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Pharmacology

Modes of action of aspirin-like drugs: Salicylates inhibit Erk activation and integrin-dependent neutrophil adhesion

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ABSTRACT **The anti-inflammatory effects of high-dose salicylates are well recognized, incompletely understood and unlikely due entirely to cyclooxygenase (COX) inhibition. We have previously reported a role for activation of the kinase Erk in CD11b/CD18 integrin-dependent adhesiveness of human neutrophils, a critical step in inflammation. We now report the effects of salicylates on neutrophil Erk and adhesion. Exposure of neutrophils to aspirin or sodium salicylate (poor COX inhibitor) inhibited Erk activity and adhesiveness of *formyl*methionyl-leucyl-phenylalanine- and arachidonic acid-stimulated neutrophils, consistent with anti-inflammation but not COX inhibition (IC_{50}s = 1–8 mM). In contrast, indomethacin blocked neither Erk nor adhesion. Inhibition of Mek (proximal activator of Erk) also blocked stimulation of Erk and adhesion by *formyl*methionyl-leucyl-phenylalanine- and arachidonic acid. Salicylate inhibition of Erk was independent of protein kinase A activation and generation of extracellular adenosine. These data are consistent with a role for Erk in stimulated neutrophil adhesion, and suggest that anti-inflammatory effects of salicylates may be mediated via inhibition of Erk signaling required for integrin-mediated responses.**

Acetyl salicylate (aspirin; ASA) has been used for a century to reduce redness, swelling, heat, and pain; recently it has been shown to prevent intravascular thrombosis, slow Alzheimer disease and prevent colon cancer (1–3). Aspirin effects vary with dose, of which three levels are generally appreciated (4). Low doses (80 mg/day; aspirin I) irreversibly acetylate serine 530 of cyclooxygenase (COX) I to inhibit platelet thromboxane A_2 generation and are antithrombotic. Intermediate doses (2–4 g/day; aspirin II) globally inhibit COX I and II, block prostaglandin production, and are analgesic and antipyretic. Finally, serum concentrations in the millimolar range (6–8 g/day; aspirin III) are as effective as cortisone in rheumatic disorders (4), but the mechanism(s) of this action is undetermined. Added to human neutrophils *in vitro*, high concentrations of aspirin (aspirin III) or its active metabolite salicylate [as sodium salicylate (NaS)] inhibit homotypic cell adhesion (5) and O_2^- generation (6), but NaS is only 1:100 as potent as aspirin at inhibiting COX (7). Millimolar concentrations of salicylates also inhibit heterotypic adhesion of stimulated neutrophils to endothelial cell monolayers, a critical early step in neutrophil-mediated injury (8). High-dose salicylates also (*i*) interfere with arachidonic acid (AA)-stimulated binding of GTP to preparations of neutrophil membranes (9), (*ii*) decrease plasma membrane viscosity (10), (*iii*) enhance intracellular cAMP levels and protein kinase A (PKA) activity in neutrophils treated with *formyl*methionyl-leucyl-phenylalanine (FMLP) (11), and (*iv*) uncouple oxidative phosphorylation, depleting ATP and increasing extracellular adenosine (8). Whether these *in vitro* effects of salicylates on neutrophils account for their anti-inflammatory action remains to be determined.

The mitogen-activated protein kinases p44Erk1 and p42Erk2 are serine/threonine kinases that, in mitotic cells, play roles in cell growth and differentiation (12). Exposure of such cells to agonists such as epidermal growth factor results in Erk activation dependent on the low molecular weight GTP-binding protein Ras, and the kinases Raf-1 and Mek (13–17). Circulating human neutrophils are postmitotic and terminally differentiated. Nonetheless, chemoattractants such as FMLP stimulate Erk activation in neutrophils in a manner likely to be mediated, at least in part, by pathways similar to those initiated by protein tyrosine kinase receptors, i.e., via Ras, Raf-1, and Mek (18–23).

We have recently reported that AA is also capable of stimulating Erk activation in neutrophils via a G protein-, Raf-1-, and Mek-dependent pathway. Such activation appears to require generation of a lipoxygenase product capable of engaging a G protein-coupled receptor (24). Moreover, we have observed a tight association between Erk activation and adhesion in neutrophils stimulated with chemoattractants or AA (20, 24, 25). Our studies of Erk in neutrophils thus reveal a proinflammatory signaling function for these enzymes. Schwenger *et al.* (26)have recently reported that NaS inhibits tumor necrosis factor-α stimulation of Erk in FS-4 fibroblasts. Although tumor necrosis factor-α itself does not stimulate Erk activity in neutrophils in suspension (27), Schwenger's observations suggest a possible mechanism for the antiadhesive effects of salicylates on neutrophils exposed to agents that do stimulate Erk activity.

We now report that aspirin and NaS inhibit neutrophil Erk activation by FMLP and AA at IC_{50}s that inhibit CD11b/CD18-dependent homotypic aggregation of neutrophils (i.e., aspirin III). The salicylate effect was upstream of Erk, and unlikely due to COX inhibition. Our data are consistent with a role for Erk in neutrophil adhesion, and suggest a novel mechanism for the anti-inflammatory effects of aspirin and other salicylates.

MATERIALS AND METHODS

Materials. Except where otherwise noted, reagents were purchased from Sigma. Accuprep was from Accurate Scientific. Dextran T500 was from Pharmacia LKB. Myelin basic protein peptide (MBPp) was from Upstate Biotechnology

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Abbreviations: ASA, acetyl salicylic acid; COX, cyclooxygenase; NaS, sodium salicylate; AA, arachidonic acid; PKA, protein kinase A; FMLP, *formyl*methionyl-leucyl-phenylalanine; PMA, phorbol myristate acetate.

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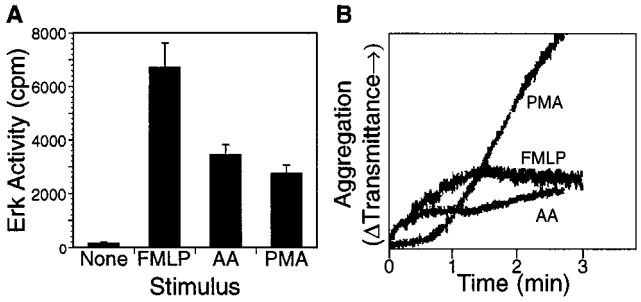

FIG. 1. Stimulation of neutrophil Erk activity and aggregation. (*A*) Neutrophils (5×10^6/condition) were stimulated for 2 min with FMLP (100 nM) or AA (20 μM) or for 10 min with PMA (1 μg/ml), and analyzed for Erk activity as described in *Materials and Methods*. Erk activity of unstimulated neutrophils at 2 and 10 min were comparable and were averaged together. (*B*) Neutrophils (1.2×10^7ml) were stimulated and analyzed online for homotypic aggregation for 2 min as described. Data shown are the mean ± SEM of ≥20 experiments (*A*), or representative of 5 experiments (*B*).

(Lake Placid, NY). ATP and anti-CD11b antiserum were from Boehringer Mannheim. [γ-^{32}P]ATP was from Amersham. Phosphocellulose circles were from Whatman. Anti-Erk1, Erk2, and Erk1/2 antisera were from Santa Cruz Biotechnology. Anti-PhosphoErk antiserum (anti-ACTIVE MAPK pAB) was from Promega.

Neutrophil Isolation. Neutrophils were isolated as described (20), suspended in cell buffer (10 mM Hepes, pH 7.4/150 mM NaCl/5 mM KOH/5 mM $CaCl_2$/1.2 mM $MgCl_2$) and used within 30 min. Microscopic examination confirmed that preparations contained >90% neutrophils.

Reagents. The sodium salt of AA was dissolved in cell buffer (stored frozen under nitrogen gas) and used within 10 min after thawing. Aspirin and NaS were dissolved in 2.5 N NaOH and cell buffer, respectively; pH of each solution was 7.4.

Erk Activity. Erk activity was measured as the ability of neutrophil lysates to phosphorylate MBPp, a synthetic peptide substrate containing the specific sequence (PRTP) of myelin basic protein that is phosphorylated by Erk, as described (20). Activation-specific phosphorylation of Erk was determined by immunoblotting neutrophil lysates with antiserum specific for phosphorylated, active state Erk1/2 and [^{125}I]protein A, and by PhosphorImaging (Molecular Dynamics). The immunoblots were then stripped (18)) and reblotted using anti-Erk1/2 antiserum indifferent to phosphorylation state (K23, Santa Cruz Biotechnology).

Neutrophil Adhesion Assays. Neutrophil homotypic aggregation was measured as increased light transmission through stirred suspensions of neutrophils (1.2×10^7 cells/ml at 37°C) (5). Neutrophil heterotypic adhesion was determined as the number of neutrophils/high-powered field (magnification, ×100; average of 5 fields counted) adherent to monolayers of a human umbilical vein endothelial cell line (ECV 304), as described (28).

Neutrophil Viability. Neutrophil viability was measured as release of lactate dehydrogenase as described by Metcalf *et al.* (29).

RESULTS

Salicylates Inhibit Erk Activation by G Protein-Linked Ligand/Receptor Pairs. FMLP, AA, and phorbol myristate acetate (PMA) each stimulated neutrophil Erk activity (Fig. 1*A*). Incubation of neutrophils with millimolar concentrations of aspirin or NaS inhibited Erk activation in response to FMLP or AA (Fig. 2*A* and *B*, and Table 1). Micromolar aspirin concentrations sufficient for COX inhibition (i.e., aspirin I and II) had no effect on FMLP or AA stimulation of Erk. Salicylates and acetaminophen had no effect on neutrophil viability as determined by lactate dehydrogenase release (7.7 ± 6% lactate dehydrogenase release for control cells, 2.3 ± 1% and 4.7% for cells treated with 25 mM ASA and 25 mM NaS, respectively, and 0.2 ± 0.2% for cells treated with 10 mM acetaminophen). Indomethacin doses sufficient for COX inhibition (1–10 μM) (30) did not inhibit FMLP or AA-stimulated Erk activity (Fig. 3*A*). Acetaminophen [analgesic and antipyretic but not anti-inflammatory *in vivo* (4)] had no effect on FMLP- or AA-stimulated Erk activity at concentrations approaching the clinically toxic dose of 3 mM (31) (Fig. 2 *A* and *B*).

PMA bypasses membrane signaling to activate protein kinase C. Preincubation with millimolar aspirin or NaS did not inhibit PMA-stimulated Erk activation in neutrophils, excluding the possibility that salicylates exert direct effects on Erk, or on assays of Erk activation. Acetaminophen also had no effect on PMA-stimulated Erk activation (Fig. 2*C*).

We tested the capacity of NaS or aspirin to inhibit Erk phosphorylation by immunoblotting with antiserum specific for phosphorylated, activated Erk. Neutrophil stimulation with 100 nM FMLP, 20 μM AA (2 min each), or 1 μg/ml of PMA (10 min) at 37°C each caused dramatic increases in phosphorylation of Erk (Fig. 4 *A–C*). Incubation with 10 mM aspirin or NaS for 10 min before stimulation inhibited Erk phosphory-

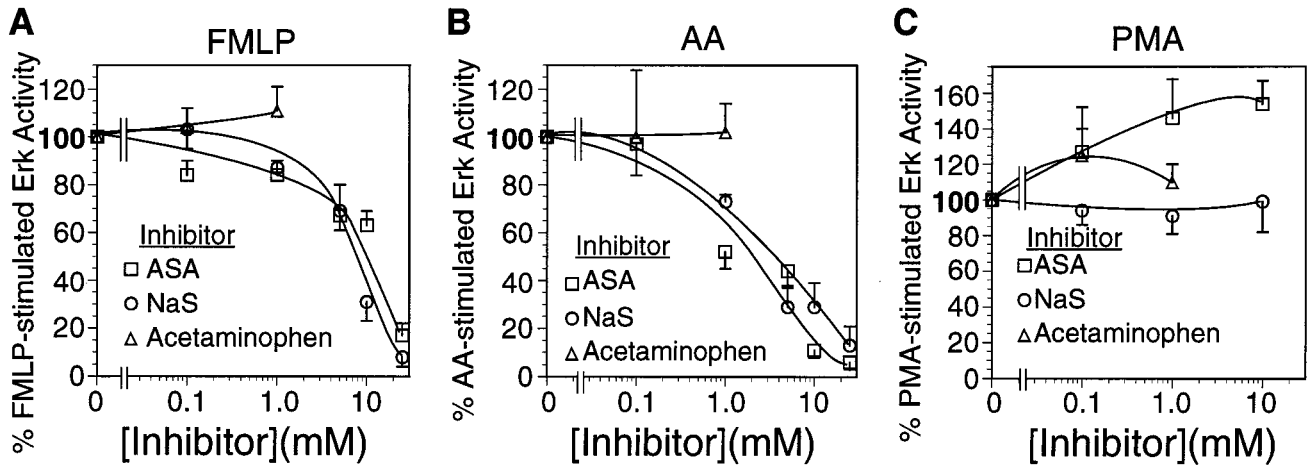

FIG. 2. Effects of salicylates and acetaminophen on neutrophil Erk activity. Neutrophils were incubated for 10 min at 37°C in the absence or presence of aspirin (ASA; □), NaS (○), or acetaminophen (△), followed by stimulation for 2 min with 100 nM FMLP (*A*) or 20 μM AA (*B*), or for 10 min with 1 μg/ml PMA (*C*), and determination of Erk activity. Results are expressed as percent of stimulated Erk activity in the absence of inhibitors, and are the mean ± SEM of three experiments.

Table 1. IC_{50}s for inhibition of stimulated neutrophil Erk activity and aggregation by salicylates

	IC_{50}, mM			
	ASA		NaS	
Stimulus	Erk Activity	Aggregation	Erk activity	Aggregation
FMLP (100 nM)	8	3	7	2
AA (20 μM)	2	2	3	1
PMA (1 μg/ml)	N.I.	10	N.I.	N.I.

Neutrophils were incubated for 10 min at 37°C with 0–10 mM ASA or NaS, stimulated with the indicated agents and analyzed for Erk activity or aggregation.

N.I., No significant inhibition at the maximum concentration tested.

lation in response to FMLP or AA but not PMA. None of the agents tested affected the absolute amount of Erk present in the lysates (Fig. 4*D-F*).

Concordance Between Erk Activation and Neutrophil Adhesion. As previously reported (20, 24), FMLP and AA each stimulated neutrophil homotypic aggregation (Fig. 1*B*); the rank order of aggregation corresponded to that of Erk activation by these agents (FML*P* > AA). Both aggregation and Erk activity provoked by FMLP and AA peaked within 1–2 min. PMA also stimulated both Erk activity and aggregation. Whereas neutrophil aggregation stimulated by PMA was greater than that of FMLP or AA, the effect of PMA on Erk activation was lower than FMLP and comparable to AA. Moreover, PMA stimulation of aggregation began as early as 1 min after stimulation, but 10 min of PMA stimulation were required to observe Erk activation. These data suggested concordance between Erk activity and aggregation in response to FMLP and AA but not PMA.

Aggregation in response to FMLP, AA, and PMA depends on the integrin CD11b/CD18 at the neutrophil surface (5, 32, 33), and preincubation of neutrophils with an anti-CD11b antibody blocked aggregation (data not shown). High but not low concentrations of aspirin and NaS inhibited neutrophil aggregation stimulated by both FMLP and AA (Fig. 5 *A* and *B*), with IC_{50}s corresponding to those for inhibition of Erk activation (Table 1). Neither indomethacin at COX-inhibitory concentrations (Fig. 3*B*) nor acetaminophen (Fig. 5 *A* and *B*) blocked aggregation stimulated by FMLP or AA. Consistent with reports by Cronstein *et al.* (8), high- but not low-dose salicylates also inhibited FMLP-stimulated neutrophil adhesion to monolayers of a human umbilical vein endothelial cell line (ECV309) (Table 2). These data are consistent with a tight linkage between Erk activation and neutrophil adhesiveness and suggest that salicylates inhibit FMLP- and AA-stimulated neutrophil adhesion by inhibiting Erk in a COX-independent

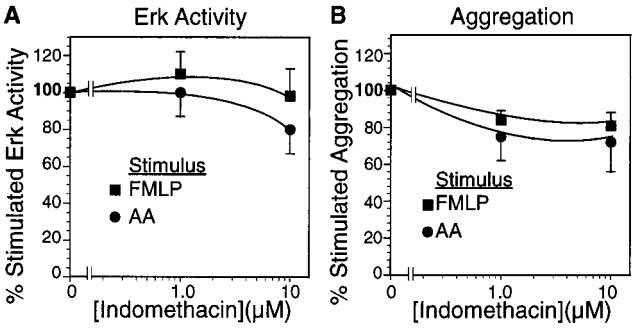

FIG. 3. Failure of indomethacin to inhibit FMLP- or AA-stimulated neutrophil Erk activity or aggregation. Neutrophils were incubated at 37°C for 10 min in the absence or presence of indomethacin, stimulated for 2 min with 100 nM FMLP (■) or 20 μM AA (●), and analyzed for Erk activity (*A*) or aggregation (*B*). Data shown are the mean ± SEM of three experiments.

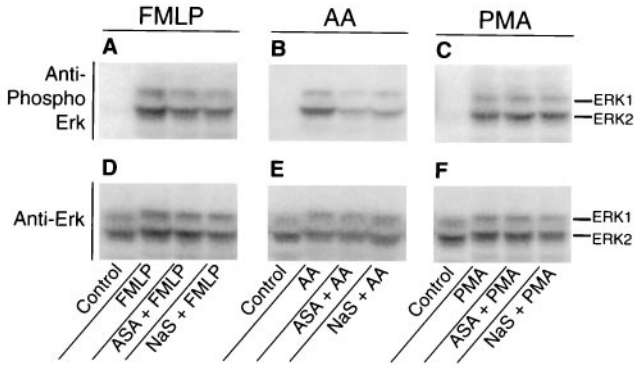

FIG. 4. Effects of aspirin and NaS on stimulated Erk phosphorylation. (*A–C*) Neutrophils were incubated in the absence or presence of 10 mM ASA or NaS, stimulated for 2 min with 100 nM FMLP (*A*) or 20 μM AA (*B*), or for 10 min with 1 μg/ml PMA (*C*), lysed, and analyzed for phosphoactivation state by SDS/PAGE and immunoblotting with an antibody specific for the phosphorylated, activated state of Erk 1 and 2. (*D–F*) The immunoblots in *A–C* were stripped and reblotted with an anti-Erk1/2 antibody indifferent to phosphorylation state. Data are representative of three experiments.

fashion. In contrast to its failure to inhibit PMA-stimulation of Erk, the highest concentrations (10 mM) of aspirin tested inhibited PMA-stimulated aggregation by 59 ± 8%, suggesting that PMA activates adhesion by an Erk-independent but salicylate-sensitive pathway.

Pharmacologic Dissection of Salicylate Action. To compare the effects of salicylates on Erk activation and neutrophil aggregation with those of a better-defined inhibitor of Erk signaling, we tested PD098059, a specific inhibitor of Mek (the proximal kinase activating Erk) (34). We have reported (24) that PD098059 inhibited both Erk activation and neutrophil aggregation stimulated by AA. We now report that PD098059 also inhibits both Erk activation and aggregation in response to FMLP (Fig. 6). PD098059 also inhibited FMLP-stimulated neutrophil adhesion to ECV309 cell monolayers (Table 2). In contrast, PD098059 inhibited Erk activation but not neutrophil aggregation stimulated by PMA.

Abramson *et al.* have reported that exposure of neutrophils to salicylates and chemoattractants increases intracellular cAMP levels (11). This observation, together with the ability of the cell-permeable cAMP analog dibutyryl cAMP to inhibit Erk activation in FMLP-stimulated neutrophil cytoplasts (20) and AA-stimulated neutrophils (24) in a PKA-dependent fashion, suggested that salicylate inhibition of Erk might be mediated through cAMP generation and PKA activation. However, the specific PKA inhibitor KT5720 (1 μM)(35) had no effect on aspirin inhibition of FMLP- or AA-stimulated neutrophil Erk activity (Fig. 7*A*) or aggregation (Fig. 7*B*). We also considered whether salicylate inhibition of Erk and aggregation might depend on the ability of these agents to cause accumulation of extracellular adenosine, which has potent anti-inflammatory effects *in vitro* and *in vivo* (36–38). Exogenous adenosine deaminase (3 units/ml) failed to reverse the effects of aspirin on Erk activity (Fig. 8*A*) or aggregation (Fig. 8*B*), in contrast to its reported ability to reverse *in vivo* inflammation by agents such as methotrexate (39).

DISCUSSION

The therapeutic effects of salicylates have been appreciated for at least three hundred years. However, it was not until 1971 that Vane found aspirin to inhibit an enzyme activity (40) soon to be identified as cyclooxygenase (41). It is now appreciated that aspirin is not equivalent in its actions on the constitutive COX1 and inducible COX2 (42), and evidence is accumulating that direct inhibition of COX is only one component of aspirin

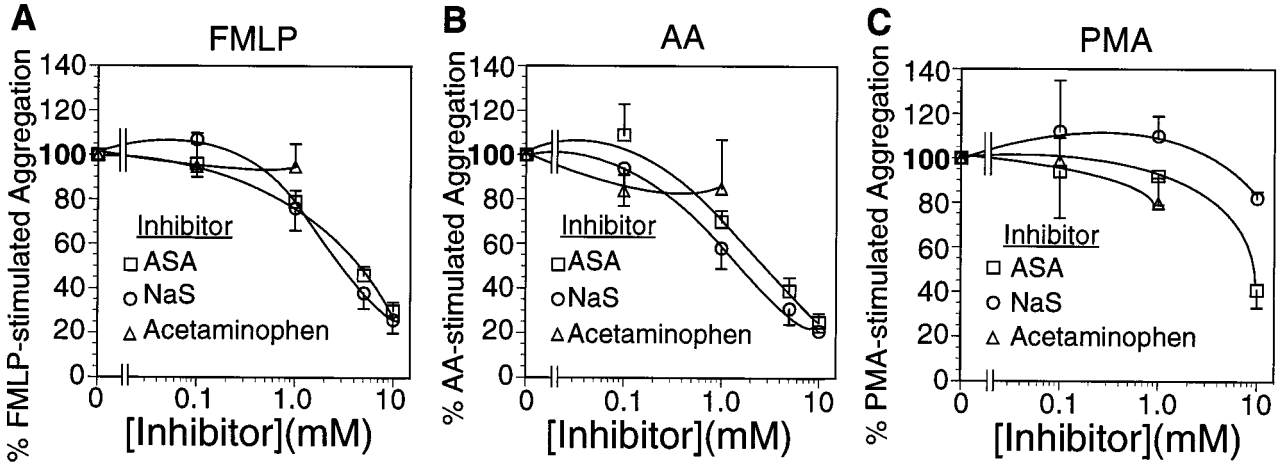

FIG. 5. Effects of aspirin, NaS, and acetaminophen on aggregation. Neutrophils were incubated for 10 min in the absence or presence of ASA (□), NaS (○), or acetaminophen (△), followed by stimulation with FMLP (*A*), AA (*B*), or PMA (*C*) for 2 min and measurement of aggregation. Results are expressed as percent of stimulated aggregation in the absence of inhibitor and are the means ± SEM of three experiments.

action. In contrast to aspirin analgesia, antipyresis, and antithrombosis, the anti-inflammatory effects of salicylates may not be entirely attributable to direct COX inhibition. Evidence for COX-independent anti-inflammatory effects of salicylates includes 1) ability of aspirin to inhibit inflammation only at concentrations dramatically higher than required for COX inhibition, 2) ability of the poor COX inhibitor NaS to block inflammation at concentrations similar to those required for aspirin, and 3) recognition that salicylates have COX-independent, pleiotropic effects whose physiologic relevance is unclear but whose targets [glucocorticoid receptors (43), NF κ-B (44, 45), heat shock proteins (46, 47), nitric oxide synthase (48)] suggest roles in inflammation. Our present data suggest that at least some of the anti-inflammatory effects of salicylates on neutrophils may be due to inhibition of Erk.

The recently documented ability of chemoattractants to stimulate Erk in post-mitotic, terminally-differentiated neutrophils was surprising in view of the reported role of Erk in cell division and differentiation. However, we have suggested that Erk activation in neutrophils is required for signaling pathways leading to adhesion (20, 24, 25). We based this suggestion on (i) tight association (kinetics and dose-response) between stimulated neutrophil Erk activation and adhesion, and (*ii*) tight correlation between pharmacologic inhibition of Erk and aggregation in response to both FMLP and AA. Most directly, we had observed that PD098059, a specific inhibitor of the proximal Erk activator Mek, inhibited AA stimulation of both Erk activation and neutrophil aggregation.

Table 2. Effects of salicylates and PD098059 on adherence of neutrophils to ECV304 cell monolayers

Inhibitor	Neutrophils adherent/high-powered field (0.05 mm^2)	
	No stimulus	FMLP (100 nM)
None	2.3 ± 0.6	9.2 ± 2.7
NaS (0.1 mM)	1.8 ± 0.9	7.7 ± 4.3
NaS (10 mM)	1.6 ± 0.5	3.8 ± 2.6
ASA (0.1 mM)	3.8 ± 0.8	16.6 ± 7.0
ASA (10 mM)	1.2 ± 0.7	3.2 ± 2.4
PD098059 (100 μM)	1.55 ± 0.4	2.3 ± 0.4

Neutrophils were incubated for 10 min at 37°C with the indicated inhibitors, then stimulated 30 min and analyzed for adhesion to ECV304 cell monolayers, expressed as the number of neutrophils adherent/high-powered field (average of 5 fields counted per experiment). Data shown are the mean ± SEM for four experiments, each done in duplicate.

To these observations we now add tight association between the capacities of salicylates, but not acetaminophen, to inhibit Erk activation and aggregation in response to FMLP and AA. Moreover, PD098059 inhibited both Erk activation and neutrophil adhesive function in response to FMLP as well as AA. In contrast, PD098059 inhibited PMA stimulation of Erk but not adhesion. The dissociation between Erk activation and adhesion in response to protein kinase C suggests that PMA stimulates adhesion downstream of Erk activity. The mechanism(s) by which Erk might signal for adhesion remain unclear. Whereas Naccache *et al.* (49) have demonstrated a possible role for tyrosine kinase activity in FMLP stimulation of CD11b/CD18 activation in neutrophils, no such evidence has been forthcoming regarding the activity of serine/threonine kinases such as Erk. It is unlikely, moreover, that the cytoplasmic domains of the CD11b/CD18 molecule serve as direct substrates for Erk, as Buyon *et al.* have shown that FMLP-stimulation of neutrophils does not affect CD11b/CD18 phosphorylation (50, 51). Interestingly, Hughes *et al.* (52) have recently reported that Erk activation in Chinese hamster ovary cells is associated with down-regulation of integrin $\alpha_{IIb}\ \beta_3$ activation, suggesting that Erk signaling may mediate either

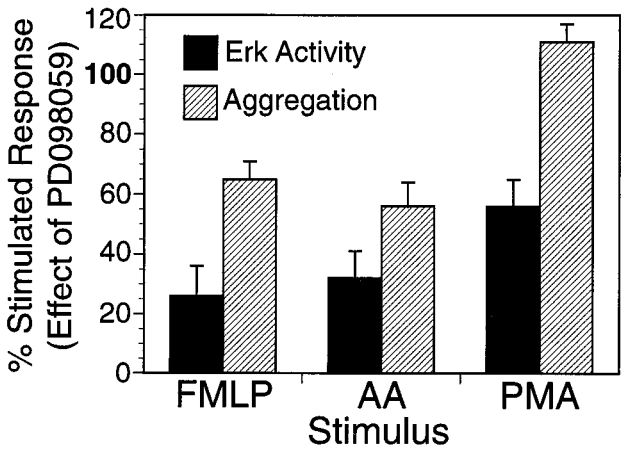

FIG. 6. Effects of the Mek inhibitor PD098059 on stimulated Erk activity and aggregation. Neutrophils were incubated for 10 min at 37°C in the absence or presence of 100 μM PD098059, stimulated with FMLP, AA (2 min) or PMA (10 min), and analyzed for Erk activity or aggregation. Results are expressed as percent of Erk activity or aggregation in PD098059-treated relative to untreated neutrophils, and are the means ± SEM of three experiments.

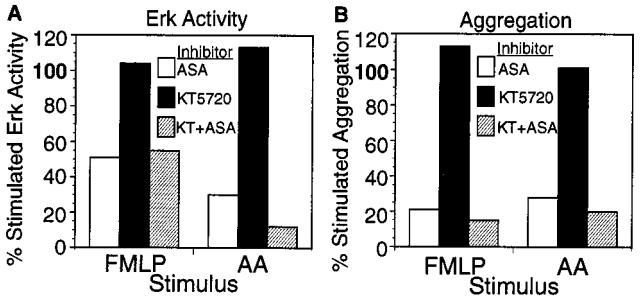

FIG. 7. The PKA inhibitor KT5720 does not rescue FMLP- or AA-stimulated neutrophil Erk activity or aggregation from inhibition by aspirin. Neutrophils were incubated for 10 min ± 1 μM KT5720, followed by incubation for 10 min ± 10 mM aspirin, stimulation for 2 min with FMLP or AA and measurement of Erk activity (*A*) or aggregation (*B*). Results are expressed as percent Erk activity or aggregation relative to neutrophils stimulated in the absence of aspirin and KT5720 and are the means of two experiments.

adhesion or antiadhesion, depending on cell and/or integrin type.

Salicylate inhibition of Erk activation is unlikely to have been due to COX inhibition. (*i*) Neutrophils have little or no COX in the absence of cytokine-stimulated up-regulation of COX-2 expression (53). (*ii*) Aspirin inhibited Erk at concentrations significantly higher than required to inhibit prostaglandin production (mM vs. μM) (54). Third, NaS inhibited Erk despite limited capacity to inhibit COX. Finally, the potent COX inhibitor indomethacin (42, 54) failed to inhibit FMLP- and AA-stimulated Erk activity. Claria and Serhan (55) have recently reported that addition of aspirin to coincubations of neutrophils and endothelial cells results in production of lipoxins, a class of compounds with potent inhibitory effects on neutrophil adhesion. Aspirin inhibition of neutrophil Erk and aggregation in our assays was unlikely due to lipoxins however, as incubation of neutrophils and aspirin in the absence of endothelial cells fails to cause lipoxin generation (55), and most of our studies were performed on isolated neutrophils.

Incubation of cells with salicylates can raise intracellular cAMP, and intracellular cAMP elevations can inhibit Erk in neutrophils and other cells in a PKA-dependent fashion (11, 20, 24, 56, 57). It is unlikely that salicylates inhibit neutrophil Erk via PKA however, because the PKA inhibitor KT5720 failed to reverse the effects of aspirin on FMLP- and AA-stimulated Erk. Salicylates also deplete ATP and release extracellular adenosine, and Cronstein *et al.* have demonstrated the capacity of adenosine to inhibit neutrophil functions including adhesion—effects reversed by exogenous adenosine deaminase (8, 58). However, adenosine release from neutrophils cannot explain neutrophil Erk inhibition by salicylates, because adenosine deaminase failed to reverse aspirin inhibition of Erk.

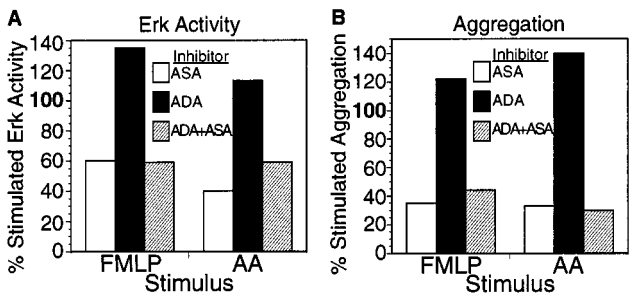

FIG. 8. Aspirin inhibition of FMLP- and AA-stimulated neutrophil Erk activity and aggregation is independent of extracellular adenosine release. Neutrophils were incubated for 10 min ± adenosine deaminase (ADA, 3 units/ml), followed by incubation for 10 min ± 10 mM aspirin, stimulation for 2 min with FMLP or AA and measurement of Erk activity (*A*) or aggregation (*B*). Results are expressed as percent Erk activity or aggregation relative to neutrophils stimulated in the absence of aspirin and ADA and are the means of two experiments.

Although AA activation of Erk requires its metabolic conversion to 5-hydroperoxyeicosatetraenoic acid (24), the ability of salicylates to inhibit both FMLP and AA stimulation of Erk suggests a site of action distal to AA metabolism and common to both FMLP and AA signaling, i.e., at or downstream of G protein-coupled receptor engagement. An alternative hypothesis–that FMLP stimulation of Erk depends on phospholipase A_2 activation and AA generation—is not formally excluded by our current data. The failure of salicylates to inhibit PMA-stimulated Erk activation suggests that their site of action is unlikely to be found among signaling elements common to both G protein (i.e., FMLP and AA) and PMA stimulation of Erk. Clearly Mek is one such element, because the Mek inhibitor PD098059 blocked Erk activation by all three stimuli.

Taken together, these data suggest that the site of action of salicylate Erk inhibition lies between receptor engagement and Raf-1 activation. The ability of salicylates—planar, hydrophobic molecules—to intercalate into the plasma membrane, altering membrane viscosity and disrupting receptor/target interactions, (59), makes disruption of Erk-activating pathways within the membrane an attractive hypothesis. It is equally plausible that salicylates disrupt the poorly-defined pathways from G protein to Ras and/or Raf-1 activation. Although Ras activation stimulated by G protein activity in mitotic cells depends on activation of phosphatidylinositol 3-kinase, we have recently excluded this pathway from FMLP activation of neutrophil Erk (28). Because salicylates and AA not only compete at discrete sites in the COXs but demonstrate antagonistic effects on G proteins (1), glucocorticoid and estrogen receptors (43, 60) and AP-1 activation (61), these planar molecules are likely to find other common targets in signaling cascades. These might include Ras itself, Ras activating proteins (GDP-exchange factors), and Ras-inhibitory proteins (GTPase activating proteins, or GAPs). The possible relevance of Ras GTPase activating proteins as salicylate targets may be emphasized by the recent finding that down-regulation of $p120^{GAP}$ is responsible for Ras activation in neutrophils stimulated with FMLP (62).

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