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Phosphorylation of Cytosolic Group IV Phospholipase A_2 Is Necessary but Not Sufficient for Arachidonic Acid Release in $P388D_1$ Macrophages[1]

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Activation of the cytosolic Group IV phospholipase A_2 ($cPLA_2$) by agonists has been correlated with the direct phosphorylation of the enzyme by members of the mitogen-activated protein kinase (MAPK) cascade. Phosphorylation of the $cPLA_2$ increases the specific activity of the enzyme, thereby stimulating the arachidonic acid release. We show here, however, that conditions that lead to full phosphorylation of the $cPLA_2$ do not lead to enhanced AA release. As the above observations were made under both Ca^{2+}-dependent and Ca^{2+}-independent conditions, they emphasize that the current paradigm for activation of the $cPLA_2$ in cells involving both phosphorylation and Ca^{2+} is incomplete and that other factors should be taken into account.

Arachidonic acid (AA)[3] is the precursor of a variety of proinflammatory mediators including the prostaglandins and leukotrienes. Production of these potent substances is often controlled by the availability of free AA. The release of AA from its phospholipid storage sites is regulated by the activation of phospholipase A_2s (PLA_2) (1), a widespread phenomenon that occurs in almost all cell types.

Mammalian cells contain multiple forms of PLA_2 capable of effecting AA release (2, 3), and it has recently become evident that in many cases, more than one PLA_2 participates in this process (3). In $P388D_1$ macrophages, two different PLA_2s, namely the Group IV cytosolic PLA_2 ($cPLA_2$) and the Group V secretory PLA_2 ($sPLA_2$), have been found to coordinately regulate AA release in response to PAF receptor stimulation (4–9). In this particular system, $cPLA_2$ activation precedes and influences the subsequent activation of the $sPLA_2$, which is ultimately responsible for the bulk of AA release and prostaglandin production (4–9). Nonetheless, the fact that $cPLA_2$ activation provides stimulatory signals for the $sPLA_2$ to act, places the $cPLA_2$ as the key regulatory enzyme in AA signaling in these cells (3). The AA release mechanism operating in mast cells and other cell types (10–17) has now been shown to be very similar in several key respects to the response in $P388D_1$ cells.

Unlike the $sPLA_2$, the $cPLA_2$ is believed to be tightly regulated by receptor-activated phosphorylation cascades and the level of intracellular Ca^{2+} (18). The latter is not believed to be required for increasing $cPLA_2$ activity, but for binding of the enzyme to the membrane. Phosphorylation of the $cPLA_2$ at Ser^{505}, mediated by proline-directed kinases of the mitogen-activated protein kinase family (MAPK), actually serves to increase enzyme activity (18). Currently, it is believed that both kinds of signals independently act to fully activate the enzyme during receptor activation (18), but to what extent $cPLA_2$ phosphorylation is required is a matter of debate (18–22). In this sense the results reported herein demonstrate that both under Ca^{2+}-dependent and -independent conditions, $cPLA_2$ phosphorylation is necessary but not sufficient for AA release to occur.

EXPERIMENTAL PROCEDURES

Materials. Iscove's modified Dulbecco's medium (endotoxin <0.05 ng/ml) was from Whittaker Bioproducts (Walkersville, MD). Fetal bovine serum was from Hyclone Labs. (Logan, UT). Nonessential amino acids were from Irvine Scientific (Santa Ana, CA). [5,6,8,9,11,12,1,15-^{3}H]Arachidonic acid (specific activity 100 Ci/mmol) was obtained from New England Nuclear (Boston, MA). LPS Re595 and PAF were from Sigma (St. Louis, MO). Group IV $cPLA_2$

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[3] Abbreviations used: AA, arachidonic acid; PLA_2, phospholipase A_2; $cPLA_2$, Group IV cytosolic Ca^{2+}-dependent phospholipase A_2; $sPLA_2$, secretory phospholipase A_2; ERK, extracellular signal-regulated kinase (p42/p44 MAPK); MAPK, mitogen-activated protein kinase; PAF, platelet-activating factor; LPS, bacterial lipopolysaccharide.

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antibodies were kindly provided by Dr. Ruth Kramer (Lilly Research Laboratories, Indianapolis, IN). Phospho-specific p42/p44^{MAPK} (Thr^{202}/Tyr^{204}), phospho-specific p38^{MAPK} (Thr^{180}/Tyr^{182}) antibody and phospho-specific SAPK/JNK (Thr^{183}/Tyr^{185}) antibody were from New England Biolabs (Beverly, MA). Antibodies that recognize both the phosphorylated and nonphosphorylated forms of the above kinases were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and labeling conditions. P388D$_1$ cells (MAB clone) (23, 24) were maintained at 37°C in a humidified atmosphere at 90% air and 10% CO_2 in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and nonessential amino acids. Cells were plated at 10^6 per well, allowed to adhere overnight, and used for experiments the following day. All experiments were conducted in serum-free Iscove's modified Dulbecco's medium.

Stimulation of P388D$_1$ cells. Our standard regimen for activating the P388D$_1$ cells has been described previously (4, 5). Briefly, radiolabeling of the cells with [^{3}H]AA was achieved by including 0.5 μCi/ml [^{3}H]AA during the overnight adherence period (20 h). The cells were placed in serum-free medium for 30–60 min before the addition of LPS (200 ng/ml) for 1 h. After the LPS incubation the cells were exposed to PAF (Ca^{2+}-dependent stimulation) or UV light (mercury lamp at 366 nm; intensity 9.6 mJ s^{-1} cm^{-2}; Spectroline Corp., Westbury, NY) (Ca^{2+}-independent stimulation) for the time indicated, in the presence of 0.1 mg/ml bovine serum. The supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid scintillation counting. More than 99% of the released radioactive material remains as unmetabolized AA under these experimental conditions.

Immunoblotting studies. Cells, serum-starved for 24 h, were stimulated as described above. Afterwards, the cells were washed, and lysed in a buffer consisting of 1 mM Hepes, 0.5% Triton, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin at 4°C. Protein was quantified and a 100-μg aliquot was analyzed by Western blot with antibodies against the phosphorylated forms of p42/p44 MAPK, p38 MAPK, and SAPK/JNK and the cPLA$_2$.

RESULTS AND DISCUSSION

P388D$_1$ cells are unique among macrophages and macrophage cell lines in that they do not mobilize arachidonic acid or produce prostaglandins in response to many inflammatory stimuli to which other macrophages are highly responsive (25–28). They respond however to Ca^{2+}-mobilizing agonists such platelet-activating factor (PAF) (27) or Ca^{2+}-independent stimuli such as UV light (Balsinde *et al.*, manuscript in preparation) only if the cells are first primed with lipopolysaccharide, which itself is ineffective under the experimental conditions employed.

The mechanism through which PAF stimulates AA mobilization in LPS-treated P388D$_1$ cells has been shown to involve participation of both the Group IV cytosolic PLA$_2$ (cPLA$_2$) and the Group V secretory PLA$_2$ (sPLA$_2$) (4–9). By interacting with its receptor at the membrane, PAF generates a burst of intracellular Ca^{2+} that allows activation of the cPLA$_2$ in an intracellular compartment (4, 5). cPLA$_2$ activation, in concert with signals related to sphingolipid metabolism (8), can provide stimulatory signals for activation of the sPLA$_2$ at the cellular surface (4, 9). A striking feature of the

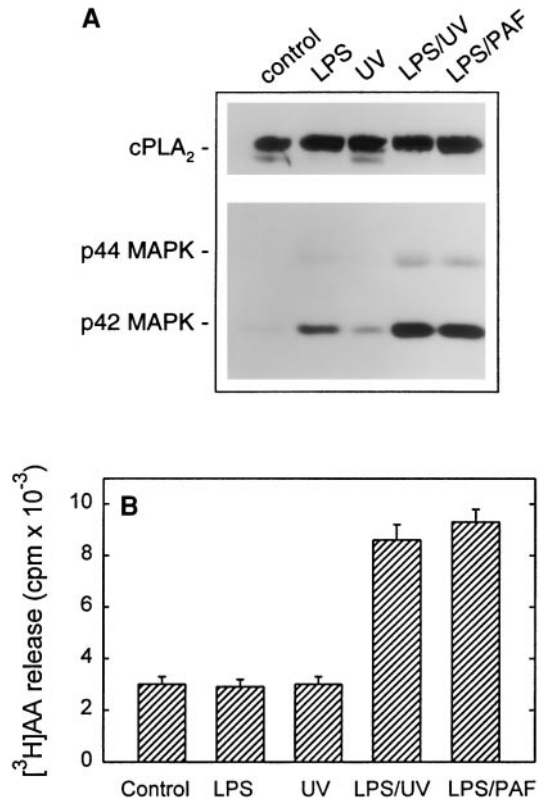

FIG. 1. (A) Phosphorylation of cPLA$_2$ and the ERKs (p42/p44^{MAPK}) by different stimuli in P388D$_1$ cells. Cells were incubated with LPS (200 ng/ml) for 1 h and then left untreated or exposed to UV radiation for 4 s or treated with 100 nM PAF for 10 min. Cells were lysed and protein was analyzed by immunoblot using antibodies against the cPLA$_2$ or the phosphorylated ERKs. (B) AA release in P388D$_1$ cells stimulated with different agonists. The [^{3}H]AA labeled cells were exposed to 100 ng/ml LPS for 1 h, UV light for 4 s, 100 ng/ml LPS for 1 h plus UV light for 4 s and 100 ng/ml LPS plus 100 nM PAF for 10 min. Afterwards the supernatants were collected and [^{3}H]AA release was determined by scintillation counting.

PAF-stimulated AA mobilization mechanism is that the sPLA$_2$ is responsible for most of the AA mobilized and PGE$_2$ produced; however sPLA$_2$ activity depends on previous cPLA$_2$ activation (4, 9). Therefore, cPLA$_2$ activation by PAF is the key regulatory step in the mechanism of AA mobilization in activated P388D$_1$ cells. Our recent investigations under Ca^{2+}-independent conditions, i.e., exposure of the cells to UV radiation have confirmed that, except for the Ca^{2+} transient, the mechanism involved is essentially the same, with the cPLA$_2$ being the key enzyme (Balsinde *et al.*, manuscript in preparation).

A major route for cPLA$_2$ regulation has long been believed to be the phosphorylation of the enzyme at Ser^{505} (18). This phosphorylation causes a characteristic retardation in the electrophoretic mobility of the enzyme on SDS gels, manifested by the appearance of a doublet (18). In resting unstimulated cells,

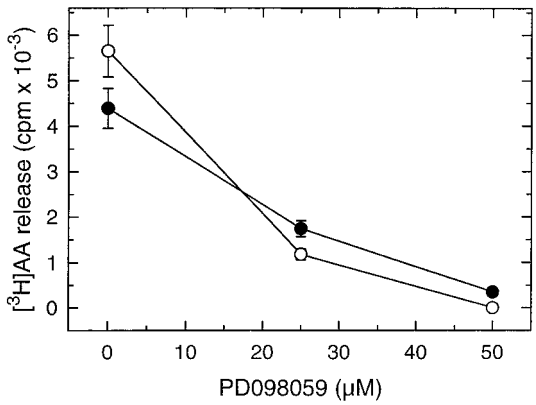

FIG. 2. Effect of the MAP kinase kinase inhibitor PD098059 on AA mobilization. The [^{3}H]AA-labeled cells were incubated with 200 ng/ml LPS for 1 h. Afterwards, the inhibitor (25 μM) was added. The cells were then exposed to UV light (open symbols) or 100 nM PAF (closed symbols) in the presence of 0.1 mg/ml bovine serum albumin. Extracellular AA release was quantitated as described under Experimental Procedures. To highlight the stimulated AA release, background values (i.e., those of unstimulated control cells) have been subtracted.

the slowly migrating cPLA$_2$ band (representing the phosphorylated form of the enzyme) represented about 70% of the protein (Fig. 1). Although LPS per se is unable to mobilize AA (25–28), the complete cPLA$_2$ mobility shift to the slowly-migrating form was detected when the cells were exposed to LPS (Fig. 1A). UV alone did not have any effect on the cPLA$_2$ mobility shift. Like LPS alone, combinations of LPS with either UV or PAF also showed complete retardation of the cPLA$_2$ (Fig. 1A). Unlike LPS alone however, the combined treatments LPS/UV or LPS/PAF leads to substantial AA release (Fig. 1B). Thus these results show a dissociation between the cPLA$_2$ mobility shift and AA release, thus demonstrating that increased cPLA$_2$ phosphorylation at Ser^{505} is not sufficient for increasing AA release in cells. Depending on cell type and stimulus, phosphorylation of the cPLA$_2$ at Ser^{505} appears to be mediated by distinct members of the MAPK family (18, 22, 29, 30). By using a specific antibody that recognizes the phosphorylated (i.e., activated) forms of the extracellular signal-regulated kinase (ERK) subfamily of MAPKs, we detected activation of both p42 MAPK and p44 MAPK in response to LPS alone (Fig. 1A), When UV radiation was applied to the cells, a slight increase in p42 MAPK phosphorylation was detected (Fig. 1A). A robust activation of both p42 MAPK and p44 MAPK was detected when LPS was combined with either UV or PAF (Fig. 1A).

The synergistic activation of the ERKs by LPS plus UV or PAF suggests that this is a key signaling event in the P388D$_1$ cells. Studies using the MAPK inhibitor PD098059 demonstrated complete inhibition of the AA release response and of p42/44 MAPK phosphorylation in response to both LPS/PAF and LPS/UV (Fig. 2). Because the phosphorylation reaction that promotes the cPLA$_2$ mobility shift is also reduced by PD098059 (32), we conclude that in the P388D$_1$ cells, cPLA$_2$ phosphorylation at Ser^{505} is mediated by the ERKs. Collectively these results, do suggest that the MAPK pathway plays a role in AA mobilization, but this role arises necessarily from its involvement in other signaling step(s) different from cPLA$_2$ phosphorylation at Ser^{505}.

In summary, we have shown that exposure of the cells to PAF—Ca^{2+}-mobilizing agonist—or to UV radiation—Ca^{2+}-independent stimulation—triggers a cPLA$_2$-dependent AA release response if the cells are previously exposed to LPS. Such an interplay between the actions of LPS and UV or PAF likely involves the synergistic activation of the ERKs, p42/p44 MAPK. However, the latter appears not to be a sufficient condition for activating the cPLA$_2$. Thus, these results challenge the currently accepted paradigm for cPLA$_2$ activation, and suggest that other signals in addition to MAPK and Ca^{2+} are involved.

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