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Cellular Regulation of Cytosolic Group IV Phospholipase A_2 by Phosphatidylinositol Bisphosphate Levels[1]

Jesús Balsinde,[2]* María A. Balboa,* Wen-Hong Li,[3] Juan Llopis,[4†] and Edward A. Dennis[2]*

Cytosolic group IV phospholipase A_2 ($cPLA_2$) is a ubiquitously expressed enzyme with key roles in intracellular signaling. The current paradigm for activation of $cPLA_2$ by stimuli proposes that both an increase in intracellular calcium and mitogen-activated protein kinase-mediated phosphorylation occur together to fully activate the enzyme. Calcium is currently thought to be needed for translocation of the $cPLA_2$ to the membrane via a C2 domain, whereas the role of $cPLA_2$ phosphorylation is less clearly defined. Herein, we report that brief exposure of $P388D_1$ macrophages to UV radiation results in a rapid, $cPLA_2$-mediated arachidonic acid mobilization, without increases in intracellular calcium. Thus, increased Ca^{2+} availability is a dispensable signal for $cPLA_2$ activation, which suggests the existence of alternative mechanisms for the enzyme to efficiently interact with membranes. Our previous in vitro data suggested the importance of phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) in the association of $cPLA_2$ to model membranes and hence in the regulation of $cPLA_2$ activity. Experiments described herein show that $PtdInsP_2$ also serves a similar role in vivo. Moreover, inhibition of $PtdInsP_2$ formation during activation conditions leads to inhibition of the $cPLA_2$-mediated arachidonic acid mobilization. These results suggest that cellular $PtdInsP_2$ levels are involved in the regulation of group IV $cPLA_2$ activation. ***The Journal of Immunology,*** **2000, 164: 5398–5402.**

Cytosolic group IV phospholipase A_2 ($cPLA_2$)[5] is a key effector of diverse pathways initiated by cytokines, growth factors, inflammatory mediators, hormones, and neurotransmitters (1, 2). The broad implication of $cPLA_2$ in cellular signaling arises from the fact that this enzyme, once activated, specifically releases arachidonic acid (AA) from membrane phospholipids (1, 2). This essential role of $cPLA_2$ in AA metabolism has been highlighted by recent experiments using $cPLA_2$ knockout mice (3, 4). Cells obtained from these animals generate significantly less AA-derived metabolites (3–5). Aside from its key role in inflammatory reactions as a precursor of the biologically active eicosanoids, AA has been recognized as an intracellular second messenger on its own, capable of activating a number of targets, including protein kinases and ion channels (6).

Regulation of $cPLA_2$ has been a key aspect in cellular signal transduction studies over the recent years. Results from these studies suggest a scenario for activation of $cPLA_2$ in which two different kinds of signals act in concert to elicit full enzyme activation. On one hand, an increase in intracellular Ca^{2+} results in the enzyme being translocated from cytosol to membrane fractions, where its substrate resides (1, 2). This process is made possible by the existence in the N-terminal half of the protein of a C2 domain, similar to the one present in many other proteins with key roles in cellular signaling such as protein kinase C (7). Thus Ca^{2+} is required for the $cPLA_2$ to act not because it is required for catalysis but because it appears to be essential for the enzyme to reach its substrate (8).

The second signal that is thought to act together with Ca^{2+} to promote full $cPLA_2$ activation is mediated by direct phosphorylation at Ser^{505} of $cPLA_2$ by members of the mitogen-activated protein kinase cascade (9). However, recent results have questioned the importance of mitogen-activated protein kinase-mediated phosphorylation of the $cPLA_2$ in terms of AA mobilization by showing that the latter response may indeed take place under circumstances where phosphorylation of the $cPLA_2$ at Ser^{505} is inhibited (10–12). Similarly, certain conditions that lead to full $cPLA_2$ phosphorylation at Ser^{505} do not result in an increased AA release response (13).

We have recently demonstrated that phosphatidylinositol bisphosphate ($PtdInsP_2$) strongly increases both $cPLA_2$ binding and activity toward phospholipid vesicles and mixed micelles. Interestingly, polyphosphoinositides decrease the requirement of $cPLA_2$ for Ca^{2+} such that under certain conditions $cPLA_2$ activity is truly Ca^{2+} independent (14). This is a remarkable finding because, as indicated above, an increase in intracellular Ca^{2+} levels is traditionally assumed to be the signal that allows the $cPLA_2$ to interact with the membrane and hence with its substrate (15). Our previous studies thus raised the very intriguing possibility that $PtdInsP_2$ might regulate a novel route for activation of the $cPLA_2$ in cells. These observations have prompted us to investigate the possible existence of such a route in cells. Data reported here demonstrate that cellular $PtdInsP_2$ levels do regulate $cPLA_2$ activation in a Ca^{2+}-independent manner.

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[5] Abbreviations used in this paper: $cPLA_2$, group IV cytosolic phospholipase A_2; AA, arachidonic acid; $PtdInsP_2$, phosphatidylinositol bisphosphate; PtdInsP, phosphatidylinositol phosphate; DBA, 2,3-dihydroxybenzaldehyde; MAFP, methyl arachidonyl fluorophosphonate; PAF, platelet-activating factor; $sPLA_2$, secretory PLA_2; $iPLA_2$, Ca^{2+}-independent PLA_2; BEL, bromoenol lactone.

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Materials and Methods

Reagents

The cell line used in this study, termed P388D_1/MAB, is a subclone of the P388D_1 cell line (TIB 63) obtained from the American Type Culture Collection (Manassas, VA), which was selected on the basis of high responsivity to LPS/platelet-activating factor (PAF) (16, 17). IMDM (endotoxin, <0.05 ng/ml) was obtained from BioWhittaker (Walkersville, MD). FBS was obtained from HyClone (Logan, UT). Nonessential amino acids were obtained from Irvine Scientific (Santa Ana, CA). [5,6,8,9,11,12,14,15-^{3}H]AA (sp. act., 100 Ci/mmol) was obtained from New England Nuclear (Boston, MA). LPS Re595 and PAF were obtained from Sigma (St. Louis, MO). Bromoenol lactone (BEL) and methyl arachidonyl fluorophosphonate (MAFP) were obtained from Biomol (Plymouth Meeting, PA). Rac1 and GTP were obtained from Calbiochem (La Jolla, CA).

Cell culture and labeling conditions

P388D_1 cells were maintained at 37°C in a humidified atmosphere at 80% air and 10% CO_2 in IMDM supplemented with 10% FBS, 2 mM glutamine, 100 U/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 IMDM.

Stimulation of P388D_1 cells

Our standard regimen for short-term activation of the P388D_1 cells has been described previously (18, 19). 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. 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 UV light (mercury lamp at 366 nm; intensity, 9.6 mJ/s · cm^2; Spectroline, Westbury, NY), PAF, or both for the time indicated in the presence of 0.1 mg/ml BSA. 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.

Intracellular Ca^{2+} determination

The cells, either LPS primed or unprimed, were loaded in HBSS containing 0.01% pluronic and 0.5 μM fura-2/AM for 30 min at room temperature. Cells were then exposed to UV light and/or PAF as indicated. Fluorescence Ca^{2+} images were obtained and calibrated as previously described (20). For experiments using fluo-3, a protocol identical to that described by Li et al. (21) was followed.

Permeabilization studies

The cells were permeabilized using 30 μM digitonin in a buffer consisting of 120 mM KCl, 30 mM NaCl, 10 mM PIPES, 1 mM KH_2PO_4, 1.03 $MgCl_2$, 0.0374 mM $CaCl_2$, and 1 mM EGTA, pH 7.0, to give a final free Ca^{2+} concentration of 15 nM (22). Immediately after adding the digitonin, the GTPase protein Rac was added, and the reactions proceeded for up to 10 min. Rac was loaded with GTP exactly as described by Hartwig et al. (23). Cell permeabilization was conducted at 37°C using adherent cells, and the total incubation time with digitonin did not exceed 10 min, as longer incubations with digitonin induced excessive detachment of the cells from the plastic culture dishes.

Determination of phosphoinositides

Cells labeled with 100 μCi/ml *myo*[^{3}H]inositol for 3 days were used. After the different treatments, the reactions were stopped and a lipidic fraction in chloroform was obtained as described (24). The chloroform was dried under a gentle stream of nitrogen, and the dried samples were applied to Silicagel G-60 TLC plates (Analtech, Newark, DE). The plates were coated with 1% potassium oxalate and heat-dried before sample application. Phospholipids were separated with chloroform/acetone/methanol/acetic acid/water (60:30:26:24:14) (25). The location of the different phosphoinositides was determined by running known standards on the same plate.

Results and Discussion

P388D_1 macrophages respond to LPS by releasing AA in a cPLA_2-dependent manner in a process that takes several hours to develop (16, 17). This process can be greatly accelerated if a Ca^{2+}-mobilizing stimulus such as PAF is added after 1 h of exposure to LPS.

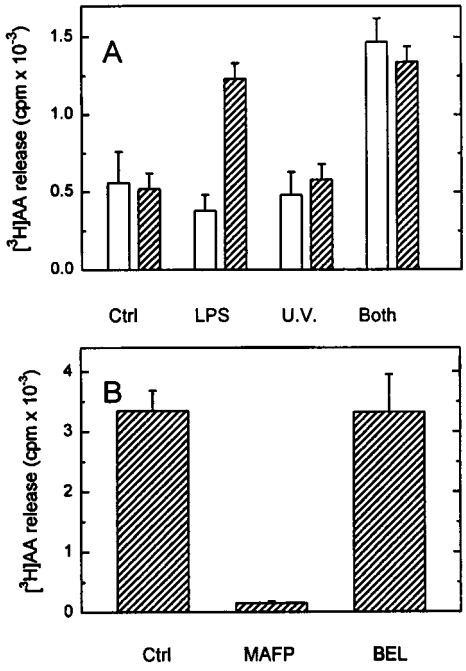

FIGURE 1. AA release in P388D_1 cells. *A*, The cells, labeled with [^{3}H]AA, were treated with LPS (200 ng/ml) for 1 h, UV light for 4 s, LPS for 1 h, followed by UV light (*Both*) or neither (*Ctrl*) as indicated. Afterward, the cells were incubated in the absence (□) or presence (▨) of 100 nM PAF for 10 min. The supernatants were then poured off and assayed for [^{3}H]AA release. *B*, Effect of PLA_2 inhibitors on AA release. The [^{3}H]AA-labeled cells were incubated with 200 ng/ml LPS for 1 h. Afterward, MAFP (25 μM), BEL (25 μM), or neither (*Ctrl*) were added, as indicated. After 15 min, the cells were then exposed to UV light for 4 s in the presence of 0.1 mg/ml BSA. Extracellular AA release was quantified as described under *Materials and Methods*.

Then, a robust AA release response is observed within 15 min of addition of PAF (26–28). Our investigations into the molecular mechanisms involved in the immediate AA release have highlighted the requirement for a rise in intracellular Ca^{2+} levels—an event that occurs within seconds after PAF addition—to fully activate the cPLA_2 (20, 28). Activation of the cPLA_2 constitutes the key step and is thought to regulate the recruitment of a novel group V secretory PLA_2 (sPLA_2) to the membrane, which ultimately results in an amplified release response (18, 19, 26).

We have now observed that AA release in LPS-treated macrophages could also be accelerated if the cells were briefly exposed to UV radiation (9.6 mJ/s · cm^2; 4 s) (Fig. 1*A*). Exposure of the LPS-treated cells to both PAF and UV did not have any effect beyond what was already induced by either of them alone (Fig. 1*A*). These data suggest that the signaling step targeted by the UV is probably the same as the one targeted by PAF. In accord with these observations, the cPLA_2 inhibitor MAFP completely abrogated the UV-induced AA release (Fig. 1*B*), indicating that the cPLA_2 is also under these settings a key component of the signaling cascade. MAFP has recently been shown to inhibit another intracellular PLA_2, i.e., the group VI Ca^{2+}-independent PLA_2 (iPLA_2) (29). The iPLA_2, but not the sPLA_2, is also strongly inhibited by BEL (18), a compound that had no measurable effect on the UV-triggered response (Fig. 1*B*). Therefore, the MAFP effects on AA release reported above are attributed to inhibition of the cPLA_2.

The above data indicate that both UV and PAF appear to signal through a common pathway involving cPLA_2, and we have previously shown that PAF signaling requires elevated Ca^{2+} (20, 28).

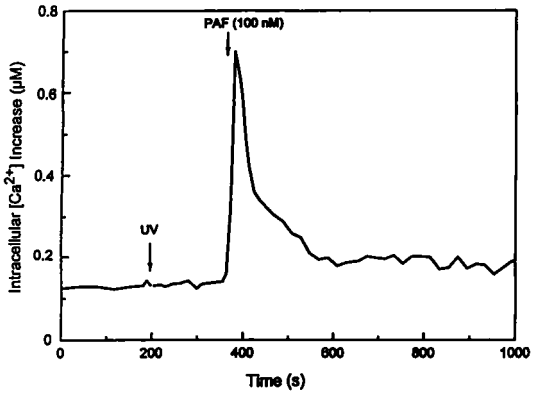

FIGURE 2. Effect of UV and PAF on intracellular Ca^{2+} rise. The LPS-treated cells, loaded with fura-2/AM, were exposed to UV for 4 s where indicated. Afterward, 100 nM PAF was added. The same profile was obtained when LPS-untreated cells were used.

Therefore, we would expect for UV to induce a transient increase in the intracellular Ca^{2+} concentration as well. Exposure of the cells, either untreated or LPS-treated, to UV did not alter the intracellular Ca^{2+} levels; however, subsequent addition of PAF did induce large alterations in the intracellular Ca^{2+} concentration, as measured with fura-2-loaded cells (Fig. 2). Identical results were obtained when fluo-3-loaded cells were used (not shown). Thus, unlike PAF, UV signaling does not involve an increase in the intracellular Ca^{2+} concentration, which demonstrates that increased Ca^{2+} mobilization is not a prerequisite for AA release to occur.

Several enzymes with key roles in cellular signaling that act on lipid surfaces, namely protein kinase C and Raf-1 kinase, dock to membranes via the "lipid anchors" diacylglycerol and phosphatidic acid, respectively (30, 31). We have recently shown that, in vitro, the cPLA$_2$ binds tightly and specifically to vesicles or micelles containing PtdInsP$_2$, resulting in dramatic increases in enzyme activity even at nanomolar Ca^{2+} levels, i.e., those present in unstimulated cells (14). Thus, higher levels of PtdInsP$_2$ in membranes targeted by the cPLA$_2$ as a consequence of cellular activation could result in increased amounts of enzyme bound to the membrane as well as increased enzyme activity (14).

To test the possibility that increased PtdInsP$_2$ levels could serve to anchor the cPLA$_2$ to membranes at resting cytosolic Ca^{2+} levels, we took advantage of the use of permeabilized cells. A useful approach to increase cellular PtdInsP$_2$ levels in the permeabilized cells is to add GTPase proteins that activate PtdInsP$_2$ synthesis (23, 32). In agreement with these previous observations, the addition of the GTPase protein Rac1 to the digitonin-permeabilized cells increased the cellular levels of PtdInsP$_2$ (Fig. 3*A*). Interestingly, this treatment also led to substantial release of AA to the incubation medium (Fig. 3*B*). The concentration-response curve of AA release corresponded well with that of PtdInsP$_2$ production (Fig. 3). Thus, these results suggest that increasing the concentration of PtdInsP$_2$ in the cells triggers an AA release response.

Fig. 4 shows that Ca^{2+}-independent activation of the macrophages by LPS and UV induced a time-dependent increase in both PtdInsP and PtdInsP$_2$ levels in cells prelabeled with [^{3}H]inositol (Fig. 4*A*). These changes were not observed if LPS-unprimed cells were used. Significant changes in the levels of phosphatidylinositol under these conditions could not be detected. Because under the LPS/UV stimulation conditions no Ca^{2+} mobilization occurs (see Fig. 2), such an elevation of PtdInsP$_2$ levels is unlikely to reflect any compensatory mechanism, but synthesis "on demand," i.e., as

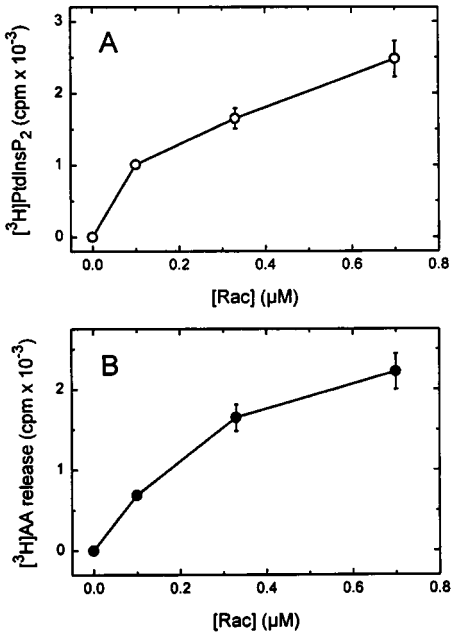

FIGURE 3. Rac1 effects on PtdInsP$_2$ production and AA mobilization. The GTPase protein Rac1 was added at the indicated concentrations to digitonin-permeabilized cells for 10 min. Afterward, PtdInsP$_2$ production (*A*) and AA release (*B*) were quantified as described in *Materials and Methods*.

a step of the LPS/UV signaling machinery. Increased PtdInsP$_2$ synthesis in the absence of intracellular Ca^{2+} increases is known to occur in cells treated with phorbol esters (33), and, coincidentally, phorbol esters are able to trigger the Ca^{2+}-independent

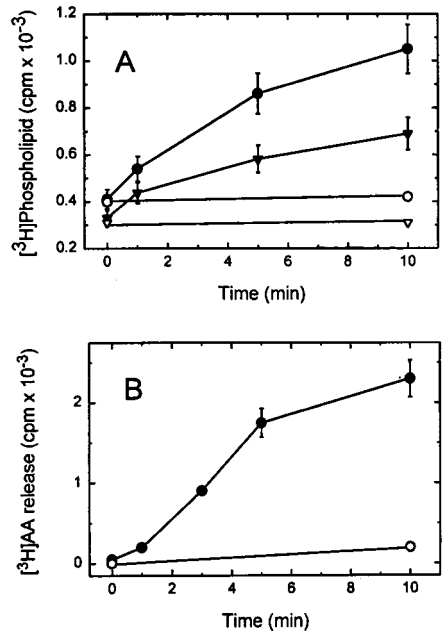

FIGURE 4. Phosphoinositide production and AA release by LPS/UV-treated cells. *A*, The LPS-treated cells, labeled with [^{3}H]inositol, were exposed (closed symbols) or not (open symbols) to UV for 4 s, and the incubations were allowed to proceed for the times indicated. PtdInsP$_2$ (circles) and PtdInsP (inverted triangles) levels were quantified by TLC. *B*, The LPS-treated cells, labeled with [^{3}H]AA, were exposed (closed symbols) or not (open symbols) to UV for 4 s, and the incubations were allowed to proceed for the times indicated. AA release was quantified as described in *Materials and Methods*.

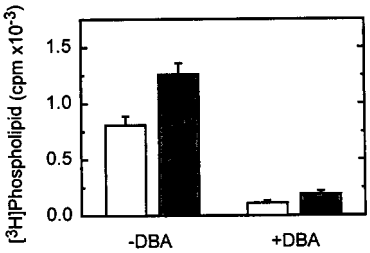

FIGURE 5. Effect of DBA on phosphoinositide levels. The cells, primed with 200 ng/ml LPS for 1 h, were exposed to UV for 4 s in the absence or presence of 25 μM DBA as indicated. The drug was added to the cells 30 min before the UV treatment. The incubations were then allowed to proceed for 10 min. PtdInsP (□) and PtdInsP$_2$ (▨) levels were quantified by TLC. To highlight the activated phosphoinositide production, unstimulated basal levels have been subtracted. These levels were 400 ± 100 cpm for PtdInsP and 650 ± 100 cpm for PtdInsP$_2$. DBA did not affect basal levels.

activation of cPLA$_2$ and concomitant AA release in certain cell types including macrophages (34). In this regard, the time course of AA release by LPS/UV, as shown in Fig. 4*B*, reflected well the time course of PtdInsP$_2$ production.

The UV-induced rises in PtdInsP$_2$ and PtdInsP could be inhibited by 2,3-dihydroxybenzaldehyde (DBA), a compound that has been shown to inhibit phosphatidylinositol 4-kinase (35, 36) (Fig. 5). DBA also blunted the UV-induced AA release (Fig. 6). At concentrations up to 50 μM, DBA had no direct effect on cPLA$_2$ activity from P388D$_1$ cell homogenates as measured toward 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine vesicles in the presence of 50 μM BEL (to block endogenous iPLA$_2$ activity (37)) and 2 mM 2-ME (to block endogenous sPLA$_2$ activity). Thus, these results directly link PtdInsP$_2$ levels with cPLA$_2$-mediated AA release under Ca^{2+}-independent activation conditions.

Collectively, the current results place the cPLA$_2$ among the growing list of proteins whose function and/or activity are regulated by PtdInsP$_2$ (38). Evidence has been presented for the existence of a route for cPLA$_2$ activation via PtdInsP$_2$ in which the final message is the mobilization of AA with Ca^{2+} levels equaling those of a quiescent cell. It should be noted that although cPLA$_2$ activation by UV light has previously been observed under biologically relevant settings (39), the use of UV light in our macrophage system should be contemplated as an experimental paradigm that allowed us to define a novel biochemical mechanism for AA mobilization. This mechanism allows one to explain the participation of cPLA$_2$ in cell regulation not involving Ca^{2+} signaling and solves the paradox of the involvement of cPLA$_2$ in the Ca^{2+}-independent delayed phase (hours) of eicosanoid generation that is characteristic of immunoinflammatory cells (16, 40).

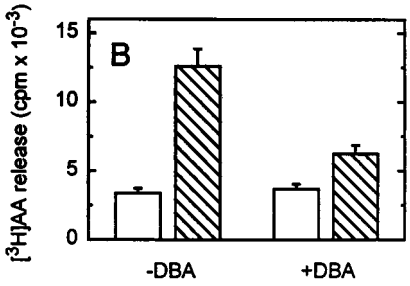

FIGURE 6. Effect of DBA on AA release. The cells, primed with 200 ng/ml LPS for 1 h, were exposed (▧) or not (□) to UV for 4 s in the absence or presence of 25 μM DBA, as indicated. The incubations were then allowed to proceed for 10 min. AA release was quantified as described in *Materials and Methods*.

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