Integrin-dependent Homotypic Adhesion of Neutrophils

Arachidonic Acid Activates Raf-1/Mek/Erk via a 5-Lipoxygenase–dependent Pathway

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Abstract

AA stimulates integrin-dependent neutrophil adhesion, a critical early step in acute inflammation. However, neither the signaling pathway(s) of AA-stimulated adhesion, nor whether AA acts directly or through the generation of active metabolites, has been elucidated. Previously, we have observed a tight association between neutrophil Erk activation and homotypic adhesion in response to chemoattractants acting through G protein–linked receptors. We now report a similar association between homotypic adhesion and Erk activation in response to AA. Erk activation was cyclooxygenase independent and required AA metabolism to 5(*S*)-hydroperoxyeicosatetraenoic acid (5-HpETE) via 5-lipoxygenase, but not the further lipoxygenase-dependent metabolism of 5-HpETE to leukotrienes. AA stimulation of Erk was accompanied by Raf-1 activation and was sensitive to inhibitors of Raf-1 and Mek. Whereas activation of Erk by AA was pertussis toxin sensitive, [^{3}H]-AA binding to neutrophils was not saturable, suggesting that an AA metabolite activates a G protein. Consistent with this hypothesis, Erk activation by 5(*S*)-hydroxyeicosatetraenoic acid (5-HETE; lipoxygenase-independent metabolite of 5-HpETE) was also pertussis toxin sensitive. These data suggest that a 5-lipoxygenase metabolite of AA, e.g., 5-HETE, is released from AA-treated cells to engage a plasma membrane–associated, pertussis toxin–sensitive, G protein–linked receptor, leading to activation of Erk and adhesion via the Raf-1/Mek signal transduction pathway. (*J. Clin. Invest.* 1998. 102: 165–175.) Key words: neutrophils • arachidonic acid • Erk • adhesion • 5-lipoxygenase

Introduction

Neutrophils are activated not only by particles (opsonized microbes, crystals) but also by diffusible chemoattractants such as bacterial signal peptides (FMLP), complement split products (C5a), chemokines (IL-8), and eicosanoids such as leukotriene B_4 (LTB_4)[1] (1). Each chemoattractant engages a G protein–linked receptor and each (in an as yet to be determined sequence) stimulates intracellular proton and calcium fluxes, subplasmalemmal actin assembly, activation of PLA_2, C, and D, and phosphatidylinositol 3-kinase, formation of IP_3, PIP_2, and PIP_3, release of arachidonate, and de novo synthesis of phosphatidate and diacylglycerol, followed by activation of protein kinase C (PKC) (2). These intracellular events mediate the several functional responses of neutrophils in inflammation: (*a*) integrin (CD11b/CD18)-mediated neutrophil aggregation (homotypic cell adhesion); (*b*) integrin- and selectin-dependent sticking of neutrophils to vascular endothelium (heterotypic cell adhesion); (*c*) directed migration to the source of the signal (chemoattraction); (*d*) phagocytosis with or without release of granule contents (degranulation); and (*e*) the generation of $O_2^{\cdot-}$ and other toxic oxygen metabolites (the respiratory burst). Specific neutrophil functions appear to be regulated, at least in part, via distinct mechanisms, as pharmacologic manipulators can inhibit one or more neutrophil responses without affecting others (3).

The mitogen-activated protein kinases (MAPKs) $p44^{Erk1}$ and $p42^{Erk2}$ are serine/threonine protein kinases that, in mitotic cells, play roles in cell growth and differentiation (4). Exposure of cells in culture to protein tyrosine kinase receptor (PTKR) agonists such as epidermal and nerve growth factors results in Erk activation via a pathway dependent upon the low molecular weight GTP-binding protein Ras, and the sequential activation of the kinases Raf-1 and Mek (MAPK or Erk kinase, also known as MAPK kinase [MAPKK]) (5–9). Circulating human neutrophils are postmitotic and terminally differentiated. Nonetheless, we and others have observed that FMLP and other chemoattractants activate Erk in neutrophils, with kinetics concordant with the rapid responses of neutrophils to these stimuli (10–12). Data from our laboratory and those of others suggest that neutrophil Erk activation by chemoattractants is mediated, at least in part, by pathways similar to those initiated by PTKRs, i.e., via activation of Ras, Raf-1, and Mek (10, 13–16). In addition, data from our laboratory and others support an association between chemoattractant stimulation of Erk and neutrophil adhesive function (10), but not $O_2^{\cdot-}$ generation (17). Studies of Erk in neutrophils thus appear to have revealed a novel, specific, nonmitotic signaling function for these enzymes.

Endogenous AA released from membrane phospholipids by PLA_2 (both secretory and cytosolic) or other phospholi-

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1. *Abbreviations used in this paper:* Bt_2cAMP and Bt_2cGMP, dibutyryl cAMP and GMP, respectively; COX, cyclooxygenase; Cp, control peptide; 5-HETE, 5(*S*)-hydroxyeicosatetraenoic acid; 5-HpETE, 5(*S*)-hydroperoxyeicosatetraenoic acid; 5-LO, 5-lipoxygenase; LT, leukotriene; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MBPp, myelin basic protein peptide; MEK, MAPK or Erk kinase; PK, protein kinase; PM, plasma membrane; PTKR, protein tyrosine kinase receptor; PTX, pertussis toxin.

pases is subject to conversion to prostaglandins by cyclooxygenases (COXs), or to sequential conversion to 5(*S*)-hydroperoxyeicosatetraenoic acid (5-HpETE) and LTA_4 after membrane assembly of 5-lipoxygenase (5-LO). The relative availabilities of 5-HpETE versus AA are important in determining the LTA_4/5-HpETE ratio (18). In addition, 5-HpETE may be converted to 5(*S*)-hydroxyeicosatetraenoic acid (5-HETE) by a 5-LO–independent pathway (19). In neutrophils, LTA_4 is metabolized further by a dual-function hydrolase to LTB_4 (20). Exogenous AA added to neutrophils mimics engagement by G protein–dependent chemoattractants: the cells aggregate, degranulate, and generate O_2^- (21, 22). Moreover, some effects of exogenous AA are inhibited by pertussis toxin (PTX) (21). Therefore, it is unclear whether AA acts directly via a G protein–linked cell surface receptor, or indirectly by serving as substrate for the generation of an intracellular product by the COXs or 5-LO (20). Indeed, AA has other effects on neutrophils that may be important for signal transduction, including activating PKC (23), enhancing the binding of GTP to neutrophil membrane preparations (suggesting a stimulatory effect on neutrophil heterotrimeric G proteins) (21), altering the physical dynamics of neutrophil membranes (24), and activating the NADPH oxidase in mixtures of neutrophil subcellular fractions (25, 26). In addition to these actions on neutrophils, AA and its metabolites have been shown recently to activate Erk in rat liver (27) and vascular smooth muscle (28) cells.

In this study we have characterized the ability of AA to stimulate Erk in neutrophils. In addition, we have explored the hypothesis that AA-stimulated neutrophil adhesion is Erk dependent. Our data indicate that, unlike all other fatty acids tested, AA stimulates neutrophil Erk in a dose- and time-dependent manner, consistent with a role in rapid neutrophil responses. AA stimulation of neutrophil Erk was COX independent and dependent on 5-LO but not LT. In contrast to the AA-stimulated Erk activity of cells in culture (27, 28), neutrophil Erk activation by AA was independent of PKC. AA stimulation of Erk was PTX and cAMP sensitive, Mek dependent, and associated with the activation of Raf-1. These data suggest that AA activates neutrophil Erk via a novel pathway dependent upon both 5-LO and the classic Erk-activating cascade (Ras, Raf-1, and Mek). These two distinct signaling sequences appear to be coupled by an AA-derived 5-LO metabolite (e.g., 5-HETE) which transmits a signal to Ras or Raf-1 via its release to the extracellular milieu and engagement of a G protein–coupled receptor. Finally, our data also support and extend the hypothesis that Erk activation in human neutrophils plays a critical role in the adhesive responses of these cells.

Methods

Materials. Except where otherwise noted, reagents were purchased from Sigma Chemical Co. Accuprep™ was from Accurate Scientific. Dextran T500 was from Pharmacia LKB Biotechnology. Myelin basic protein (MBP) was from Life Technologies. Myelin basic protein peptide (MBPp; APRTPGGRR) and hexahistidine-tagged Mek1/glutathione-*S*-transferase (6His-Mek1-GST) fusion protein were from Upstate Biotechnology. Control peptide (Cp; APRVPGGRR) was synthesized by Chiron Mimotopes. Antisera specific for $p44^{Erk1}$ (C-16), $p42^{Erk2}$ (C-14), and Raf-1 (C-12) were from Santa Cruz Biotechnology. 5-HpETE and 5-HETE were from Cayman Chemical. Calphostin C, rottlerin, Gö6976, and a myristoylated PKC pseudosubstrate/inhibitor peptide (myr-ψPKC) were from Calbiochem. ATP and a mAb against CD11b were from Boehringer Mannheim. [γ-^{32}P]ATP was from Amersham. [^{3}H]-AA was purchased from Moravek Biochemicals. Phosphocellulose circles and Gf/C glass microfiber filters were from Whatman. PTX was from List Biological Laboratory. Zileuton (SC-54324) and SC-53228 were the kind gift of Dr. Donald Fretland (Searle). PD098059 was the kind gift of Dr. Alan Saltiel (Parke-Davis).

Neutrophil isolation. Neutrophils were isolated as described previously (29). Microscopic examination confirmed that preparations contained > 90% neutrophils. Isolated neutrophils were suspended in cell buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM KOH, 5 mM $CaCl_2$, and 1.2 mM $MgCl_2$) and used within 30 min of preparation.

Reagent preparation. The sodium salt of AA was dissolved in cell buffer (see above), stored in aliquots frozen under nitrogen gas, and used within 10 min after thawing. Fatty acids other than AA and SC-53228 were dissolved in DMSO and stored and used as described for AA. PGE_1 was dissolved in methanol. 5-HpETE and 5-HETE, provided in 100% ethanol, were dried under nitrogen stream, redissolved in cell buffer, and used within 10 min of solvation as recommended by the manufacturer. Solvent (DMSO, methanol, or ethanol) control experiments confirmed no independent effect on Erk activity at concentrations equivalent to those present in the actual experiments (data not shown). PTX potency was confirmed before use by its ability to inhibit FMLP-stimulated O_2^- generation, previously shown to be PTX sensitive (21, 30).

Erk (MBPp kinase) activity assay. Neutrophils (5×10^6), preincubated in the absence or presence of inhibitor, were stimulated with eicosanoid, AA, FMLP, or PMA as described in Results. Reactions were stopped by the addition of ice-cold lysis buffer (final concentrations, 20 mM Tris, pH 7.4, 1 mM EGTA, 2 mM sodium vanadate, 25 mM sodium fluoride, 0.5% [vol/vol] Triton X-100, 2 mM PMSF, 5 KU/ml aprotinin, and 10 μg/ml each of chymostatin, antipain, and pepstatin). Lysates were incubated on ice for 15 min and centrifuged at 4°C for 10 min at 14,000 *g*. Supernatants were incubated for 15 min at 37°C in phosphorylation buffer (40 mM Tris, pH 7.4, 20 mM $MgCl_2$, 200 μM NaEGTA, 2 mM sodium fluoride, 800 μM sodium vanadate, 3 mM DTT, 350 μM ATP, and 25 μCi/ml [γ-^{32}P]ATP) containing 500 μM MBPp or Cp. Reactions were stopped by the addition of formic acid (final concentration 6%). The reaction mixtures were spotted onto phosphocellulose circles which were washed thoroughly in distilled water and counted. Duplicate assays in the absence of MBPp were performed to determine non-Erk background kinase activities. Erk activity was calculated as the difference between samples containing MBPp and samples containing no peptide.

To confirm that the activity measured as MBPp phosphorylation was due to Erk, lysates of unstimulated, FMLP (100 nM)-stimulated, and AA (20 μM)-stimulated neutrophils were immunodepleted overnight with anti-Erk antisera ($p44^{erk1}$ and $p42^{erk2}$, in equimolar concentrations) or a control antiserum in varying concentrations (expressed as the ratio of antisera added in picograms versus neutrophil equivalents) in the presence of protein A–Sepharose. Supernatants were measured for Erk activity as described above and expressed as Erk activity in supernatants immunodepleted with anti-Erk antibodies relative to Erk activity in the control supernatants.

Gel renaturation Erk (MBP kinase) activity assay. Neutrophils (5×10^6) were stimulated with FMLP or AA as described in Results and lysed as above. Cell lysates were analyzed using a gel renaturation method as described previously (31). Erk activity was measured by phosphorimaging.

Neutrophil homotypic aggregation. Neutrophil homotypic aggregation was measured on-line as the increase in light transmittance through a stirred suspension of neutrophils (1.2×10^7 cells/ml) in a platelet aggregometer (Peyton Industries), and quantitated as the area under the aggregation curve for the first 2 min after stimulation (32).

[^{3}H]-AA binding to neutrophils. Neutrophils (4×10^5 cells/ml) were incubated with [^{3}H]-AA (210 Ci/mmol) in the absence (total

counts) or presence (nonspecific counts) of 100-fold excess of unlabeled AA for 1 h at 0°C. Incubations were stopped by diluting the samples 40-fold with ice-cold wash buffer (150 mM NaCl, 0.01% BSA, and 20 mM Tris, pH 7.4). The cells were isolated on GF/C glass microfiber filters which were washed extensively under vacuum aspiration and counted. Specific binding was calculated as the difference between total and nonspecific counts. In other experiments, binding studies were performed on neutrophil plasma membranes (PMs; isolated as previously described [33]) in lieu of intact cells.

Raf-1 kinase assay. Raf-1 kinase activity was determined by a modification of the method of Knall et al. (34). Lysates of unstimulated or AA (20 μM)-stimulated neutrophils (prepared as described above) were incubated with anti–Raf-1 antiserum or a control antiserum for 1 h at 4°C. Antigen–antibody complexes were isolated by incubation with protein A–Sepharose overnight at 4°C. Protein A–Sepharose was washed three times with lysis buffer containing 0.5% Triton X-100 and once with 20 mM Tris, pH 7.4, then incubated in the presence of 6His-Mek1-GST (800 ng) and phosphorylation buffer (see Erk activity assay). The resultant phosphoproteins were analyzed by 10% PAGE and Mek-1 phosphorylation was quantitated by phosphorimaging (Molecular Dynamics).

Results

AA activates Erk in human neutrophils. Erk activity was measured as the ability of lysates of stimulated or unstimulated neutrophils to phosphorylate MBPp, a nonapeptide containing the specific target phosphorylation sequence for Erk (PR**T**P) previously identified in MBP (35). Incubation of human neutrophils with AA (20 μM for 2 min at 37°C) induced a 1,127±144% increase in MBPp phosphorylation relative to unstimulated controls (Fig. 1 *A*). This degree of Erk activation was similar to, but somewhat less than, that stimulated by FMLP (1,697±319% control). Substitution of MBPp with Cp, a control peptide in which valine was substituted for threonine (PR**V**P), resulted in little or no increase of measured counts above background levels (i.e., in the absence of peptide) (Fig. 1 *A*). Immunodepletion of lysates using antisera specific for the Erks $p44^{Erk1}$ and $p42^{Erk2}$ resulted in complete reduction of AA- and FMLP-stimulated MBPp phosphorylation, confirming that the measured kinase activity was due exclusively to Erk (Fig. 1 *B*). We have reported previously the use of a gel renaturation assay in which neutrophil lysates are analyzed on SDS-PAGE gels impregnated with MBP (10). In situ renaturation of proteins so analyzed, followed by incubation of the gel in the presence of [γ-^{32}P]ATP and phosphorimaging, permits visualization of MBP phosphorylation coincident with the presence of MBP kinases. Analysis of lysates of AA-stimulated neutrophils by this assay confirmed the presence of AA-dependent 44- and 42-kD MBP kinases, consistent with the molecular weights of Erk 1 and 2 (Fig. 1 *B*, *inset*), and suggested that the predominant Erk isoform activated by AA was Erk 2. This impression was confirmed by immunoprecipitating Erk 1, Erk2, or both from lysates of AA-stimulated neutrophils and analyzing for MBPp kinase activity: Erk 2 and Erk 1/2, but not Erk 1, immunoprecipitates were observed to contain kinase activity (Table I). Erk activation by AA was rapid (peak at 2 min), concordant with the kinetics of AA-stimulated neutrophil homotypic aggregation (Fig. 1 *C*). Maximal

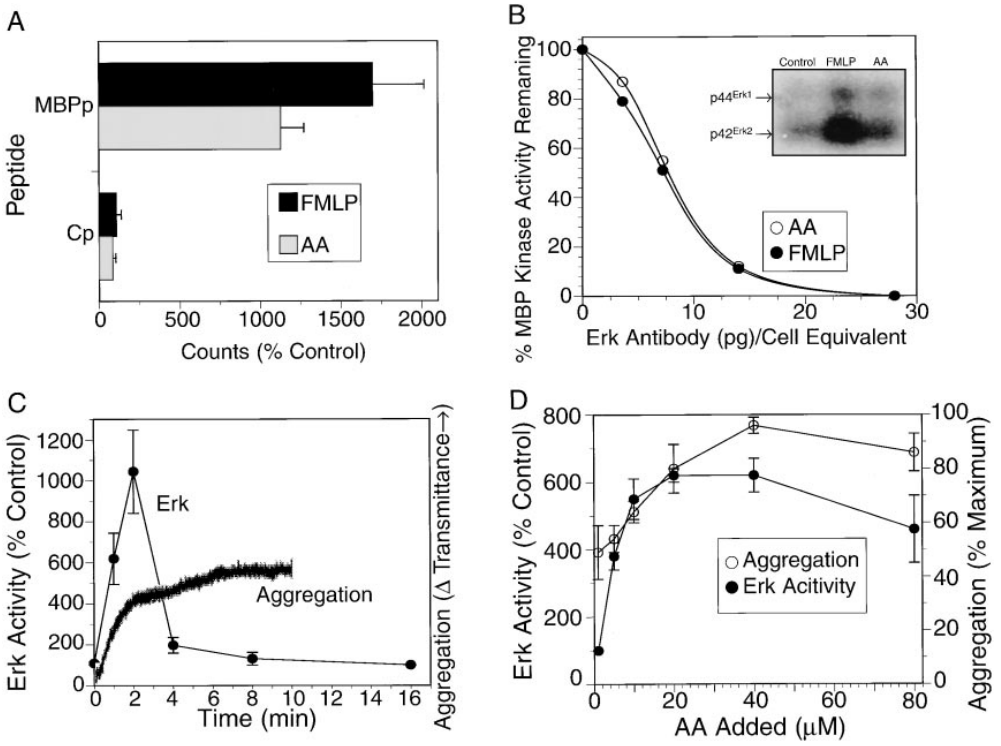

Figure 1. AA stimulates neutrophil Erk activity in a time- and dose-dependent manner. (*A*) Neutrophils were stimulated with FMLP (100 nM) or AA (20 μM) for 2 min at 37°C, lysed, incubated in the absence or presence of the Erk peptide substrate (MBPp) or Cp, and analyzed for peptide-directed kinase activity as described in Methods. (*B*) Lysates of neutrophils stimulated with FMLP (100 nM) (*closed circles*) or AA (20 μM) (*open circles*) were incubated overnight with increasing proportions of antisera specific for $p44^{Erk1}$ and $p42^{Erk2}$, cleared of antigen–antibody complexes using protein A–Sepharose, and analyzed for the ability to phosphorylate MBPp. (*Inset*) Neutrophils were incubated in the absence (*Control*) or presence of FMLP (100 nM) or AA (20 μM) for 2 min at 37°C, lysed, and analyzed for Erk activity using a gel renaturation assay (see text). (*C*) The thin line depicts neutrophils stimulated with AA (40 μM) at 37°C, lysed at the indicated times, and analyzed for Erk activity. The thick line depicts neutrophils stimulated as above and assayed on-line for homotypic aggregation. (*D*) Neutrophils were stimulated with the indicated concentrations of AA for 2 min at 37°C, lysed, and analyzed for Erk activity (*closed circles*) or aggregation (*open circles*). Results shown are the mean±SEM or representative of three experiments, except *B* (mean of two experiments).

Table I. Erk2 but Not Erk1 Activation in AA-stimulated Human Neutrophils

	Neutrophil Erk activity (counts)					
	Experiment 1		Experiment 2		Mean of two experiments	
Antisera	Unstimulated	AA-stimulated	Unstimulated	AA-stimulated	Unstimulated	AA-stimulated
Erk1 + Erk2	199	3297	68	1965	134	2631
Erk1 only	86	2	87	59	87	31
Erk2 only	41	3971	56	2264	49	3118
Control (rabbit anti–mouse)	44	42	103	100	74	71

Erk1, Erk2, or both were immunoprecipitated from lysates of unstimulated or AA-stimulated neutrophils and analyzed for MBP kinase activity as described in Methods. Data shown are the individual results and averages for two separate experiments.

activation of Erk by AA was observed at concentrations between 20 and 40 μM (EC_{50} = 5 μM), again concordant with its effects on homotypic aggregation (Fig. 1 *D*). Since the critical micellar concentration for AA is ~ 40 μM (36, 37), all subsequent experiments were performed using 20 μM AA unless otherwise indicated.

Like AA, both linoleic (18:2 *cis*) and oleic (18:1 *cis*) acid can stimulate neutrophil O_2^- generation in intact cells (21). In contrast, these agents failed to activate neutrophil Erk at concentrations of 20 or 80 μM (Fig. 2 *A*), and had little or no effect on aggregation (Fig. 2 *B*). Other fatty acids tested (arachidic [20:0], linolelaidic [18:2 *trans*], and myristic [14:0]) also failed to stimulate Erk or aggregation. The anionic detergent SDS shares with AA the ability to stimulate NADPH oxidase in a cell-free system (25, 26). SDS, at concentrations of 20 and 80 μM, had little or no effect on Erk activity or aggregation of intact neutrophils (Fig. 2, *A* and *B*). Consistent with a previous observation (17), these data suggest a lack of association between neutrophil Erk activity and O_2^- generation. These results also demonstrate that Erk activation by AA is specific and not due to a general lipid or detergent effect.

AA-stimulated Erk activation is dependent upon 5-LO, but

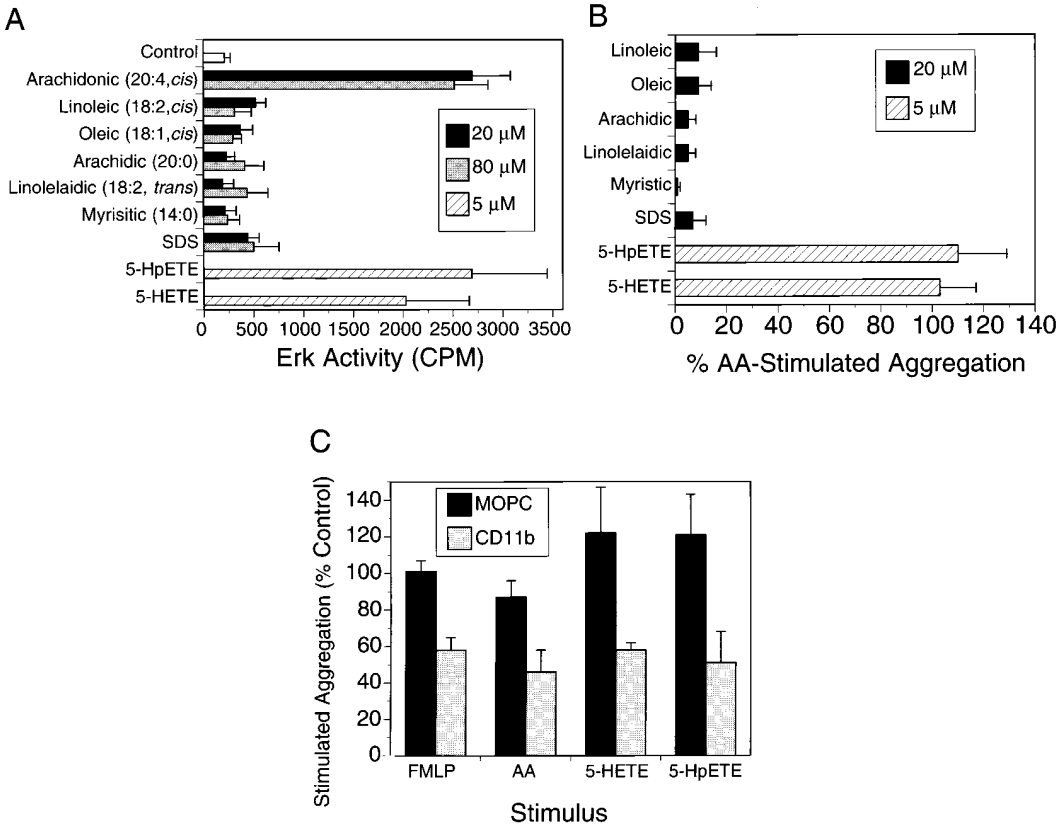

Figure 2. 5-HpETE and 5-HETE but not SDS or fatty acids other than AA stimulate Erk activity and homotypic aggregation. (*A*) Neutrophils were incubated in the absence or presence of the indicated concentrations of fatty acids, SDS, or 5-HpETE or 5-HETE for 2 min at 37°C, followed by lysis and determination of Erk activity. (*B*) Neutrophils were incubated in the absence or presence of the indicated concentrations of fatty acids, SDS, or 5-HpETE or 5-HETE and assayed for homotypic aggregation relative to AA. (*C*) Neutrophils were preincubated in the absence or presence of anti-CD11b or an isotype control mAb (*MOPC*), stimulated with the indicated agent(s) and assayed for aggregation relative to cells stimulated in the absence of antibody. Results shown are the mean±SEM of three or more experiments for each condition.

independent of COX activity. When Erk 1 and/or Erk 2 were immunoprecipitated from lysates of unstimulated neutrophils and incubated with AA, no increase over baseline kinase activity was observed (not shown). These data suggested that either (*a*) AA must undergo metabolism to some active form upon exposure to intact neutrophils; (*b*) activation of Erk by AA requires intact signal transduction pathways not present in disrupted cells; or (*c*) both of the above. Free AA may be metabolized by COX to form prostaglandins, or by 5-LO to form 5-HpETE and LTs. To determine whether the generation of AA metabolites is required for Erk activation or neutrophil homotypic aggregation, we tested the effect of COX and 5-LO inhibitors on AA-stimulated Erk activity and aggregation (Fig. 3, *A* and *B*). Consistent with the observation that neutrophils contain little COX activity (38), concentrations of the nonsteroidal antiinflammatory drug indomethacin sufficient to inhibit COX ($IC_{50} \leq 2.8$ μM) in intact neutrophils (39) as well as COX1 in an in vitro system (40) had little or no effect on Erk activity or homotypic aggregation in response to AA. Higher concentrations of indomethacin (25 μM), which have nonspecific effects including $cPLA_2$ inhibition (41), markedly inhibited both Erk activation and aggregation in response to AA (64±13 and 47±15% inhibition, respectively). In contrast, the specific 5-LO inhibitor zileuton blocked both AA-stimulated Erk activity and homotypic aggregation with an IC_{50} (1 mM) comparable to that required for inhibition of LT generation in rat leukocytes (42). Zileuton had no effect on FMLP-stimulated Erk activity (data not shown). Two other inhibitors of 5-LO, nordihydroguaiaretic acid (43) and the potent zileuton derivative A-79175 (44), also inhibited AA-stimulated Erk activity (data not shown). 5-HpETE, the product of the action of 5-LO on AA, independently stimulated Erk in neutrophils (Fig. 2 *A*). These data indicate that AA stimulation of neutrophil Erk depends upon its metabolism by 5-LO to 5-HpETE or a metabolite of 5-HpETE.

In addition to its effect on AA, 5-LO mediates the conversion of 5-HpETE to LTA_4, a rate-limiting step in the generation of LTs such as the neutrophil chemoattractant LTB_4 (20). We and others have shown that LTB_4 activates Erk in neutrophils (10, 34). However, AA activation of Erk did not depend upon LTB_4 generation, since the specific LTB_4-receptor antagonist SC-53228 (45) inhibited Erk activation by LTB_4 but not by AA (Fig. 3 *C*). To determine whether any LT might be responsible for AA-stimulated Erk activation, we tested the effect of zileuton on 5-HpETE–stimulated Erk activity. In contrast to its effects on Erk activity stimulated by AA, zileuton had no effect on that stimulated by 5-HpETE (Fig. 3 *D*). Like AA, 5-HpETE also stimulated neutrophil aggregation (Fig. 2 *B*) and zileuton had no effect on 5-HpETE–stimulated aggregation (Fig. 3 *D*). Thus, neither AA- nor 5-HpETE stimulation of Erk or aggregation can depend upon the generation of LTs. In contrast to the LTs, 5-HETE is derived from 5-HpETE by a 5-LO–independent reaction (19). Like 5-HpETE, 5-HETE stimulated both Erk activity and aggregation (Fig. 2, *A* and *B*), and both Erk activity and aggregation were minimally inhibited by zileuton (Fig. 3 *D*). Consistent with a role for Erk in adhesion, both 5-HpETE and 5-HETE stimulated neutrophil homotypic aggregation at concentrations similar to those that activated Erk. Moreover, neutrophil aggregation in response to AA, 5-HpETE, and 5-HETE shared with FMLP a final common pathway dependent upon the β_2 integrin CD11b/CD18, as mAbs to CD11b blocked aggregation by all four stimuli (Fig. 2 *C*). These data suggest that 5-LO–derived AA metabolites other than LTs (e.g., 5-HpETE and 5-HETE) are the

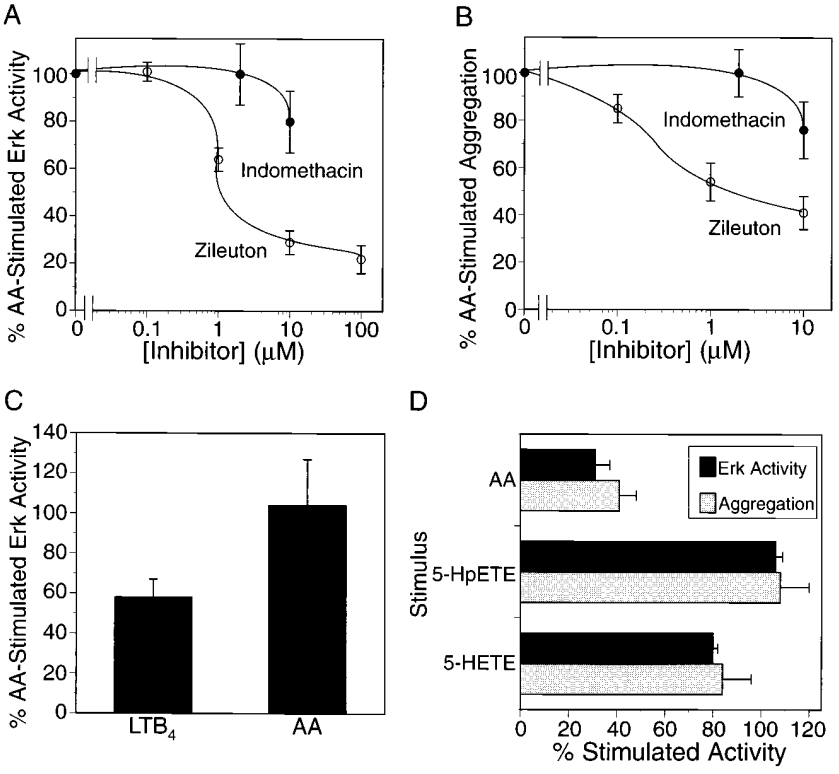

Figure 3. 5-LO, but not COX, mediates AA stimulation of both Erk activity and homotypic aggregation. (*A* and *B*) Neutrophils were incubated for 10 min at 37°C with increasing concentrations of indomethacin or zileuton (SC-54324), stimulated for 2 min with AA (20 μM), and assayed for Erk activity (*A*) or homotypic aggregation (*B*). (*C*) Neutrophils were preincubated for 2 min with the LTB_4 receptor antagonist SC-53228 (100 nM), stimulated for 2 min with LTB_4 (300 nM) or AA (20 μM), and assayed for Erk activity. (*D*) Neutrophils were incubated for 10 min in the absence or presence of zileuton, stimulated for 2 min with AA (20 μM), 5-HpETE (5 μM), or 5-HETE (5 μM), and assayed for Erk activity or homotypic aggregation. Results are expressed as the percentage of stimulated Erk activity or aggregation in the absence of inhibitors and are the mean±SEM of three experiments for each condition.

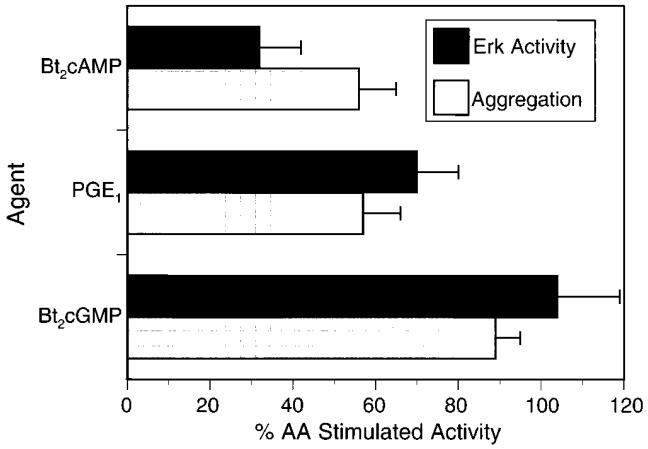

Figure 4. Agents that raise intracellular cAMP levels inhibit AA-stimulated Erk activity and aggregation. Neutrophils were incubated for 10 min at 37°C in the absence or presence of Bt_2cAMP (1 mM), PGE_1 (1 mM), or Bt_2cGMP (1 mM), stimulated for 2 min with AA (20 μM), and assayed for Erk activity or homotypic aggregation. Results are expressed as the percentage of AA-stimulated Erk activity or aggregation in the absence of agent and are given as the mean±SEM of three experiments.

mediators of AA-stimulated Erk activity and homotypic aggregation.

Inhibition of Erk activation by agents elevating intracellular cAMP levels. Erk activity in response to growth factors and some G protein–linked receptor agonists depends upon the sequential activation of Ras and the kinases Raf-1 and MEK (5–9, 46). Previous studies (47–49) have demonstrated that cAMP inhibits PTKR-stimulated Erk activity via PKA-dependent phosphorylation of Raf-1, resulting in diminished Ras/Raf-1 interactions. To examine whether AA stimulation of Erk is similarly Ras, Raf-1, and MEK dependent, we tested the effect of cAMP on AA-stimulated Erk activity. Preincubation of neutrophils with the cell permeant cAMP analogue dibutyryl cAMP (Bt_2cAMP, 1 mM) resulted in a 68±10% inhibition of AA-stimulated Erk activity and a 43±9% inhibition of homotypic aggregation (Fig. 4). Fig. 4 also shows that PGE_1 (1 mM), which stimulates increased intracellular cAMP levels in human neutrophils and inhibits FMLP-stimulated Erk activation in enucleate neutrophil cytoplasts (31), inhibited AA-stimulated Erk activity (30±10% inhibition) and aggregation (44±9% inhibition). In contrast, the cell-permeable cyclic GMP analogue Bt_2cGMP (1 mM) did not inhibit either AA-stimulated Erk activity or homotypic aggregation (Fig. 4). These data suggest that activation of Ras, Raf-1, and (presumably) the Raf substrate MEK may play a role in AA-stimulated Erk activation.

Role of MEK and Raf-1 in AA-stimulated Erk activation. To further examine the possibility that AA stimulation of neutrophil Erk is mediated via Raf-1 and MEK, we determined whether PD098059, a specific inhibitor that binds to Mek and prevents its phosphorylation and activation by Raf-1 or other kinases (50), has an effect on Erk activation. Preincubation of neutrophils with PD098059 (100 μM) resulted in 68±9% inhibition of AA-stimulated Erk activity. PD098059 also inhibited AA-stimulated homotypic aggregation by 44±8% at 100 μM (Fig. 5).

The ability of both PD098059 and Bt_2cAMP to inhibit AA-stimulated Erk activation suggested a central role for Raf-1 in the AA signaling pathway. To directly test whether AA stimulates Raf-1 in neutrophils we immunoprecipitated Raf-1 from lysates of resting and AA-stimulated neutrophils and assayed for phosphorylation of purified recombinant MEK-1 (Fig. 6). AA activated Raf-1 kinase from human neutrophils (191± 29% control), and this observation was confirmed in an alternate Raf-1 activity assay using the peptide Syntide 2 (51, 52) as a substrate for Raf-1 (not shown). Consistent with the effects of cyclic nucleotides on Erk activation, both 1 mM Bt_2cAMP and 1 mM PGE_1 inhibited AA-stimulated Raf-1 activation. In contrast, 1 mM Bt_2cGMP had no effect on AA-stimulated Raf activation, although the effects of this agent were less consistent than those of Bt_2cAMP.

AA-stimulated Erk activity of neutrophils is PKC independent. AA stimulates neutrophil PKC activity (23), and active PKC has been reported to stimulate Erk, Ras, and Raf-1 (53–55) in a number of cell types. Moreover, exposure of vascular smooth muscle cells (28) or rat liver cells (27) to AA results in Erk activation in a PKC-dependent fashion. These observations led us to test the hypothesis that AA-dependent generation of 5-HpETE, and AA activation of Raf-1 and MEK in neutrophils, may be coupled via PKC activation. Consistent with previous reports (17), the direct PKC activator PMA stimulated neutrophil Erk activity (1,250±186% control). Preincubation of neutrophils with the specific PKC inhibitor calphostin C (specific for Ca^{2+}-dependent PKC isoforms α, β_1, β_2, and γ [56, 57]) inhibited PMA-stimulated Erk activity by 44±13% and PMA-stimulated aggregation by 33±3% (Table II). Several other PKC inhibitors with specificities broader or narrower than calphostin C were also tested. Rottlerin (inhibits isoforms α, β, γ, as well as Ca^{2+}-independent isoforms δ, ϵ, ν, ζ [58]) and Gö6976 (α and β_1 but not Ca-independent isoforms [59]), but not the myristoylated pseudosubstrate peptide myr-ψPKC (reported to inhibit α and β [60]), inhibited both Erk activation and aggregation of neutrophils in response to

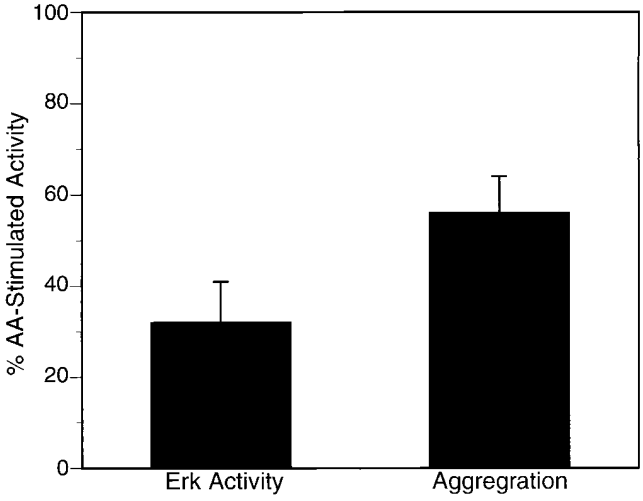

Figure 5. AA stimulation of neutrophils is Mek dependent. Neutrophils were incubated for 10 min at 37°C in the absence or presence of the specific inhibitor of Mek activation PD098059 (100 μM), stimulated for 2 min with AA (20 μM), lysed, and analyzed for Erk activity. Results shown are the mean±SEM of three experiments.

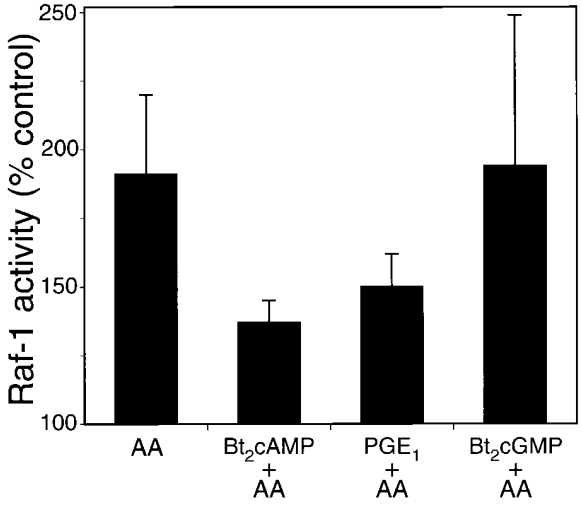

Figure 6. AA stimulates Raf-1 activity in a cAMP-sensitive fashion. Neutrophils were preincubated for 15 min at 37°C in the absence or presence of 1 mM Bt_2cAMP, Bt_2cGMP, or PGE_1, incubated in the absence or presence of AA (20 μM) for 2 min at 37°C, lysed and immunoprecipitated with Raf-1 antiserum, and measured for Raf-1 kinase activity as described in Methods. Control experiments performed in the absence of substrate or using a nonspecific control antiserum resulted in levels of Raf-1 activity below those of unstimulated lysate. Results shown are the mean±SEM of three or more experiments for each condition.

PMA. However, neither calphostin C nor any of the other PKC inhibitors tested inhibited AA stimulation of either Erk activity or homotypic aggregation (Table II). These data suggest that AA stimulation of both Erk and neutrophil adhesion is PKC independent.

Erk activation by AA is PTX sensitive. Previous studies from our laboratory have suggested that at least some effects of AA on neutrophils may depend upon the activation of a heterotrimeric G protein (21). Therefore, we examined whether AA-stimulated Erk activity was G protein dependent by testing the effects of PTX on Erk activity (Fig. 7 *A*). Consistent with a previous report (61), preincubation of neutrophils with PTX inhibited Erk activation by FMLP (30±8% inhibition). Surprisingly, pretreatment with PTX inhibited AA-stimulated Erk activity to an even greater extent (55±2% inhibition). These results are compatible with a model whereby AA directly engages a G protein–linked receptor. However, we were unable to demonstrate specific, saturable binding of [^{3}H]-AA to intact neutrophils (Fig. 7 *B*), arguing against an AA receptor. Because of the possibility that [^{3}H]-AA binding might be affected by AA uptake and metabolism, these studies were performed at 0°C. Moreover, studies of AA binding to preparations of isolated neutrophil PMs at 0°C yielded similar results (not shown).

Table II. Effects of PKC Inhibitors on AA- and PMA-stimulated Erk Activation and Homotypic Aggregation of Human Neutrophils

	Neutrophil assay*			
	Erk activity		Aggregation	
PKC inhibitor	% AA-stimulated	% PMA-stimulated	% AA-stimulated	% PMA-stimulated
None	100	100	100	100
Calphostin C	108±8	66±8	108±10	59±4
Rottlerin	97±4	65±5	102±10	85±4
Gö6976	90±7	68±2	93±6	78±10
myr-ΨPKC	116±17	116±5	Not done‡	Not done‡

*Neutrophil Erk activity and homotypic aggregation are reported as the percentage of maximal activity observed in response to AA or PMA in the absence of PKC inhibitors, and expressed as the mean±SEM of three or more experiments for each condition. ‡The effect(s) of myr-ΨPKC on aggregation could not be measured as addition of the peptide to suspensions of neutrophils resulted in spontaneous neutrophil aggregation in the absence of stimuli.

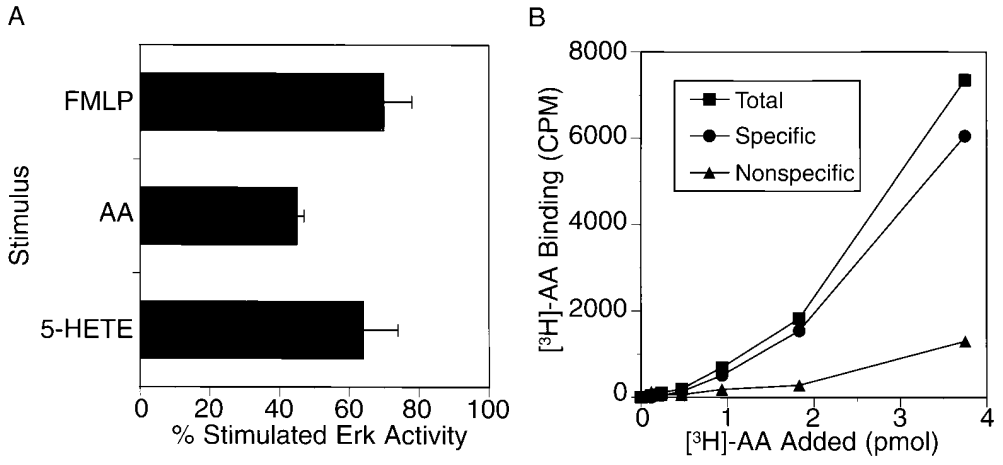

Figure 7. PTX inhibits AA-, FMLP-, and 5-HETE–stimulated Erk activity. (*A*) Neutrophils were incubated for 1 h at 37°C with PTX (10 μg/ml), stimulated for 2 min with FMLP (100 nM), AA (20 μM), or 5-HETE (5 μM), lysed, and analyzed for Erk activity. Results are expressed as percent inhibition of stimulated Erk activity in the absence of PTX. (*B*) [^{3}H]-AA binding to neutrophils was measured in the presence (nonspecific binding) or absence (total binding) of 100-fold excess unlabeled AA. Specific binding was calculated and expressed as described in the text. Results shown are the mean±SEM of three experiments (*A*) or representative of five experiments (*B*).

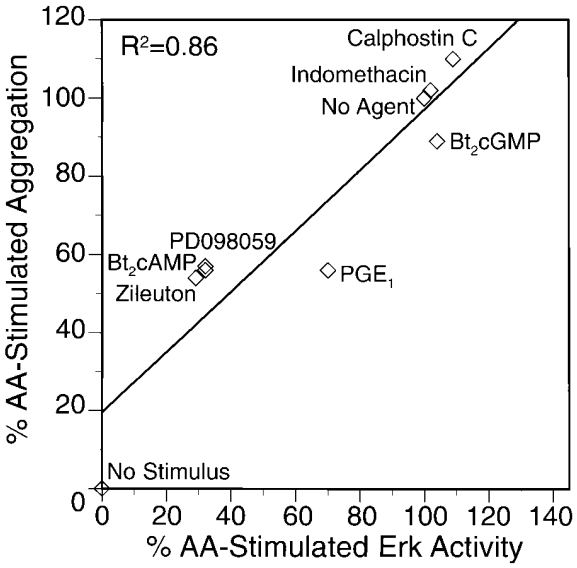

Figure 8. Agents that inhibit AA-stimulated Erk activity also inhibit homotypic aggregation. Regression analysis comparing the effect of putative inhibitors (indomethacin, 2 μM; zileuton, 10 μM; Bt_2cAMP, PGE_1, and Bt_2cGMP, all at 1 mM; calphostin C, 100 μM; PD098059, 100 μM) of AA-stimulated Erk activity versus their effects on AA-stimulated homotypic aggregation.

PTX sensitivity, combined with nonsaturable binding of AA, suggested that a metabolite of AA (e.g., 5-HETE), and not AA itself, may interact with a G protein–coupled receptor. Consistent with this hypothesis, Erk activation by 5-HETE was PTX sensitive (36±10% inhibition) (Fig. 7 *A*).

AA stimulation of Erk activity correlates with AA-stimulated neutrophil homotypic aggregation. Previously, we have reported an association between chemoattractant-stimulated Erk activity and neutrophil adhesive function (10). To examine this relationship further we compared the effects of agents on AA-stimulated Erk to their effects on AA-stimulated aggregation. As shown in Fig. 8, the effects of these agents on AA-stimulated aggregation correlated well (R^2 = 0.86) with their effects on AA stimulation of Erk. It was not possible to determine the effects of PTX on eicosanoid-stimulated neutrophil aggregation, as PTX possesses intrinsic CD11b/CD18-directed hemagglutinin activity (62, 63) that resulted in rapid agglutination of unstimulated as well as stimulated neutrophils (not shown). Indeed, incubation of paraformaldehyde-fixed neutrophils with PTX also resulted in rapid agglutination of cells, confirming that PTX-stimulated aggregation did not require intracellular signaling.

Discussion

In these studies we examined the mechanisms of AA signaling in human neutrophils, observing a possible role for Erk in AA-stimulated neutrophil responses. AA stimulation of Erk was dose and time dependent. The effects of AA on Erk were unlikely to be due to a nonspecific lipid effect, since other *cis*-polyunsaturated fatty acids, as well as *trans*-polyunsaturated and saturated fatty acids, all failed to stimulate Erk activity. Whereas the fatty acids tested were selected because they differed from AA in defined physicochemical properties, we did not study the effects of other, 20–carbon chain unsaturated fatty acids such as 20:5ω3 and 20:3; studies with these and other lipids will be required to more definitively establish that AA is specifically serving to yield a 5-LO product. The effects of AA were also unlikely due to a detergent effect, since SDS, which stimulates neutrophil O_2^- generation, had no effect on Erk activity, and maximal Erk activation was observed at concentrations of AA below its critical micellar concentration. Thus, it appears that AA stimulates Erk by acting in a specific manner, either intracellularly or at the PM.

The ability of AA to stimulate neutrophil PKC (23), together with recent reports that AA activates Erk in mitotic cells via PKC (27, 28), led us to test whether AA activation of neutrophil Erk was similarly PKC dependent. However, calphostin C and other PKC inhibitors blocked PMA- but not AA-stimulated Erk activation, suggesting that neutrophil Erk activation by AA is PKC independent. Indeed, the kinetics of Erk activation by AA in neutrophils and mitotic cells are also distinct, peaking at 2 and 5–10 min, respectively (27, 28).

The capacity of AA to serve as a substrate for eicosanoid generation suggested other possible mechanisms for Erk activation. The failure of indomethacin to inhibit Erk activation at concentrations sufficient for inhibition of COX in neutrophils indicated that prostaglandin generation is not required for AA stimulation of Erk. Future evaluation of the effects of other COX inhibitors, particularly COX-2 inhibitors since indomethacin is largely COX-1 specific (40), will be useful in confirming COX independence of Erk activation by AA. However, the apparent COX independence of Erk activation was not unexpected, given that resting neutrophils exhibit little COX activity and that expression of COX-2 is observed only after several hours of cytokine stimulation (64). In contrast, we observed a requirement for 5-LO activity in AA stimulation of Erk. 5-LO catalyzes two reactions in AA metabolism: AA conversion to 5-HpETE, and 5-HpETE conversion to LTA_4. Only the first of these steps appears to be required for Erk activation, since the 5-LO inhibitor zileuton blocked Erk activation by AA but not 5-HpETE. Thus, AA activation of Erk does not depend upon the generation of LTs. In contrast, 5-HpETE can be metabolized to 5-HETE by a 5-LO–independent pathway. As both 5-HpETE and 5-HETE stimulated neutrophil Erk, it is likely that each of these eicosanoids (and/or their metabolites) participates in AA-stimulated Erk activation.

Previous data from our laboratory have suggested the involvement of a G protein(s) in AA-stimulated neutrophil O_2^- generation (21). The ability of PTX to inhibit AA stimulation of Erk suggests a similar G protein dependence in the activation of this enzyme. One model to explain this observation invokes the direct binding of AA to a specific G protein–linked receptor. Although studies of lipid binding to membrane receptors are made difficult by nonspecific ligand integration into PM (65), several groups have successfully demonstrated specific lipid ligand–neutrophil receptor interactions, including those between LTB_4 (66) and platelet-activating factor (67) and their respective receptors. In contrast, we were unable to find evidence for AA–receptor interactions on intact neutrophils or isolated PM. These studies should be interpreted with caution, however. Although our binding studies were performed at 0°C and recapitulated in isolated neutrophil membranes, we cannot rule out the possibility that AA metabolism

may have obscured our capacity to observe saturation binding. It is also unlikely that AA directly activated a G protein via PM perturbation, since fatty acids with sizes and configurations similar to AA had no effect on Erk. Therefore, our data support the hypothesis that a product of AA metabolism may bind to, and activate, a G protein–coupled receptor. A possible candidate is 5-HETE. In addition to its role as an AA metabolite, and its ability to activate Erk, 5-HETE has been shown to bind specifically, and to stimulate PTX-sensitive GTPγS binding, to neutrophil membranes, as well as to stereospecifically stimulate a variety of neutrophil functions (68). Our data indicate that 5-HETE activation of Erk is indeed PTX sensitive, consistent with a recent demonstration by O'Flaherty et al. that preincubation of neutrophils with PTX blocks a 5-HETE–induced mobility shift of a 40-kD protein recognized by immunoblotting with an anti-Erk antibody (69, 70).

Erk stimulation in response to PTKRs and some G protein–linked receptors proceeds via Ras, Raf-1, and MEK. Our data suggest that AA stimulation of Erk also involves this "classical" pathway, since (*a*) AA stimulated Raf-1 activity; (*b*) Bt_2cAMP, which blocks Raf-1 activation by Ras in mitotic cells (46–48), inhibited AA stimulation of both Raf-1 and Erk; and (*c*) the specific Mek inhibitor PD098059 also inhibited Erk activation by AA, although this inhibition was incomplete ($< 70\%$).

Based on these observations we propose a model of Erk activation by AA (Fig. 9). We suggest that AA serves as a substrate for 5-LO, leading to the generation of 5-HpETE. 5-HpETE is metabolized, independently of 5-LO, to 5-HETE which diffuses to the extracellular face of the PM to engage a G protein–linked receptor. G protein activity then initiates Erk activation via Ras, Raf-1, and Mek, as previously observed in other systems. To our knowledge, this is the first example in which the generation of eicosanoid via lipoxygenase is coupled to a specific intracellular kinase cascade via extracellular, autacoid engagement of a G protein–coupled receptor. Although this model represents a best-fit for the available data, it fails to provide a mechanism whereby G protein activation leads to Ras and/or Raf-1 activation, a subject of ongoing investigation in a number of signal transduction laboratories.

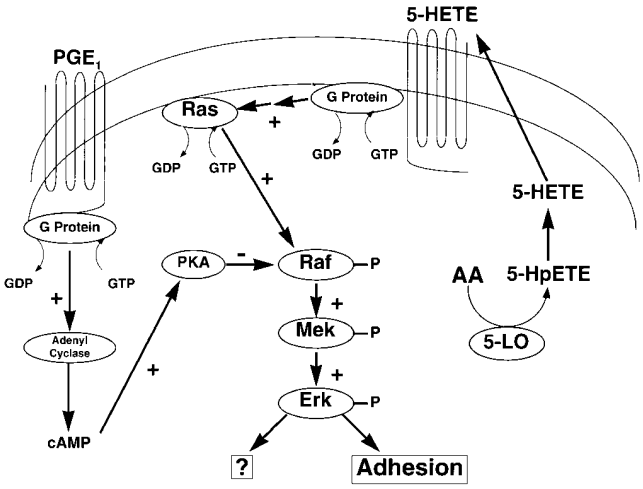

Figure 9. A model of Erk activation by AA in neutrophils. See text for explanation. Broken arrow indicates an undefined pathway.

Our data further suggest that Erk activation is required for AA stimulation of neutrophil adhesion. Previously, we have observed a correlation between Erk activation and neutrophil aggregation in response to chemoattractants (10), and now report a similar correlation between aggregation and Erk activity in response to AA. AA stimulated Erk activity and aggregation with similar dose–responses and kinetics. The 5-LO metabolites 5-HpETE and 5-HETE were also each capable of stimulating both Erk activity and aggregation. Finally, we observed a tight correlation between the ability of a variety of agents, including Bt_2cAMP and PD098059, to inhibit Erk activity and aggregation. The degree of Erk inhibition by both Bt_2cAMP and PD098059 was greater than the degree of inhibition of aggregation by these agents, suggesting that other, Erk-independent pathways may also be involved in adhesion signaling. In contrast, a similar association between Erk and O_2^- has not been observed: (*a*) unlike Erk activation, the ability of AA to stimulate O_2^- is shared by a number of lipids as well as SDS (21, 25, 26); (*b*) the kinetics of O_2^- generation are distinct from those of Erk and aggregation (17); (*c*) in contrast to Erk activation and aggregation, 5-HETE does not independently stimulate O_2^- generation (63); (*d*) Yu et al. have reported a dissociation between neutrophil Erk activation and O_2^- generation (17); and (*e*) Mocsai et al. have reported that PD098059 does not inhibit O_2^- generation (71). Thus, the role of Erk in rapid physiologic responses of terminally differentiated neutrophils may be limited to specific functions including adhesion. The recent observation that L-selectin ligation on neutrophils (such as would be observed during adhesion) stimulates Erk (72) suggests a possible mechanism for further amplification of neutrophil adhesive responses.

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