21) measured the incorporation of ^{32}P from [γ-^{32}P]ATP into type H1 histone and was performed at 30 °C in the presence or absence of 0.6 mM added Ca^{2+} (0.2 mM free Ca^{2+}), 20 μg/ml phosphatidylserine, and the indicated concentration of diglyceride. PKC activity is expressed as pmol of ^{32}P incorporated/min/10^7 cell equivalents.

Statistical Analysis—Data are expressed as means ± S.E. The number of determinations (N) refers to the number of separate PMNL preparations studied. Nonlinear regression employed a modification of the Marquardt algorithm (42) (Graph-Pad, ISI Software). Significance was tested with the Student's t test (paired or unpaired) or Wilcoxon rank sum test, as appropriate. A $p < 0.05$ was considered significant.

RESULTS

Stimulation of the Respiratory Burst by fMLP—The chemotactic peptide, fMLP, caused a transient stimulation of H_2O_2 release from normal PMNL (Fig. 1). Cytochalasin B has been reported to slightly prolong the response to fMLP (43); however, even with cytochalasin preincubation, the response began to decrease within 1 min and had completely ceased within 2.5 min after stimulation (Fig. 1). Most investigators studying fMLP stimulation of PMNL employ cytochalasin B to obtain an optimal and reproducible response. In this study, unless specified otherwise, cytochalasin B, 5 μg/ml, was added 2 min before fMLP. Under these conditions, 10 nM fMLP (the concentration used in the priming experiments) directly stimulated 5.07 ± 0.35 nmol/min H_2O_2 release (initial rate; $n = 18$), and 1 μM fMLP (a maximally effective concentration) stimulated 24.4 ± 1.54 nmol/min ($n = 5$).

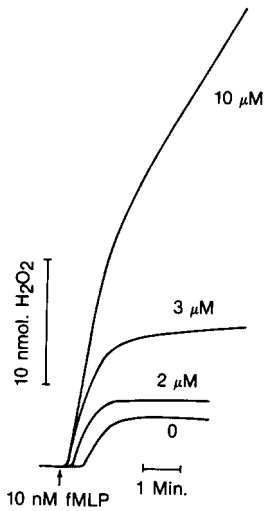

FIG. 1. **Tracing of H_2O_2 release induced by 10 nM fMLP and modulation of the response after preincubation with the diacylglycerol, AAG(18:1–2:0).** Prior to stimulation by fMLP, PMNL were preincubated for 10 min with the indicated concentrations of AAG(18:1-2:0) (0, 2, 3, or 10 μM) and for 2 min with 5 μg/ml cytochalasin B. The *vertical bar* represents the equivalent of 10 nmol of H_2O_2 released from 10^7 PMNL. Similar results were observed in 14 separate PMNL preparations.

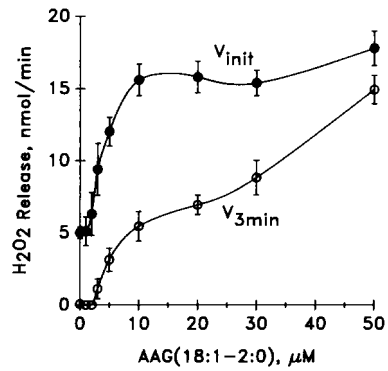

FIG. 2. **Priming of fMLP-stimulated H_2O_2 release by preincubation with varying concentrations of AAG(18:1–2:0).** The initial velocity (V_{init}) of H_2O_2 release is the slope usually measured in such responses. Since responses to fMLP alone were always flat (0) by 3 min, the velocity after 3 min ($V_{3\,min}$) was used as an indicator of prolongation of the response. Experimental conditions were as described for Fig. 1. Data are means ± S.E. from 14 PMNL preparations and are nmol H_2O_2 released/min/10^7 PMNL.

Priming of the Respiratory Burst by Diacylglycerols, AAG(18:1-2:20) and AAG(8:0-8:0)—Preincubation of PMNL with AAG(18:1-2:0) caused a shortening of the lag time and up to a 3-fold enhancement of the initial rate of the response to subsequent fMLP stimulation (Figs. 1 and 2). AAG(18:1-2:0) also caused a concentration-related prolongation (or lack of termination) of the response. With priming by 50 μM AAG(18:1-2:0), H_2O_2 release continued nearly unabated for the 6-min duration of the experiments (data not shown). To provide a means of quantitating duration, the rate of H_2O_2

release was determined 3 min after fMLP stimulation. In unprimed PMNL, the $V_{3\,min}$ was always zero. However, in PMNL primed with AAG(18:1-2:0), the concentration-related prolongation, reflected in the increasing $V_{3\,min}$, is shown in Fig. 2. Under the conditions employed (preincubation with diglyceride prior to addition of cytochalasin B), there was little direct stimulation by AAG(18:1-2:0) up to 50 μM (at which the rate was 2.91 nmol/min).

AAG(8:0-8:0) has been reported to stimulate the respiratory burst of PMNL (32), presumably by activation of PKC (44). In the present study, AAG(8:0-8:0) was much more potent as a direct stimulant than AAG(18:1-2:0), with significant stimulation observed at ≥10 μM. However, at low concentrations (2-4 μM), AAG(8:0-8:0) caused minimal direct stimulation (approximately 1 nmol H_2O_2/min), but caused priming of both initial velocity and duration of the response to fMLP. Priming with 3 μM AAG(8:0-8:0) increased the fMLP V_{init} from 3.9 ± 0.56 (controls) to 12.9 ± 1.4 (primed) nmol H_2O_2/min (n = 3). The $V_{3\,min}$ was increased from 0 to 4.49 ± 0.71 nmol/min.

Priming by 1-O-Alkyl-2-acyl-glycerols—The EAG analog, 1-*O*-Δ9-octadecenyl-2-acetyl-*sn*-glycerol (EAG(18:1-2:0)) also shortened the lag time (Fig. 3) and primed the initial rate of the oxidative burst induced by fMLP (Figs. 3 and 4). However, EAG(18:1-2:0) had little or no effect on the duration of the response (Fig. 3). With EAG(18:1-2:0) priming, the velocity after 3 min was always 0 (13 separate PMNL preparations).

In contrast to the potent direct stimulatory effects of AAG(8:0-8:0), EAG(8:0-8:0) had no effect as a direct stimulant of H_2O_2 release (data not shown). However, it was able to prime the initial rate of the response to fMLP, although

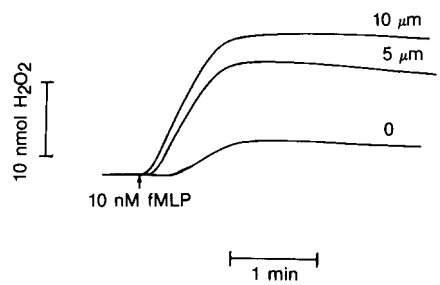

FIG. 3. **Modulation of the fMLP-induced H_2O_2 release caused by preincubation with the 1-*O*-alkyl-2-acyl diglyceride, EAG(18:1-2:0).** PMNL were preincubated with 0 (buffer alone), 5 or 10 μM EAG(18:1-2:0), as indicated adjacent to the tracings, and were stimulated with 10 nM fMLP. Similar responses were consistently observed in 13 PMNL preparations.

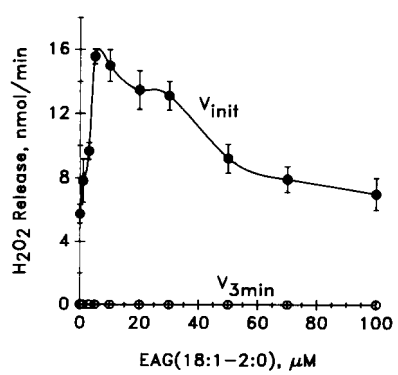

FIG. 4. **Priming of fMLP-stimulated H_2O_2 release by preincubation with varying concentrations of EAG(18:1-2:0).** Conditions were as described for Fig. 1. Data are means ± S.E. from 13 PMNL preparations.

less potently than EAG(18:1-2:0). After preincubation with buffer alone or with 20, 30, or 50 μM EAG(8:0-8:0), 10 nM fMLP elicited H_2O_2 release (V_{init}) of 4.6 ± 0.14, 6.8 ± 0.4, 7.1 ± 0.8, and 11.25 ± 1.1 nmol/min, respectively (n = 5). The kinetics of H_2O_2 release were similar to that observed with priming by EAG(18:1-2:0), except that EAG(8:0-8:0) did cause slight prolongation of H_2O_2 release, with $V_{3\,min}$ of 0.66 ± 0.44, 0.97 ± 0.48, and 1.0 ± 0.48 nmol/min after priming with 20, 30, or 50 μM diglyceride, respectively.

Two other alkylacylglycerols EAG(16:0-2:0) and EAG(18:1-3:0) had priming activity comparable to that of EAG(18:1-2:0). EAG(16:0-6:0) caused only slight priming, perhaps due to the longer acyl chains hindering permeability into the cell. The 1,3-isomer of EAG(8:0-8:0) and the dialkyl diglyceride, 1-*O*-hexadecyl-2-*O*-ethyl-*sn*-glycerol were inactive (data not shown).

Effect of Platelet Activating Factor on Stimulation by fMLP—Diglyceride:CDP-choline phosphotransferase, which is present in PMNL (45), could convert EAG(18:1-2:0) to 1-*O*-octadecenyl-2-acetyl-*sn*-glycerophosphocholine (PAF(18:1-2:0)). Since PAF has been reported to prime the respiratory burst of PMNL (9, 11), it was considered possible that priming by EAG(18:1-2:0) was mediated by conversion of the diglyceride to PAF(18:1-2:0). However, with the experimental protocol used in these studies, including cytochalasin B, PAF had little priming effect on the response of fMLP. After preincubation with 0, 1, 10, 100, and 1000 nM PAF, stimulation by 10 nM fMLP caused a H_2O_2 V_{init} of 7.4 ± 0.3, 6.2 ± 0.6, 6.1 ± 0.9, 8.3 ± 1.2, and 9.7 ± 0.8 nmol/min (n = 8), respectively, with no prolongation of the response. In separate preliminary experiments, we have found that PAF will prime to the weaker fMLP-induced response when cytochalasin B is not included in the preincubation medium (data not shown), as reported previously (9, 11). In contrast, as noted above, the diglycerides were able to prime the optimal fMLP response, generated with inclusion of cytochalasin B. The significance of this remains under investigation. However, these data would suggest that priming by PAF and by the diglycerides involve different mechanisms.

Effect of Removal of Extracellular Diglyceride Prior to Primed Stimulation—The experimental protocol used for the above priming studies employed 10-min preincubation with diglyceride before stimulation with fMLP. Preliminary experiments had shown that 4-10-min preincubation was optimal, but significant priming occurred even when diglyceride and fMLP were added simultaneously (data not shown). Since residual diglyceride might remain after the 10-min preincubation, these data could not distinguish whether priming involved 1) cellular alterations which persist with time, or 2) synergy between residual diglyceride and other fMLP-induced events (in particular, transiently increased cytosolic free Ca^{2+}) which would require interaction of diglyceride and other mediators (*e.g.* Ca^{2+}) at the time of fMLP stimulation. Two experiments were used to test these possibilities. The first involved washing the cells after priming, to remove extracellular diglyceride. The second involved reversal of the priming/stimulation sequence.

If diglyceride-mediated priming involved synergy with Ca^{2+} to induce PKC translocation to cellular membranes (46-48), then washing of PMNL to reduce diglyceride concentration should be followed by dissociation of membrane-bound PKC (49) and loss of the priming effect. If PMNL were preincubated with diglyceride for 2.5 min and then washed free of extracellular diglyceride by centrifugation, both AAG(18:1-2:0) and EAG(18:1-2:0) still caused significant priming of the

responses induced by fMLP. Whereas 10 nM fMLP elicited a V_{init} of 4.81 ± 1.0 nmol H_2O_2/min, priming by 20 μM AAG(18:1-2:0) or 20 μM EAG(18:1-2:0), and washing by centrifugation, increased the V_{init} to 15.1 ± 0.9 and 18.5 ± 0.9 nmol/min, respectively (n = 3). After AAG(18:1-2:0) priming and washing, the $V_{3\,min}$ remained elevated, although it was somewhat decreased compared with unwashed PMNL. The ratio of $V_{3\,min}/V_{init}$ of unwashed, AAG-primed PMNL was 0.40 ± 0.06 and after washing was 0.27 ± 0.06, n = 3. In two experiments, similar priming persisted even for 60 min after washing PMNL free of diglyceride, but after such prolonged preincubation the $V_{3\,min}$ was reduced to nearly zero (data not shown). These experiments could not absolutely exclude contribution by residual intracellular diglyceride but do indicate that continuous presence of the relatively high concentrations of diglyceride, added initially, are not needed for priming.

Priming of fMLP and Stimulation by AAG or EAG—If fMLP were used to prime PMNL, the fMLP-induced increase in cytosolic Ca^{2+} would decay to control levels well before exposure of the cells to diglyceride, 10 min later. This agonist sequence (fMLP priming, followed after 10 min with diglyceride stimulation) could therefore be used to test whether a transient increase in cytosolic Ca^{2+} is necessary for primed stimulation. Whereas 10 μM AAG(18:1-2:0) alone caused minimal direct stimulation (1.31 ± 0.93 nmol/min), priming by 10 nM fMLP markedly enhanced H_2O_2 release to 15.1 ± 2.05 nmol/min/10^7 PMNL, n = 6. In contrast, EAG(18:1-2:0), which had no ability to directly stimulate PMNL at any concentration tested (up to 300 μM), also caused little H_2O_2 release from PMNL even after priming with fMLP (0.46 ± 0.28 nmol/min after primed stimulation with 10 μM EAG(18:1-2:0), similar results after 20, 30, or 50 μM diglyceride, n = 3).

Interaction of 1,2-Diacyl and 1-Alkyl-2 Glycerides in Priming and in Direct Stimulation of PMNL—Since EAGs could not prime for a prolongation of the fMLP-induced response, and higher concentrations (50–100 μM) of EAG(18:1-2:0) lost ability to prime initiation, it was considered possible that EAG, especially at higher concentrations, might inhibit priming or stimulation of PMNL. PMNL were preincubated with a constant amount (*e.g.*, 10 μM) of AAG(18:1-2:0) and varying concentrations of EAG(18:1-2:0) (Fig. 5). At 5 μM EAG(18:1-2:0), there was a modest but significant ($p < 0.01$) increase in both V_{init} and $V_{3\,min}$ responses to fMLP (although the effects of AAG plus EAG were always less than additive). At higher concentrations of EAG(18:1-2:0), there was concentration-related inhibition of the AAG-primed responses. The inhibitory effect appeared to have the greatest influence on the prolongation of the response. At 70 μM EAG(18:1-2:0), the $V_{3\,min}$ was reduced to zero, whereas the initial velocity was reduced by 52%.

Direct stimulation of unprimed PMNL by higher concentrations of AAG(18:1-2:0) was inhibited by EAG(18:1-2:0), in a concentration-related manner (Fig. 6). A Dixon plot (*inset* to Fig. 6) indicated that EAG competitively inhibited AAG with an effective K_i of about 20 μM EAG(18:1-2:0).

Inhibition of Activation of the PKC of Human PMNL by EAG(18:1-2:0)—Direct stimulation of PMNL by AAG(18:1-2:0), ≥50 μM, probably involves activation of PKC, since the stimulation has been shown to be markedly reduced by an isoquinoline sulfonamide inhibitor of PKC (14, 50) and AAG stimulation correlated with translocation of PKC to the plasma membrane of PMNL (14). Since EAG inhibited AAG-mediated priming and stimulation, the stimulatory and inhibitory effects of EAG on activity of PKC were examined *in vitro*.

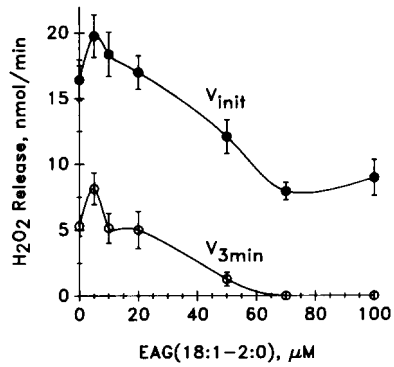

FIG. 5. **Modulation of H_2O_2 release after preincubation with both AAG(18:1-2:0) and EAG(18:1-2:0).** PMNL were preincubated with a constant amount of AAG(18:1-2:0) combined with varying concentrations of EAG(18:1-2:0) and were then stimulated with 10 nM fMLP. Data are from eight PMNL preparations employing 10 μM AAG(18:1-2:0), two preparations using 20 μM AAG(18:1-2:0), and three preparations with 30 μM AAG(18:1-2:0). Since the effect of EAG(18:1-2:0) appeared independent of AAG(18:1-2:0) concentrations within this range (see Fig. 2), the data were combined in the figure. The effects on initial velocity (V_{init}) and velocity after 3 min ($V_{3\,min}$) (as an indicator of prolongation) are shown. In these experiments, fMLP alone (without priming) induced a V_{init} of 6.4 ± 0.9 nmol/min; $V_{3\,min}$ was always 0.

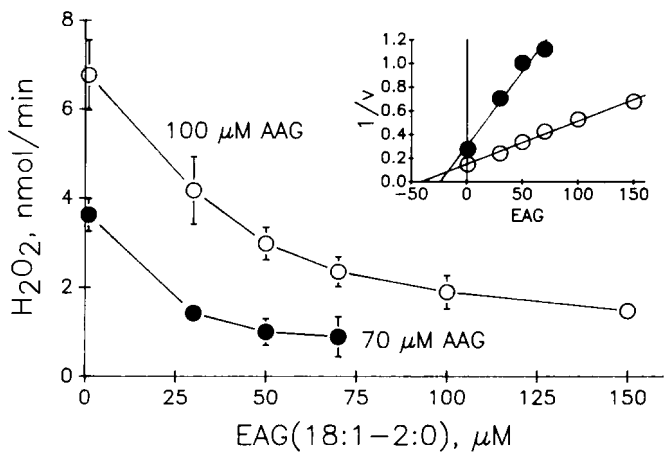

FIG. 6. **Inhibition by EAG(18:1-2:0) of the direct stimulation by AAG(18:1-2:0).** PMNL were preincubated with 5 μg/ml cytochalasin B for 2 min and were then stimulated with 70 (●) or 100 μM (○) AAG(18:1-2:0) in the presence of the indicated concentrations of EAG(18:1-2:0). Data are means ± S.E., n = 3. In the *inset*, the same data are plotted according to the method of Dixon (53) to allow determination of the effective K_i of EAG(18:1-2:0).

EAG(18:1-2:0), 0.26–78 μM (Fig. 7), and EAG(16:0-2:0) 25 μM (data not shown) had no demonstrable ability to activate PKC(s) in cytosolic fractions from PMNL. In contrast, high concentrations of EAG(8:0-8:0) did cause histone phosphorylation (Fig. 7). However, a 100-fold greater concentration of EAG(8:0-8:0) was required to cause phosphorylation equivalent to that induced by the diacyl species AAG(8:0-8:0) and AAG(18:1-2:0) (Fig. 7). The increased activity induced by AAG(16:1-2:0) and EAG(8:0-8:0) was completely lost if either Ca^{2+} or phosphatidylserine were not included in the incubation. Activation by AAG(8:0-8:0) required presence of phosphatidylserine, but partial activation (30–40% of the complete Ca^{2+}/phosphatidylserine/AAG(8:0-8:0) system) occurred in absence of added Ca^{2+} (data not shown).

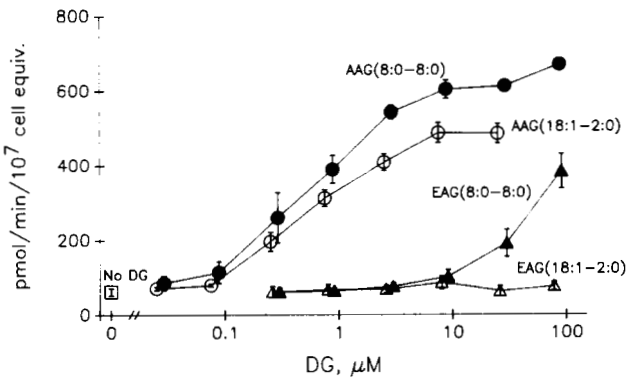

FIG. 7. **Activation of protein kinase C in cytosolic fractions of human PMNL by diglycerides.** PKC activity was assayed as described under "Experimental Procedures." Data shown are for histone phosphorylation in the presence of 0.2 mM free Ca^{2+} and 10 μg/ml phosphatidylserine without addition of diglyceride (□) or with addition of the indicated concentrations of AAG(18:1–2:0) (○), AAG(8:0–8:0) (●), EAG(18:1–2:0) (△) or EAG(8:0–8:0) (▲). Phosphorylation in absence of both Ca^{2+} and phosphatidylserine (60 pmol/min) was unchanged by diglycerides and has been subtracted from all points shown. Data are expressed as pmol of ^{32}P incorporated/min/10^7 cell equivalents and are means ± S.E., $n \geq 3$.

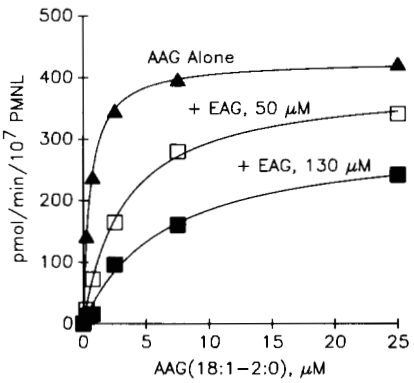

FIG. 8. **Effect of EAG(18:1–2:0) on PKC activation by AAG(18:1–2:0).** Assays were conducted in the presence of the indicated concentrations of AAG alone (▲) or with further addition of 50 μM (□) or 130 μM (■) EAG(18:1–2:0). The curves were drawn by nonlinear regression analysis, employing a rectangular hyperbolic (Michaelis-Menten) model. Histone phosphorylation in the absence of diglyceride activator was subtracted from each point. Data are means of closely agreeing triplicate determinations from a representative experiment, with similar results with three separate PMNL cytosol preparations. In all instances, the regression model fit with a high correlation coefficient ($r^2 > 0.99$).

Nonlinear regression analysis indicated that AAG(18:1–2:0) activated PKC with rectangular hyperbolic kinetics, with an effective K_m of 0.45 ± 0.04 μM AAG and a V_{max} of 412 pmol/min/10^7 PMN equivalents ($n = 3$) (Fig. 8). Addition of 50 or 130 μM EAG(18:1–2:0) reduced the activation of PKC by AAG(18:1–2:0). The predominant effect of 50 μM EAG(18:1–2:0) was to increase the apparent K_m for AAG more than 10-fold, to 6.3 ± 1.8 μM AAG(18:1–2:0), with a slight (8%) decrease in the V_{max} to 380 ± 8 pmol/min/10^7 cell equivalents ($n = 3$) (Fig. 8). Higher (130 μM) EAG(18:1–2:0) had little further effect on the K_m but did cause greater decrease in the V_{max} (247 ± 31 pmol/min/10^7 cell equivalents) ($n = 3$).

DISCUSSION

Both AAGs and EAGs are released following stimulation of human PMNL (29–31) or HL-60 myeloid leukemia cells (51). AAGs can directly stimulate the respiratory burst of PMNL (20, 50) in correlation with translocation and activation of PKC (14). However, the bioactivities of EAGs in PMNL have not been defined. In this study, EAGs had no ability to directly stimulate the respiratory burst of PMNL. However, at low concentrations, EAGs were capable of priming the initiation of H_2O_2 release when PMNL were stimulated with fMLP. At higher concentrations, EAGs lost this priming effect and actually inhibited priming induced by AAGs. Thus, EAGs may have bimodal modulatory effects on PMNL responses. At low concentrations, EAGs may facilitate initiation of functions such as the respiratory burst. At higher concentrations, EAGs may be involved in termination of the response. The distinct effects of AAGs and EAGs also revealed an important aspect of priming of the respiratory burst of PMNL. Priming occurs in at least two phases, initiation and duration. AAG and EAG(18:1–2:0) primed initiation of the burst with comparable efficiencies, yet only the diacyl species had a major effect on the duration of the response. Thus, activation of the NADPH oxidase and ongoing maintenance of oxidase activity appear to involve different transductional events.

The mechanisms of priming are largely unknown. AAGs are endogenous activators of PKC. However, several lines of data suggest that diglyceride priming, at least of initiation, may involve mechanisms other than PKC activation. We recently reported that priming by the diacylglycerol, AAG(18:1–2:0) was not influenced by the isoquinoline sulfonamide inhibitor of PKC, C-I (14), and that priming occurred at AAG concentrations which did not induce detectable translocation of PKC to PMNL membranes (14). Those data suggested that priming by AAG did not require PKC activation, but other interpretations of the data remained possible. It could be that priming requires activation of only a small portion of total cellular PKC, which might be incompletely blocked by the PKC inhibitor. The priming effect might not require PKC translocation, or (perhaps more likely) the biochemical assay of total cellular PKC might not be sufficiently sensitive to detect shifts in a small subcompartment of the kinase. The current studies significantly extend the previous data.

One proposed mechanism regarding enhancement of PKC activation by low diglyceride concentrations would involve a diglyceride-mediated increase in affinity of PKC for cytosolic Ca^{2+}, perhaps by translocation of PKC to the plasma membrane (46, 48). Transiently increased cytosolic Ca^{2+}, induced by fMLP, would then cause enhanced stimulation of the cell through PKC-mediated events. Phorbol esters cause such translocation and increased Ca^{2+} affinity of PKC (46). A similar enhanced responsiveness mediated by diglycerides would be anticipated. After decreasing the phorbol ester concentration, the effects were rapidly reversed (46, 49). Conversely, Dougherty and Niedel (48) studied HL-60 myeloid leukemia cells and reported that agonists which increase cytosolic Ca^{2+}, including fMLP, cause increased affinity of PKC for phorbol esters and result in a lower concentration of phorbol ester required for cellular stimulation. The duration of upregulation of phorbol ester binding of PKC correlated with the transient increase in Ca^{2+} induced by fMLP (3–4 min of duration) (48). This mechanism of diglyceride/Ca^{2+}/PKC interaction is perhaps better considered as synergy, since both diglyceride-mediated and Ca^{2+}-mediated events occur concomitantly. In contrast, priming implies sequential events, with the primed condition persisting beyond the immediate application of the priming agent. Thus, diglyceride priming was effective for 10–60 min after removal of extracellular

diglyceride. And fMLP-priming was effective (for PMNL stimulated with AAG) after the fMLP-induced changes in cytosolic Ca^{2+} had returned to resting levels.

EAG(18:1–2:0), which had no demonstrable ability to activate PKC, caused priming of initiation with a potency identical to that of AAG(18:1–2:0). This further supports the hypothesis that priming of initiation occurs by mechanisms other than activation of PKC.

Whether priming of a prolonged response is also PKC independent is not proven. Priming of prolongation occurred with diacylglycerols, which could activate PKC, but did not occur with alkylacylglycerols, EAG(18:1–2:0) and EAG(16:0–2:0), which do not activate PKC. Interestingly, EAG(8:0–8:0), which had a weak ability to activate PKC, also caused slight prolongation of the response to fMLP. Higher concentrations, 50–100 μM, of EAG(18:1–2:0) inhibited priming (especially of prolongation) induced by AAG(18:1–2:0), and inhibited activation of PKC *in vitro*. Thus, priming of prolongation and the ability to activate PKC occur, at least to some degree, in parallel.

The abilities of 50–100 μM EAG(18:1–2:0) to terminate priming, to inhibit direct stimulation by 70–100 μM AAG(18:1–2:0), and to inhibit activation of PKC by AAG(18:1–2:0), suggest that elevated concentrations of endogenous EAGs might act to terminate certain cellular functions, especially those involving PKC activation. EAGs are released following fMLP stimulation of PMNL, but EAG release is delayed for about 2 min following stimulation.[2] Thus, detectable increases in EAGs may occur at about the time of termination of the normal response to fMLP. It is possible that the termination is influenced by EAG-mediated inhibition of PKC.

Observations of effects of EAGs on activity of PKC have not been entirely uniform. Variable results could relate to different EAGs examined or to differences in PKC isozymes in the different tissues studied. It has been suggested that different isozymes of PKC are differentially modulated by fatty acids (52). Whether specific PKC isozymes might be selectively influenced by EAGs has not been reported. Cabot and Jaken (33) reported that EAG(16:0–2:0) did not activate PKC from mouse brain, but did not test for inhibition of AAG activity. Daniel *et al.* (35) recently found that EAG(18:1–2:0) and EAG(16:0–2:0) had no ability to activate PKC and inhibited AAG(18:1–2:0) activation of PKC(s) from HL-60 cells. In contrast, Heymans *et al.* (34) studied phosphorylation of a 43-kDa protein in platelets and reported that EAG(10:0–10:0) had about 10% of the stimulatory activity of AAG(10:0–10:0); EAG(16:0–2:0) had only slight activity, and EAG(18:0–2:0) was inactive. This suggests that the ability of EAGs to activate or inhibit PKC might relate to the fatty acid composition of the diglyceride. This hypothesis is supported by the observation that EAG(8:0–8:0), but not EAG(18:1–2:0), was able at high concentrations to activate PKC in cytosolic fractions of human PMNL. Endogenous species of alkyl-linked PCs contain primarily long chain residues (18:0, 16:0, 18:1) in the *sn*-1 position (28). Since such long chain EAGs have been found not to activate PKC and, where tested, to inhibit PKC activity, the hypothesis that inhibition of PKC might be a natural function of such EAGs is reasonable, albeit speculative at this time.

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