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TLR-4 mediated group IVA phospholipase A2 activation is phosphatidic acid phosphohydrolase 1 and protein kinase C dependent [#]

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Abstract

Group IVA phospholipase A_2 (GIVA PLA_2) catalyzes the release of arachidonic acid (AA) from the *sn*-2 position of glycerophospholipids. AA is then further metabolized into terminal signaling molecules including numerous prostaglandins. We have now demonstrated the involvement of phosphatidic acid phosphohydrolase 1 (PAP-1) and protein kinase C (PKC) in the Toll-like receptor-4 (TLR-4) activation of GIVA PLA_2. We also studied the effect of PAP-1 and PKC on Ca^{+2} induced and synergy enhanced GIVA PLA_2 activation. We observed that the AA release induced by exposure of RAW 264.7 macrophages to the TLR-4 specific agonist Kdo_2-Lipid A is blocked by the PAP-1 inhibitors bromoenol lactone (BEL) and propranolol as well as the PKC inhibitor Ro 31-8220; however these inhibitors did not reduce AA release stimulated by Ca^{+2} influx induced by the P2X7 purinergic receptor agonist ATP. Additionally, stimulation of cells with diacylglycerol (DAG), the product of PAP-1 mediated hydrolysis, initiated AA release from unstimulated cells as well as restored normal AA release from cells treated with PAP-1 inhibitors. Finally, neither PAP-1 nor PKC inhibition reduced GIVA PLA_2 synergistic activation by stimulation with Kdo_2–Lipid A and ATP.

INTRODUCTION

Bacterial sepsis and septic shock result from the exacerbated production of inflammatory mediators by the immune system in response to bacterial endotoxins such as lipopolysaccharide (LPS) (1). Exposure of macrophages to LPS initiates the Toll-like receptor-4 (TLR-4) signaling pathway, resulting in the release of a host of cytokines and eicosanoids, including various prostaglandins (PGs). PGs are known to contribute to a number of normal and pathological physiological processes, including inflammation, pain, and vascular permeability and are also implicated as major contributors to endotoxic shock (2).

Group IVA phospholipase A_2 (GIVA PLA_2), also known as the cytosolic PLA_2 ($cPLA_2$), is the proinflammatory phospholipase responsible for the release of arachidonic acid (AA) which

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is further metabolized into eicosanoids (3,4). GIVA PLA_2 catalyzes the hydrolysis of AA from the *sn*-2 position of membrane phospholipids, resulting in the release of free AA from the membrane and formation of lysophospholipids (5,6). GIVA PLA_2 has been implicated in a variety of inflammatory disease models, including collagen induced arthritis, autoimmune encephalomyelitis and acute lung injury; therefore, obtaining a complete understanding of the molecular mechanism of its activation is essential. (7–9).

Phosphatidic acid phosphohydrolase 1 (PAP-1) is a Mg^{+2}-dependent, cytosolic enzyme that associates with phosphatidic acid (PA) containing membrane surfaces, where it converts PA into inorganic phosphate and diacylglycerol (DAG) (10–17). With the discovery by Carman and coworkers (17) in 2006 that yeast PAP-1 is the yeast ortholog of Lipin-1 discovered by Reue and coworkers (18); the interest in PAP-1 has escalated. For a recent review of the lipins, see (19). Our laboratory has utilized two different immortalized mammalian cell lines to characterize distinctive signaling pathways in which PAP-1 inhibition leads to the loss of cyclooxygenase 2 (COX-2) expression and PGE_2 release. The first study was conducted in the human amnionic WISH cell line utilizing the protein kinase C (PKC) activator phorbol myristate acetate (PMA) (18). The second study was conducted in the human U937 macrophage-like cell line stimulated with the Toll-like receptor (TLR)-4 agonist lipopolysaccharide (LPS) (19). In the macrophage cells, we (21) found in a Western blot that antibody to Lipin-1, and not antibodies to Lipin-2 or Lipin-3, crossreacted with protein at a molecular mass of 140 kDa on SDS-PAGE electrophoresis, the correct molecular mass for Lipin-1 (22), suggesting its presence and that the macrophage PAP-1 is Lipin-1, in analogy to the Carman finding in yeast (16,17). Drawing on these studies and other published reports, we hypothesized that DAG evolved from PAP-1 is likely to activate PKC, which has been shown to cause the phosphorylation of GIVA PLA_2 in cellular studies conducted with phorbol esters (20–23).

In this manuscript, we provide further evidence that inhibition of PAP-1 or PKC results in reduced GIVA PLA_2 activation in macrophages stimulated with the TLR-4 specific agonist, Kdo_2-Lipid A (24). Furthermore, we observed that exposure of cells to the inhibitors did not reduce GIVA PLA_2 -mediated AA release initiated with Ca^{+2} influx via ATP. Finally, our data suggests that inhibition of PAP-1 or PKC does not affect synergistic activation of GIVA PLA_2, despite reducing the absolute amount of AA released from cells that have been stimulated with both Kdo_2-Lipid A and ATP stimuli.

EXPERIMENTAL PROCEDURES

Materials

Murine RAW 264.7 macrophages were purchased from American Type Culture Collection (Manassas, VA). DMEM cell culture medium was obtained from Gibco (Grand Island, NY). Fetal bovine serum was from VWR International (Bristol, CT). DTT and lipopolysaccharide (LPS), from *E. coli* 0111:B4, were obtained from Sigma Chemical Company (St. Louis, MO). Kdo_2-Lipid A, 1-palmitoyl-2-arachidonoyl phosphatidylcholine was from Avanti Polar Lipids (Alabaster, AL). Propranolol, Dioctoyl-DAG and Ro 31-8220 were from Biomol (Plymouth Meeting, PA). Bromoenol lactone (BEL) was from Cayman Chemical (Ann Arbor, MI). 1-palmitoyl-2-(1-^{14}C)-palmitoyl phosphatidylcholine, 1-palmitoyl-2-(1-^{14}C)-arachidonoyl phosphatidylcholine, [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid (specific activity = 100 Ci/mmol) and [9,10-^{3}H] oleic acid (specific activity = 23 Ci/mmol) were obtained from NEN Life Science Products (Boston, MA). Phosphatidylinositol 4,5-bisphosphate was from Roche (Basel, Switzerland). The specific $cPLA_2$ inhibitor, pyrrophenone, was kindly provided by Dr. Kohji Hanasaki (Shionogi Research Laboratories of Shionogi & Co., Ltd). 20 cm × 20 cm × 250 μm K6 Silica gel thin layer chromatography plates were from Whatman (Clifton, New Jersey).

Cell Culture and Stimulation Protocol

RAW 264.7 macrophages were maintained at 37 °C in a humidified atmosphere at 90% air and 5% CO_2 DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, 100 μg/ml streptomycin, and non-essential amino acids. Cells were plated at a confluency of 2×10^6 cells/6 well tissue culture plate and 1×10^6/12 well tissue culture plate at the time of experimentation. Following plating, they were allowed to adhere overnight, and then used for experiments the following day, with the exception of cells that were radiolabeled, described below. When DAG was added to the cells, it was done immediately after the addition of Kdo_2-Lipid A. Whenever cellular experiments required the usage of chemical inhibitors; the inhibitors were initially diluted into media at a stock concentration that was 100 times more concentrated than that of the final cellular concentration. From this stock concentration, the inhibitors were then applied to the cells to achieve the desired final cellular concentration. The final concentration of DMSO in the supernatants never exceeded .05% (volume/volume). When synergy experiments were performed, ATP was added to cells treated with Kdo_2-Lipid A 50 minutes after Kdo_2-Lipid A stimulation, and supernatants were collected at 1 hour following stimulation. Cell viability was assessed visually by the Trypan Blue Dye exclusion assay (Gibco, Grand Island, NY) and through the usage of the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI).

Sample Preparation

Media was analyzed for extracellular eicosanoid release, as described previously (25). After stimulation, 1.8 ml of media was removed and supplemented with 100 μl of internal standards (100 pg/μl, EtOH) and 100 μl EtOH to bring the total EtOH to 10% by volume. Samples were centrifuged for 5 minutes at 3000 rpm to remove cellular debris, and the supernatants were decanted into solid phase extraction columns. All eicosanoid extractions were conducted using Strata-X SPE columns (Phenomenex). The columns were washed with 2 ml MeOH and then 2 ml H_2O prior to the samples being applied. After applying the sample, the columns were washed with 10% MeOH to remove non-adherent debris, and eicosanoids were then eluted off the column with 1 ml MeOH. The eluant was dried under vacuum and redissolved in 100 μl of solvent A [water-acetonitrile-formic acid (63:37:0.02; v/v/v)] for LC/MS analysis.

Cell Quantitation

Eicosanoid levels were normalized to cell number using DNA quantitation. After the extracellular media was removed, the cells were scraped in 500 μl PBS and stored at 4°C for DNA quantitation using the Broad Range DNA Quant-Kit (Invitrogen).

HPLC and Mass Spectrometry

The analysis of eicosanoids was performed by LC/MS/MS (26). Eicosanoids were separated by reverse-phase HPLC on a C18 column (2.1 mm × 150 mm, Grace-Vydac) at a flow rate of 300 μl/min at 25°C. The column was equilibrated in Solvent A [water-acetonitrile-formic acid (63:37:0.02; v/v/v)], and samples were injected using a 50 μl injection loop and eluted with a linear gradient from 0%–20% solvent B [acetonitrile-isopropyl alcohol (50:50; v/v)] between 0 to 6 min; solvent B was increased to 55% from 6 to 6.5 min and held until 10 min. Solvent B was increased to 100% from 10 to 12 min and held until 13 min; solvent B was dropped to 0% by 13.5 min and held until 16 min.

Eicosanoids were analyzed using a tandem quadrupole mass spectrometer (ABI 4000 Q Trap®, Applied Biosystems) via multiple-reaction monitoring (MRM) in negative-ion mode. The electrospray voltage was −4.5 kV, the turbo ion spray source temperature was 525°C. Collisional activation of eicosanoid precursor ions used nitrogen as a collision gas. While arachidonic acid and its major metabolites (referred to as eicosanoids) were detected, by far

the major species at one hour was arachidonic acid, which was the focus of this study; for quantitation of the major eicosanoids produced, see (30).

Quantitative eicosanoid determination was performed by stable isotope dilution, as previously described (26). Results are determined as pmol of AA per million cells (mean ± standard deviation).

RESULTS

PAP-1 inhibition reduces Kdo_2-Lipid A stimulated AA release

We initiated this study by characterizing GIVA PLA_2-mediated AA release from RAW 264.7 macrophages that were stimulated with Kdo_2-Lipid A. Kdo_2-Lipid A is a truncated LPS structure which is a specific binding-agonist of TLR-4 (24). The AA release time-course experiments were conducted over a period of 24 hours. As described in Figure 1, the maximal release of AA was observed to be 300–400 pmol AA/10^6 cells at 1 hour after stimulation, after which AA release gradually returned to baseline levels. Subsequent AA release experiments were conducted at one hour following stimulation as this was determined to be the most optimal time point.

To ensure that GIVA PLA_2 is the enzyme that is responsible for mediating AA release under the experimental conditions employed, cells were pretreated with the GIVA PLA_2 specific inhibitor pyrrophenone (31) prior to stimulation. Pretreatment of the cells with pyrrophenone reduced Kdo_2-Lipid A stimulated AA release to background levels, as shown in Figure 2A; this result indicates that the Kdo_2-Lipid A stimulated AA release is mediated through the activity of GIVA PLA_2.

To evaluate whether PAP-1 may participate in the activation of GIVA PLA_2, we preincubated cells with the PAP-1 inhibitors BEL and propranolol prior to stimulation of the cells. Preincubation of the cells with bromoenol lactone (BEL) resulted in a ~70% decrease of Kdo_2-Lipid A stimulated AA release into the supernatant, as seen in Figure 2B. This result was corroborated by repeating the experiment with another inhibitor of PAP-1, propranolol, as shown in Figure 2C.

To address the possibility that PLC may play a role in this pathway, cells were treated with the classic PLC inhibitors D609 and U-71322 prior to stimulation with Kdo_2-Lipid A; neither inhibitor reduced AA release, suggesting that PLC is not involved in this signaling cascade (data not shown).

BEL and propranolol do not inhibit ATP stimulated AA release

The inhibitor data summarized in Figures 2B and 2C suggest that PAP-1 participates in the TLR-4 mediated activation of GIVA PLA_2. Cellular GIVA PLA_2 activity can also be induced by the treatment of cells with Ca^{+2} mobilizing agonists, such as the purinergic P2X7 receptor agonist ATP. Inhibited and uninhibited cells were treated with ATP prior to AA release quantitation. Stimulation of macrophages with ATP increased AA release into the supernatant (Figure 3). To ensure that the Ca^{+2} agonists had induced AA release via participation of GIVA PLA_2, cells were also treated with pyrrophenone prior to stimulation; exposure to pyrrophenone resulted in reduction of AA release to background levels. Neither BEL nor propranolol reduced AA release induced by this stimulus, indicating that the inhibitors do not affect GIVA PLA_2.

As an additional control, to ensure that an extracellular Ca^{+2} influx into the cell is not required in the TLR-4 mediated activation of the GIVA PLA_2, cells were cultured in the presence of EDTA and EGTA prior to stimulation with Kdo_2-Lipid A. Neither EDTA nor EGTA reduced

the AA release, indicating that Ca^{+2} influx is not required for TLR-4 mediated activation of the GIVA PLA_2 (Figure 4).

PAP-1 chemical inhibition does not reduce the synergistic activation of GIVA PLA_2

Recently, we reported that cells that were stimulated with both Kdo_2-Lipid A and ATP released more AA than the sum of AA released from cells stimulated separately, a phenomena referred to as synergy (27). The extent to which AA release is enhanced by joint Kdo_2-Lipid A and ATP stimuli can be calculated from:

$$\text{Synergistic Activation} = \frac{[\text{Kdo}_2 - \text{Lipid A} + \text{ATP}]}{[\text{Kdo}_2 - \text{Lipid A}] + [\text{ATP}]} \qquad \text{Equation 1}$$

When synergy is present, the synergistic activation value is greater than 1.0, as joint Kdo_2-Lipid A and ATP stimulation result in AA release that exceeds the sum of the individual stimulations. If no synergy is present, and therefore joint stimulation equals the sum total of separate stimulations, the value will equal 1.0. Synergy-enhanced GIVA PLA_2 activation results in enhanced AA release that exceeds the sum total of TLR-4 and Ca^{+2} mediated AA releases.

In the current study, sequential treatment of cells with Kdo_2-Lipid A and ATP resulted in a synergistic activation value of 2.5±0.4, as shown in Figure 5A. In this particular example, cells that were treated with Kdo_2-Lipid A and ATP released 1,600 pmol AA/10^6 cells, which is four times greater than the amount of AA released by either Kdo_2-Lipid A or ATP alone and approximately 2.5 times greater than the sum total of separate Kdo_2-Lipid A and ATP stimulations. It is important to note that the absolute level of AA released via the [Kdo_2-Lipid A + ATP] stimulation shown in Figure 5A is not an indicator of synergy. To determine the extent to which synergy activation is present, one must compute the synergistic activation value through equation 1 and then evaluate it relative to a value of “1”.

Figures 2 and 3 show that preincubation of macrophages with either BEL or propranolol prior to Kdo_2-Lipid A treatment reduces AA release, whereas neither inhibitor affects ATP stimulated AA release. Similarly, preincubation of the cells with BEL or propranolol reduces the AA released from cells treated with [Kdo_2-Lipid A + ATP], as shown in Figure 5A. However, despite the reduction in [Kdo_2-Lipid A + ATP] stimulated AA release, the synergistic activation value was not reduced by the presence of the inhibitors (Figure 5B). This suggests that PAP-1 inhibition reduces AA release from cells treated with [Kdo_2-Lipid A + ATP] by reducing the Kdo_2-Lipid A portion of the stimulation, while not affecting the contribution of the synergy stimulus.

Exogenous DAG stimulates GIVA PLA_2-mediated AA release

Since PAP-1 converts cellular PA into DAG, macrophages were treated with exogenous DAG to assess whether this would elicit AA release. The addition of DAG to unstimulated cells slightly increased AA release into the supernatant. Additionally, the supplementation of DAG to Kdo_2-Lipid A treated cells resulted in a further increased AA release from the cells relative to that of cells that were treated with only Kdo_2-Lipid A (Figure 6). To ensure that the enhanced AA release was due to additional activation of GIVA PLA_2, we preincubated cells with pyrrophenone prior to stimulation. Pyrrophenone inhibited AA release to background levels in both cases, indicating that the enhanced AA release elicited by DAG is mediated through the action of GIVA PLA_2.

Exogenous DAG restores AA release from macrophages inhibited by propranolol or BEL

We investigated whether the addition of exogenous DAG in an "add-back" fashion would restore AA release from cells that had been pretreated with BEL or propranolol. The addition of DAG to cells that had been pretreated with the PAP-1 inhibitors prior to stimulation with Kdo_2-Lipid A resulted in full restoration of AA release to levels seen in uninhibited cells (Figure 7A and B). This result suggests that the effect of PAP-1 inhibition is reversible by the addition of DAG, which is the product of PAP-1 mediated hydrolysis.

PKC chemical inhibition inhibits Kdo_2-Lipid A stimulated AA release, but not synergistic activation

Since our data suggests that inhibition of PAP-1 results in decreased Kdo_2-Lipid A stimulated AA release but does not affect the synergistic activation of GIVA PLA_2, we next investigated whether inhibition of PKC would corroborate the PAP-1 inhibitory data. Cells were treated with the general PKC inhibitor Ro 31-8220 prior to stimulation with Kdo_2-Lipid A, ATP and [Kdo_2-Lipid A + ATP] and the synergistic activation value was computed. Similar to that of the PAP-1 inhibitor results, inhibition of PKC resulted in baseline reduction of Kdo_2-Lipid A stimulated AA release and also significantly reduced [Kdo_2-Lipid A + ATP] stimulated AA release, while not having a significant affect on ATP stimulation (Figure 8A). Also, consistent with the PAP-1 inhibitory data, the synergistic activation value was not reduced relative to that of the uninhibited cells (Figure 8B).

DISCUSSION

The focus of this research was to investigate the relationship between PAP-1, PKC and the activation of GIVA PLA_2 in the TLR-4 signaling cascade. To evaluate cellular GIVA PLA_2 activity, macrophages were stimulated with the TLR-4 specific agonist Kdo_2-Lipid A and then AA release into the supernatant was quantitated as a metabolic indicator of GIVA PLA_2 function. In previous studies in which AA release was quantitated from macrophages, our laboratory and others made use of scintillation counting of radioactive AA as well as EIA quantitation of PGE_2 secreted to the supernatant as an indicator of cellular GIVA PLA_2 activity (19,28–31). Both techniques have important limitations that must be accounted for. Scintillation counting does not discriminate between AA release and AA-derived metabolites, such as eicosanoids. Utilization of commercial EIA kits to measure PGE_2 release as an indicator of AA release makes the assumption that PGE_2 release is a direct indicator of cellular AA release. In the current study, we make use of HPLC-MS methodology to quantitate AA release specifically from unlabelled cells.

We began this study by conducting time courses of AA release from macrophages stimulated with Kdo_2-Lipid A to evaluate the optimal time to measure AA release following cellular activation. Maximal AA release into the supernatant was observed at one hour following stimulation, and therefore this was chosen to be the time point at which subsequent experiments were conducted. Since AA release was utilized as an indicator of cellular GIVA PLA_2 activity, it was imperative to ensure that Kdo_2-Lipid A stimulated AA release was mediated via GIVA PLA_2. To achieve this, macrophages were pretreated with the GIVA PLA_2 specific inhibitor, pyrrophenone, prior to stimulation with Kdo_2-Lipid A. Pyrrophenone reduced AA release to background levels, indicating that GIVA PLA_2 is responsible for Kdo_2-Lipid A stimulated AA release.

To investigate whether PAP-1 participates in the Kdo_2-Lipid A stimulated GIVA PLA_2 activation, cells were treated with the PAP-1 inhibitors BEL and propranolol prior to stimulation. Preincubation of cells with BEL or propranolol resulted in a marked reduction of AA release into the supernatant. Since this result could be explained by direct inhibition of

GIVA PLA_2, we studied the effect of the inhibitors on AA release from cells stimulated with the Ca^{+2} mobilizing agent, ATP. ATP activates GIVA PLA_2 by raising the cellular Ca^{+2} levels, rather than through a TLR-4-mediated mechanism (30). Neither BEL nor propranolol reduced AA release from cells stimulated with ATP, indicating that the reduced Kdo_2-Lipid A stimulated AA release is not due to direct inhibition of cellular GIVA PLA_2 activity. It is worth noting that we have shown in a previous publication that activation of TLR-4 in macrophages does not increase intracellular Ca^{+2} levels (32). Additionally, we confirmed in this manuscript that AA release from cells stimulated with ATP is mediated through GIVA PLA_2 by pyrrophenone inhibition. For the sake of completion, we investigated whether DAG generated by PLC may play a role in GIVA PLA_2 activation by dosing cells with the PLC inhibitors D609 and U-71322 prior to stimulation; neither inhibitor had an affect, suggesting that PLC is not involved.

Since our data suggests that PAP-1 inhibition reduced GIVA PLA_2 activation in Kdo_2-Lipid A treated cells, it was logical to assess what the effect of introducing DAG would be on GIVA PLA_2 activation. To evaluate whether DAG would directly activate cellular GIVA PLA_2, cells were stimulated with exogenous DAG prior to the measurement of AA release into the supernatant. The addition of DAG alone significantly increased AA release, relative to that of control cells. Additionally, supplementation of DAG to macrophages that were also pretreated with Kdo_2-Lipid A resulted in enhanced AA release into the supernatant. The AA release was confirmed to be mediated through GIVA PLA_2 by pyrrophenone inhibition. Taken in conjunction with the PAP-1 inhibitory data, the fact that DAG evoked AA release suggests that DAG formation is an essential step in the stimulation of GIVA PLA_2 catalysis (33–37). Furthermore, considering the fact that the PAP-1 and PKC inhibitory data are similar would suggest that PKC is a likely molecular target of PAP-1 that also participates in the activation mechanism (38). Additionally, since PAP-1 inhibition reduced AA release, exogenous DAG was added to PAP-1 inhibited cells to discern whether this would restore AA release. It did restore AA release to levels released from uninhibited cells, suggesting that introduction of the DAG had compensated for the blocked PAP-1.

In a recent publication, our laboratory described the synergistic activation of GIVA PLA_2 in macrophages that had been treated with Kdo_2-Lipid A and ATP, resulting in AA release that exceeded the sum total of separate Kdo_2-Lipid A and ATP stimulations. This phenomenon has been referred to as synergy (27), whereby in combination Kdo_2-Lipid A activates GIVA PLA_2 by eliciting TLR-4 signaling and ATP does it through increased cellular Ca^{+2}; but somehow leading to more product than the sum of each acting alone. Our current data suggest that these two activation mechanisms are quite distinct. First, it is shown that PAP-1 and PKC inhibition do not affect ATP stimulation, whereas they have a pronounced affect on Kdo_2-Lipid A mediated activation. Second, by adding EGTA and EDTA to chelate extracellular Ca^{+2}, we have shown that Kdo_2-Lipid A does not require an influx of Ca^{+2}, unlike ATP.

In the current manuscript, we have expanded upon our previous knowledge of GIVA PLA_2 synergistic activation by studying the chemical inhibition of the TLR-4 signaling while observing the effect upon synergy. Initially, PAP-1 chemical inhibitors were employed with the observation that PAP-1 inhibition resulted in a reduction of [Kdo_2-Lipid A and ATP] stimulated AA release, while the synergistic activation was unaffected. A likely explanation for this observation is that PAP-1 participates in the TLR-4 mediated signaling mechanism leading to GIVA PLA_2 activation, but does not participate in the synergistic activation. Possibilities for other independent effects of the TLR-4 activation are discussed elsewhere (30). Furthermore, this data suggests that blockage of the TLR-4 signaling mechanism does not inhibit activation of GIVA PLA_2 through synergy. To confirm this data, the general PKC inhibitor Ro 31-8220 was employed to block PKC, which lies downstream of PAP-1 in this pathway. Interestingly, inhibition of PKC resulted in total blockage of AA release elicited from

cells stimulated with Kdo_2-Lipid A while not reducing the synergistic activation value. This demonstrates that full inhibition of the TLR-4 signaling cascade leading to GIVA PLA_2 does not result in a reduction in synergistic activation, further suggesting that GIVA PLA_2 synergistic activation is the result of a unique signaling mechanism.

It is now known that the mammalian lipins 1, 2 and 3 exhibit PAP activity similar to the PAH1 gene product of yeast (43). Using qRT-PCR we have found in RAW cells that lipin 1 is expressed at significant levels. We also found that lipin 2 is expressed at even higher levels, but did not detect lipin 3 to any significant extent (data not shown). Therefore, we did not differentiate the extent to which each of the lipins contributes to the observed PAP-associated effects.

In summary, we present data that suggests that the activation of GIVA PLA_2 by Kdo_2-Lipid A requires the participation of PAP-1; it is likely that the function of PAP-1 in this model is to activate PKC. Four lines of evidence support such a theory. First, it is known that GIVA PLA_2 undergoes phosphorylation and activation involving PKC through a number of agonist-induced signaling pathways, including TLR-4 via LPS (42,44–46). Gene deletion or chemical inhibition of PKC in these studies resulted in loss of agonist-induced GIVA PLA_2 phosphorylation with reduced arachidonic acid release. Direct activation of PKC by the agonist PMA has been shown to result in the release of arachidonic acid and GIVA PLA_2 activation in a number of macrophage cell lines, including RAW 264.7 and U937 (20,42,43).

Second, all three proteins have been observed to co-localize on the nuclear membrane surface. PAP-1 contains a nuclear localization sequence and the protein expression is concentrated to the nuclear membrane region of HEK293 and 3T3-L1 cells (49,50). PKC has been shown to translocate to the nuclear membrane with rising membrane levels of DAG (51,52). Likewise, GIVA PLA_2 associates with the nuclear membrane after phosphorylation (53).

Third, PAP-1 was found to be associated with PKCε and epidermal growth factor (EGF) receptor in EGF receptor immunoprecipitates. Following treatment with EGF and a second immunoprecipitation with an anti-PKCε antibody, PAP-1 was found to be associated with PKCε (54). GIVA PLA_2 is known to undergo phosphorylation and activation with subsequent AA release by way of an oxidative stress EGF receptor mediated pathway (55).

Finally, we have previously described that PAP-1 participates in TLR-4 mediated COX-2 expression, and our current results suggest that PAP-1 also participates in the TLR-4 activation of the GIVA PLA_2 (18,19). Collectively, these results suggest that PAP-1 plays a significant role in inflammatory signaling within the TLR-4 activated macrophage. Of course, these conclusions about the role of PAP-1 are based heavily on the inhibitor studies reported herein and, of course, “a specific inhibitor is one that has not yet been shown to lack specificity”. While BEL and propranolol undoubtedly have other activities in cells, the overall argument for a PAP-1 role depends on their being specific for the specified steps in the TLR-4 activation of GIVA PLA_2. The observation that these inhibitors do not affect the activation with ATP bolsters the argument, but does not prove specificity, so additional approaches to test this hypothesis are certainly warranted.

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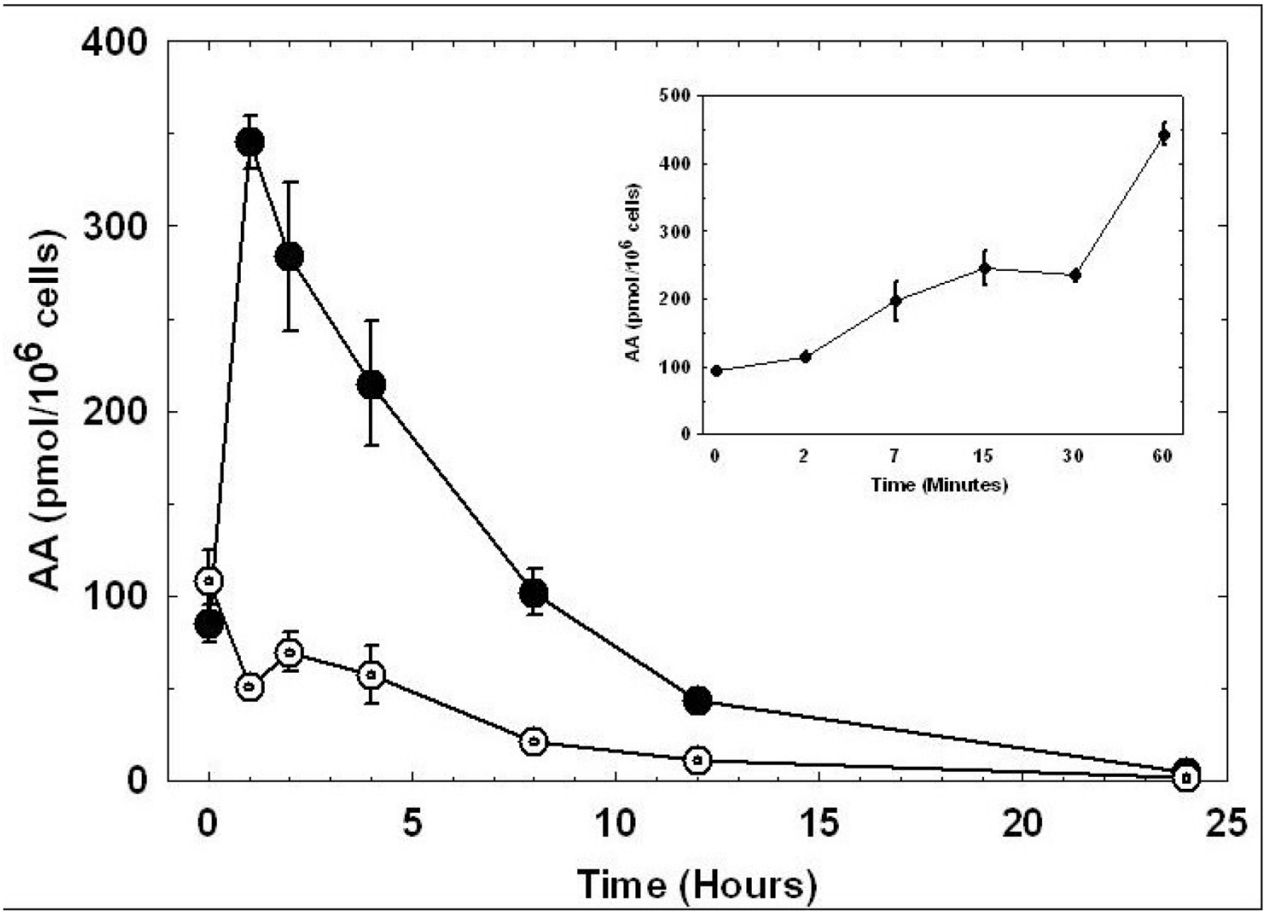

Figure 1. Kdo_2-Lipid A stimulated AA release time-course

RAW 264.7 macrophages were cultured in the presence (•) or absence (○) of 100 ng/mL Kdo_2-Lipid A, the media was collected at the indicated time points and then subsequently analyzed for AA release by HPLC-MS. The insert is a separate experiment showing Kdo_2–Lipid A stimulated AA release over a 60 minute time-course. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

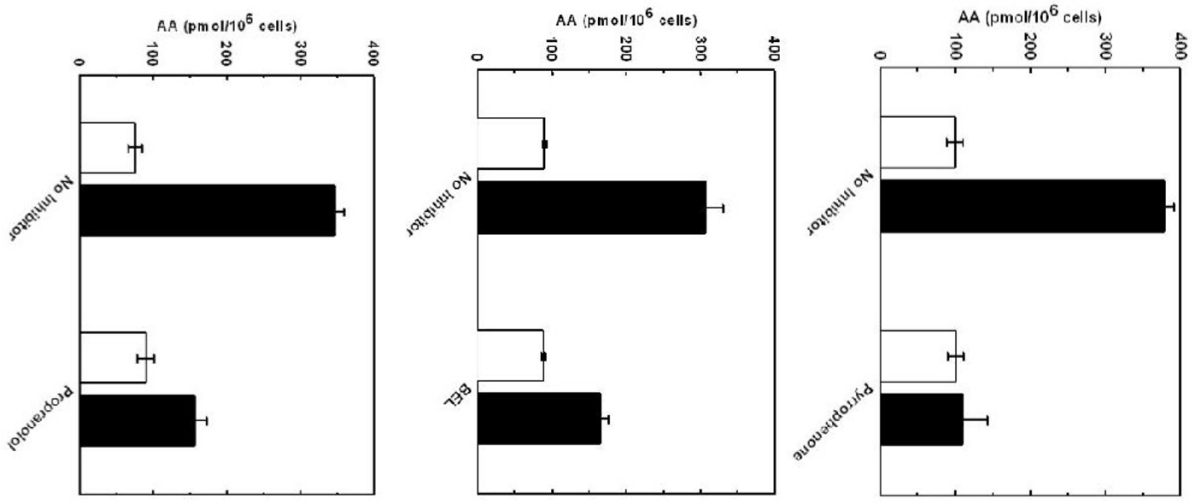

Figure 2. Inhibition of PAP-1 blocks the Kdo_2-Lipid A stimulated release of AA

AA release was measured from RAW 264.7 macrophages preincubated with and without (*A*) 1 μM pyrrophenone, (*B*) 25 μM BEL, or (*C*) 50 μM propranolol prior to stimulation with (■) and without (□) 100 ng/mL Kdo_2-Lipid A. The media was collected at 1 hour following Kdo_2-Lipid A stimulation and analyzed for AA release by HPLC-MS. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

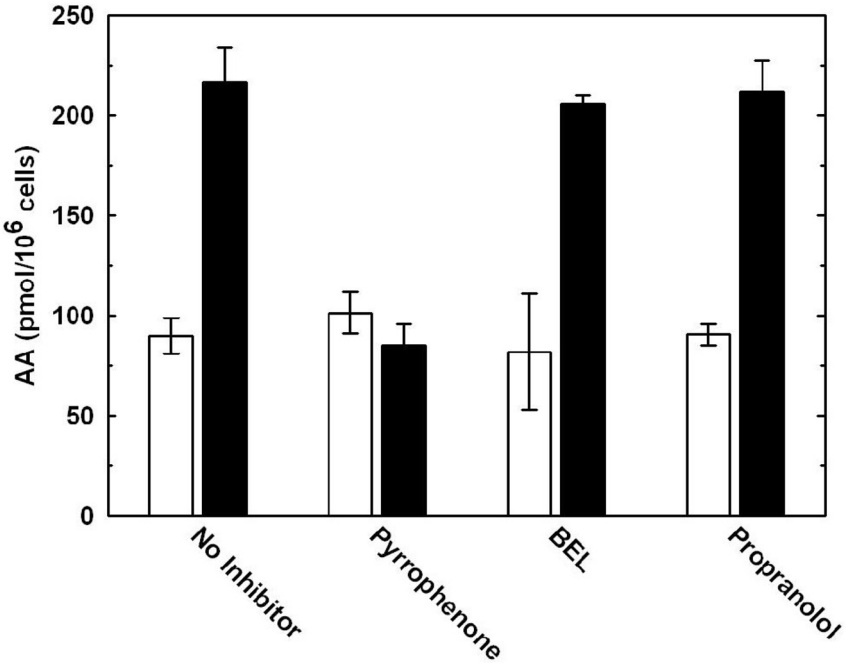

Figure 3. Inhibition of PAP-1 does not block the ATP stimulated release of AA

AA release was measured from RAW 264.7 macrophages preincubated with and without 1 μM pyrrophenone, 25 μM BEL or 50 μM propranolol prior to stimulation with (■) and without (□) 2 mM ATP. The media was collected at 10 minutes following stimulation and analyzed for AA release. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

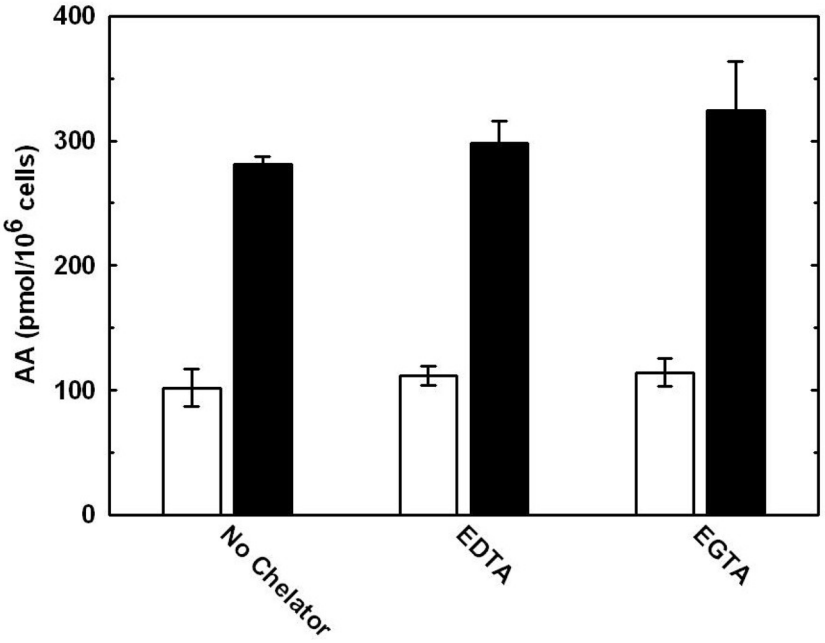

Figure 4. The Ca^{+2} chelators EDTA and EGTA do not reduce Kdo_2-Lipid A stimulated AA release

A, AA release was measured from RAW 264.7 macrophages preincubated with and without 2 mM EDTA or EGTA prior to stimulation with (■) and without (□) 100 ng/mL Kdo_2-Lipid A. The media was collected at 1 hour following stimulation and analyzed for AA release. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

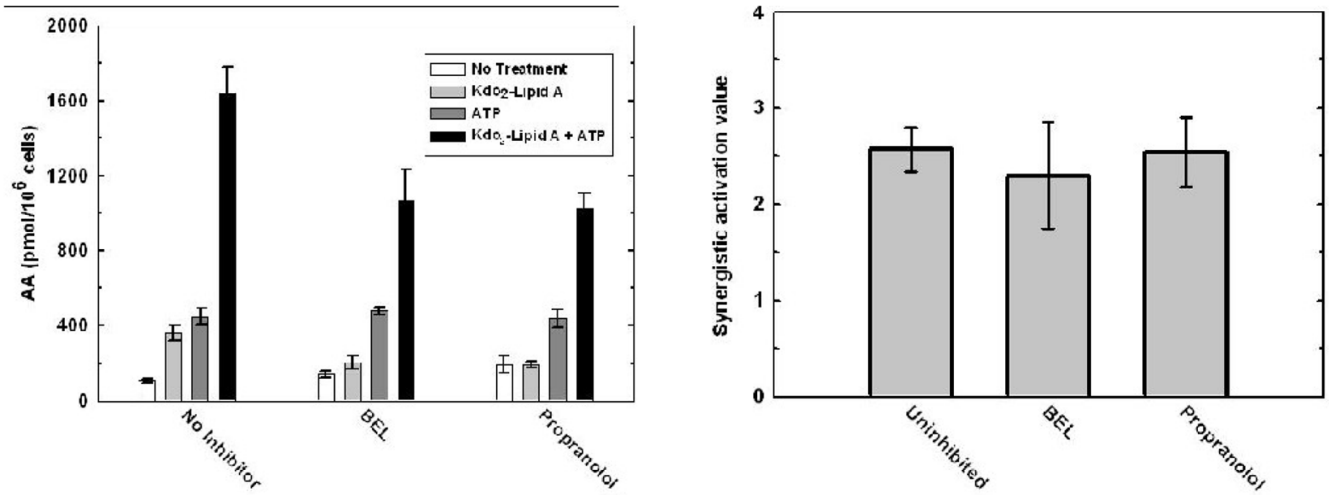

Figure 5. Inhibition of PAP-1 blocks synergy-enhanced AA release, but does not reduce the synergistic activation value

A, AA release was measured from RAW 264.7 macrophages preincubated with and without 25 μM BEL or 50 μM propranolol prior to stimulation with and without 100 ng/mL Kdo_2-Lipid A for 50 minutes and with and without 2 mM ATP for 10 minutes (as indicated in the legend). The media was collected at 1 hour following the addition of the 100 ng/mL Kdo_2-Lipid A agonist and analyzed for AA release. *B*, Synergistic activation values were calculated for uninhibited cells, preincubated with 25 μM BEL or 50 μM propranolol. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

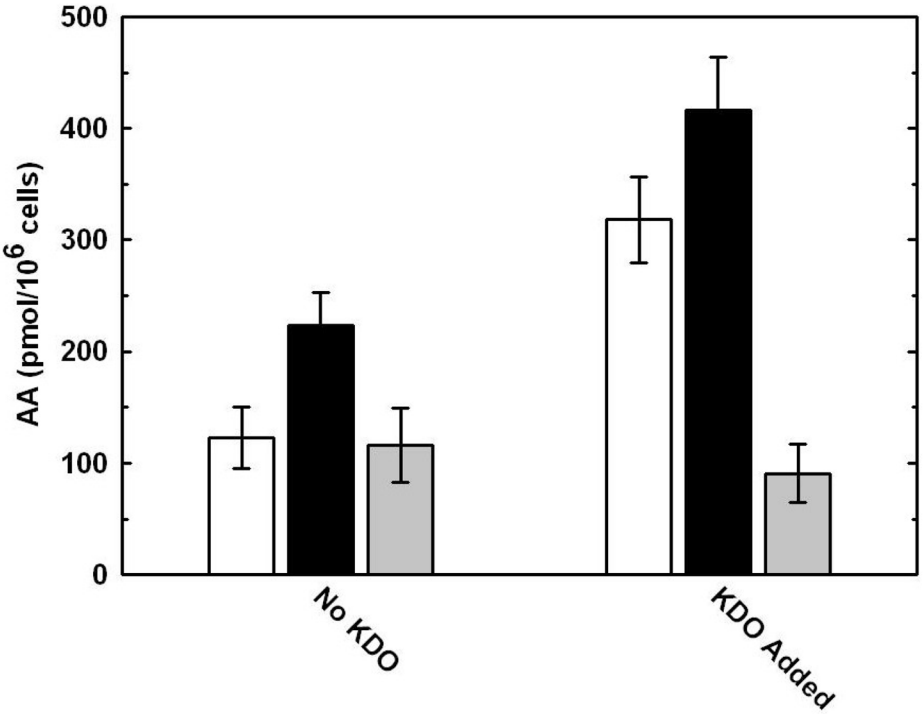

Figure 6. Exogenous DAG bolsters the Kdo_2-Lipid A stimulated release of AA

AA release into the media was measured from RAW 264.7 macrophages that were stimulated with and without 100 ng/mL Kdo_2-Lipid A (as indicated) and in the absence (white bars) and presence (black bars) of 50 μM DAG for 1 hour. Additionally, grey bars indicate AA release from cells preincubated with 1 μM pyrrophenone for 30 minutes prior to the addition of DAG. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

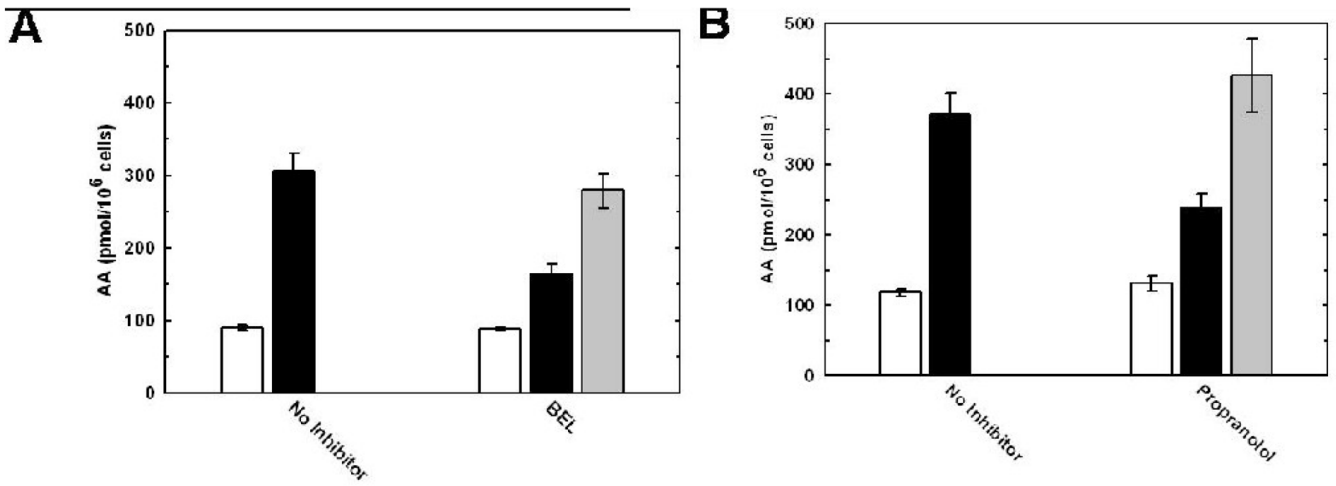

Figure 7. DAG supplementation restores AA release from Kdo_2-Lipid A stimulated RAW 264.7 macrophages pretreated with PAP-1 inhibitors

AA release was measured from cells preincubated with and without (*A*) 25 μM BEL or (*B*) 50 μM propranolol (as indicated) prior to stimulation with 100 ng/mL Kdo_2-Lipid A (black bars) and 50 μM DAG (grey bars). The media was collected at 1 hour following stimulation and analyzed for AA release. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

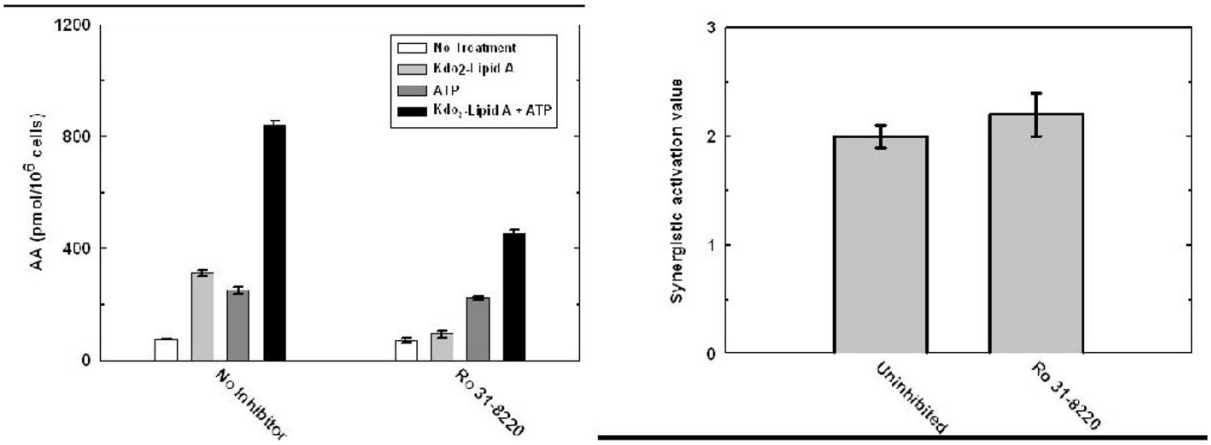

Figure 8. Inhibition of PKC blocks synergy-enhanced AA release, but does not reduce the synergistic activation value

A, AA release was measured from RAW 264.7 macrophages preincubated with and without 5 μM Ro 31-8220 prior to stimulation with and without 100 ng/mL Kdo_2-Lipid A for 50 minutes and with and without 2 mM ATP for 10 minutes (as indicated in the legend). The media was collected at 1 hour following the addition of the 100 ng/mL Kdo_2-Lipid A agonist and analyzed for AA release. *B*, Synergistic activation values were calculated for uninhibited cells and those preincubated with 5 μM Ro 31-8220. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.