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Group V Phospholipase A_2-mediated Oleic Acid Mobilization in Lipopolysaccharide-stimulated $P388D_1$ Macrophages*

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$P388D_1$ macrophages prelabeled with [^{3}H]arachidonic acid (AA) respond to bacterial lipopolysaccharide (LPS) by mobilizing AA in a process that takes several hours and is mediated by the concerted actions of the group IV cytosolic phospholipase A_2 and the group V secretory phospholipase A_2 ($sPLA_2$). Here we show that when the LPS-activated cells are prelabeled with [^{3}H]oleic acid (OA), they also mobilize and release OA to the extracellular medium. The time and concentration dependence of the LPS effect on OA release fully resemble those of the AA release. Experiments in which both AA and OA release are measured simultaneously indicate that AA is released 3 times more efficiently than OA. Importantly, LPS-stimulated OA release is strongly inhibited by the selective $sPLA_2$ inhibitors 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxy)propane sulfonic acid and carboxymethylcellulose-linked phosphatidylethanolamine. The addition of exogenous recombinant $sPLA_2$ to the cells also triggers OA release. These data implicate a functionally active $sPLA_2$ as being essential for the cells to release OA upon stimulation with LPS. OA release is also inhibited by methyl arachidonyl fluorophosphonate but not by bromoenol lactone, indicating that the group IV cytosolic phospholipase A_2 is also involved in the process. Together, these data reveal that OA release occurs during stimulation of the $P388D_1$ macrophages by LPS and that the regulatory features of the OA release are strikingly similar to those previously found for the AA release.

Using the murine macrophage-like cell line $P388D_1$, we have recently shown that arachidonic acid (AA)[1] mobilization and prostaglandin production stimulated by platelet-activating factor and/or lipopolysaccharide (LPS) involves the participation of three effectors, namely group IV cytosolic PLA_2 ($cPLA_2$), secretory group V PLA_2 ($sPLA_2$), and COX-2. In this system, the $cPLA_2$ fundamentally plays a regulatory role, whereas the $sPLA_2$ plays an augmentative role by providing most of the AA metabolized by COX-2 (1–6).

The different roles for both $cPLA_2$ and $sPLA_2$ during stimulus-response coupling have now been recognized in a number of different systems (7–14). Interestingly, in some instances the $sPLA_2$ involved is not a group V $sPLA_2$ but rather a closely related group IIA enzyme (15). Nevertheless, group IIA $sPLA_2$ appears to serve in the same augmentative role (9, 10, 16, 17).

Recently, a surface receptor that recognizes certain $sPLA_2$ forms with high affinity has been cloned (18). In line with the existence of putative $sPLA_2$ receptors, it has been suggested that $sPLA_2$-mediated AA release in some systems may not involve the hydrolytic activity of the $sPLA_2$. Rather, the $sPLA_2$ would act as a ligand-like agonist that stimulates the $cPLA_2$ for an increased AA release response (19–21). A major argument in favor of the above scenario is the finding that no fatty acids other than AA are detected in the extracellular medium (19–21). Specific AA release would be inconsistent with the involvement of a $sPLA_2$, since this enzyme shows little or no fatty acid preference (22).

Since the augmentative role that group V $sPLA_2$ plays in LPS-activated $P388D_1$ macrophages appears to depend on enzyme activity (5, 6), we have now examined the hypothesis of whether these cells mobilize other fatty acids in addition to AA. Our results show that the activated cells do release measurable amounts of oleic acid (OA), that this release appears to be due to the hydrolytic action of the $sPLA_2$ acting on the cellular surface, and that the regulatory features of the OA release are strikingly similar to those previously found for the AA release.

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 Laboratories (Logan, UT). Nonessential amino acids were from Irvine Scientific (Santa Ana, CA). [9,10-^{3}H]Oleic acid (specific activity 55 Ci/mmol) and [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (specific activity 100 Ci/mmol) were from NEN Life Science Products. [1-^{14}C]Oleic acid (specific activity 56 mCi/mmol) was from Amersham Pharmacia Biotech. LPS (*E. coli* 0111:B4) was from Sigma. Methyl arachidonyl fluorophosphonate (MAFP) and (*E*)-6(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one (bromoenol lactone (BEL)) were from Biomol (Plymouth Meeting, PA). The $sPLA_2$ inhibitor 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxy)propane sulfonic acid (LY311727) was generously provided by Dr. Edward Mihelich (Lilly). Human recombinant group V $sPLA_2$ was produced in our laboratory utilizing the *Pichia pastoris* expression system (6). The $sPLA_2$ inhibitor CMPE (phosphatidylethanolamine covalently linked to carboxymethylcellulose) was synthesized in Dr. Yedgar's laboratory by Arie Dagan and Miron Krimsky (23).

Cell Culture and Labeling Conditions—$P388D_1$ cells (MAB clone) (5, 6) 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. $P388D_1$ cells were plated at 10^6/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. When required, radiolabeling of

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[1] The abbreviations used are: AA, arachidonic acid; OA, oleic acid; PLA_2, phospholipase A_2, $cPLA_2$, group IV cytosolic PLA_2; $sPLA_2$, secretory PLA_2; $iPLA_2$, group VI Ca^{2+}-independent PLA_2; MAFP, methyl arachidonyl fluorophosphonate; BEL, bromoenol lactone; CMPE, phosphatidyletanolamine linked to carboxymethyl cellulose; LY311727, 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxy)propane sulfonic acid; LPS, lipopolysaccharide.

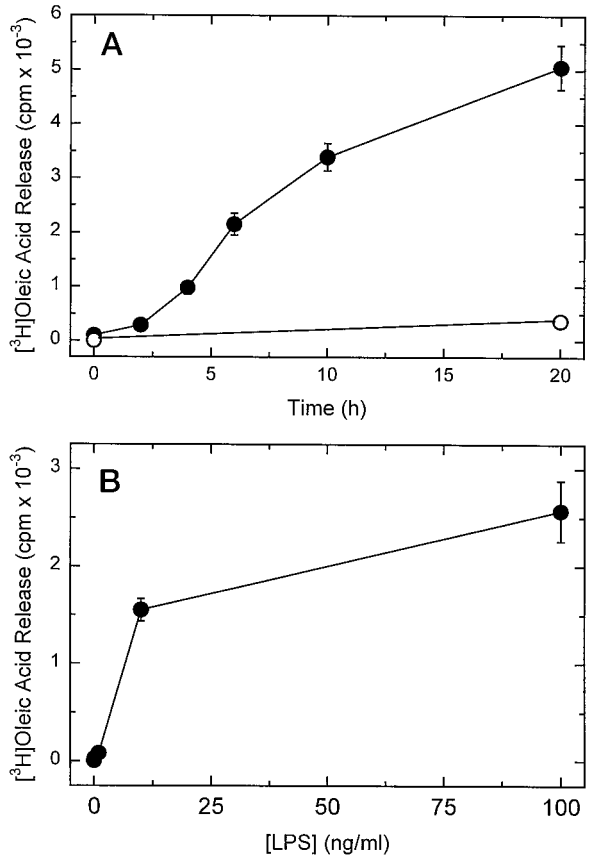

FIG. 1. **LPS-stimulated [^{3}H]OA release in P388D$_1$ macrophages.** *A*, time course of [^{3}H]OA release upon stimulation with 100 ng/ml LPS (*closed circles*) and in the absence of stimulation (*open circles*). *B*, dose response of the LPS effect (20-h incubation).

the P388D$_1$ cells was achieved by including 0.5 μCi/ml [^{3}H]OA or 0.1 μCi/ml [^{14}C]OA plus 0.5 μCi/ml [^{3}H]AA during the overnight adherence period (20 h). Labeled fatty acid that had not been incorporated into cellular lipids was removed by washing the cells six times with serum-free medium containing 1 mg/ml albumin.

Measurement of Extracellular Fatty Acid Release—The cells were placed in serum-free medium for 30 min before the addition of LPS or exogenous sPLA$_2$ for different periods of time in the presence of 0.5 mg/ml bovine serum albumin. The supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid scintillation counting. When inhibitors were used, they were added 30 min before the addition of LPS.

Data Presentation—Except for the data in Fig. 2, which are given as percentage of release with respect to total cellular radioactivity levels, agonist-stimulated OA release is expressed by subtracting the basal rate observed in the absence of agonist and inhibitor. These background values were in the range of 1000–2000 cpm. Each set of experiments was repeated at least three times with similar results. Unless otherwise indicated, the data presented are from representative experiments.

RESULTS

OA Release in LPS-stimulated P388D$_1$ Cells—We have previously shown that exposure of P388D$_1$ macrophages (MAB clone) to LPS induces a concentration-dependent release of AA to the extracellular medium that spans several hours (5). We began the current study by determining whether LPS was able to cause the extracellular release of OA as well. To this end, the cells, labeled with 0.5 μCi/ml [^{3}H]oleic acid, were exposed to different concentrations of LPS for various periods of time. As shown in Fig. 1, LPS did induce a time- (Fig. 1*A*) and concentration- (Fig. 1*B*) dependent release of [^{3}H]OA from the cells. As shown in Fig. 1*A*, the kinetics of the LPS effect on OA release was very similar to that previously found for the LPS-induced AA release (5). Thus, after a lag of about 3 h, OA release proceeded linearly up to about 10 h, after which it continued at a slower rate. The concentration dependence of the LPS-induced OA equally resembled that of the LPS-induced AA release (5).

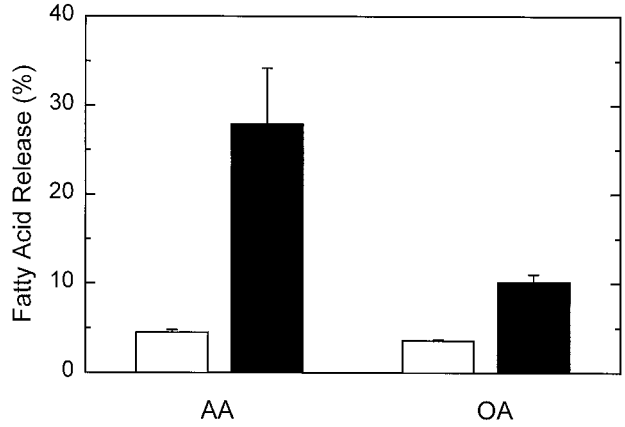

FIG. 2. **Simultaneous measurement of OA and AA in activated P388D$_1$ macrophages.** The cells, prelabeled with [^{3}H]AA and [^{14}C]OA, were stimulated with 100 ng/ml LPS for 20 h (*closed bars*). Afterward, the supernatants were collected and assayed for ^{3}H radioactivity (AA release) and ^{14}C radioactivity (OA release). *Open bars* denote control incubations (*i.e.* those that did not receive LPS).

By simultaneously labeling the cells with [^{3}H]AA and [^{14}C]OA, it was possible to measure under identical settings the release of these two fatty acids in response to LPS. To allow for a direct comparison, the results are given as the percentage of labeled fatty acid incorporated into cells that is released. Fig. 2 shows that despite the fact that the LPS-activated cells released OA to a significant level (2-fold above basal), AA was released about 3 times more efficiently.

PLA$_2$ Inhibition Studies—To address the involvement of the different PLA$_2$ forms in LPS-induced OA release, we first utilized MAFP (2), a dual cPLA$_2$/iPLA$_2$ inhibitor that has previously been found to block the cPLA$_2$-dependent release of AA from LPS-stimulated P388D$_1$ macrophages (2, 5). As shown in Fig. 3*A*, MAFP strongly blocked the LPS-induced [^{3}H]OA release.

iPLA$_2$ involvement was studied with BEL, a compound that manifests a 1000-fold selectivity for inhibition of the iPLA$_2$ *versus* the cPLA$_2$ *in vitro* (2). As shown in Fig. 3*B*, BEL had no measurable inhibitory effect on LPS-induced [^{3}H]OA release. Nevertheless, it completely inhibited iPLA$_2$ activity in homogenates prepared from LPS-treated cells (not shown). In turn, these data indicate that the effects of MAFP shown above are due to inhibition of the cPLA$_2$.

To assess the involvement of sPLA$_2$, we utilized two structurally unrelated inhibitors, namely LY311727 and CMPE (Fig. 4). The first compound is an indole derivative (24), and the second one is composed of *N*-derivatized phosphatidylethanolamine covalently linked via the head group to carboxymethyl cellulose (23). Both of these compounds strongly inhibited [^{3}H]oleic acid release (Fig. 4). When, in the experiment shown in Fig. 4*B*, carboxymethyl cellulose alone was added instead of CMPE, no effect on LPS-induced [^{3}H]OA release was observed at all (data not shown).

Exogenous Group V sPLA$_2$ Triggers OA Release—CMPE is a cell-impermeable inhibitor that prevents the sPLA$_2$ from attacking the phospholipids on the outer surface (23). Thus, the data shown in Fig. 4*B* imply that the extracellular sPLA$_2$ pool is the one that participates in OA release in the LPS-treated cells. Given that group V sPLA$_2$ is active *per se* toward cell membranes (*i.e.* no "membrane rearrangement" is needed for this enzyme to attack the outer membrane) (6, 25), we reasoned

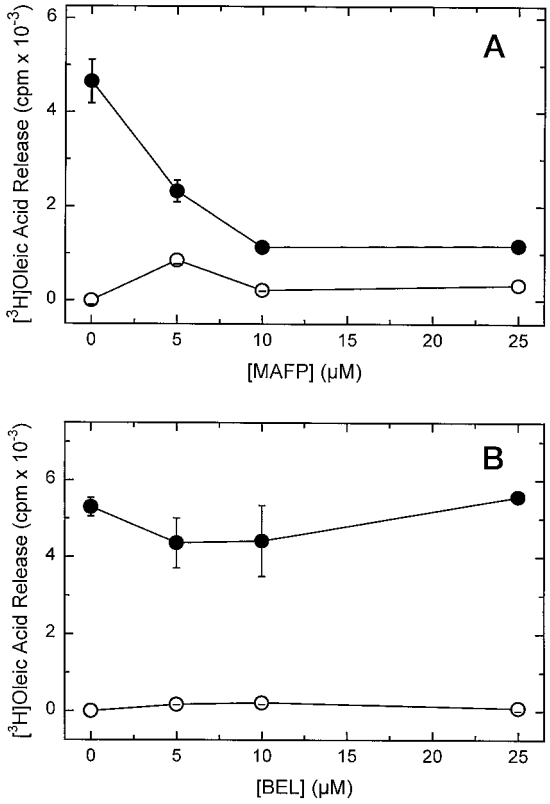

FIG. 3. **Effect of MAFP and BEL on LPS-induced OA release.** The cells were treated with the indicated concentrations of MAFP (*A*) or BEL (*B*) for 30 min before the addition of 100 ng/ml LPS (*closed symbols*), and the incubations proceeded for 20 h. *Open circles* denote control incubations (*i.e.* those that did not receive LPS).

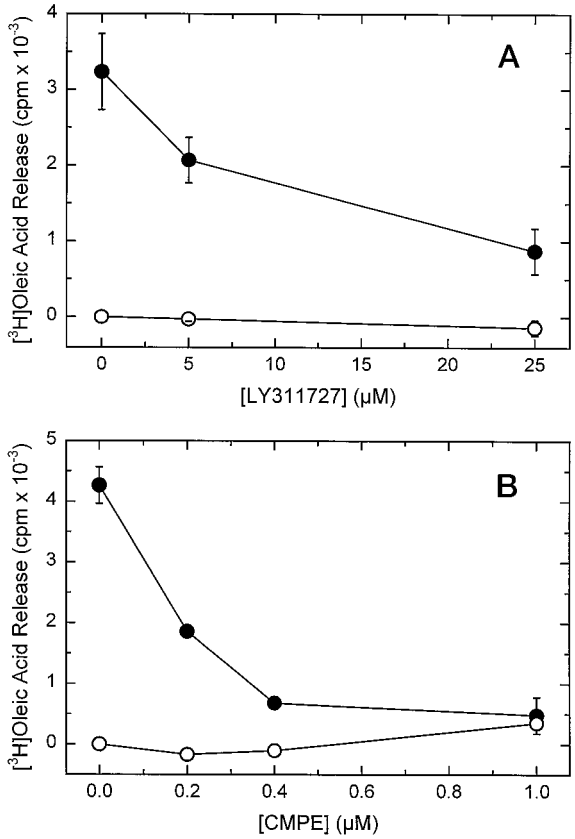

FIG. 4. **Effect of LY311727 and CMPE on LPS-induced OA release.** The cells were treated with the indicated concentrations of LY311727 (*A*) or CMPE (*B*) for 30 min before the addition of LPS (*closed symbols*), and the incubations were allowed to proceed for 20 h. *Open circles* denote control incubations (*i.e.* those that did not receive LPS).

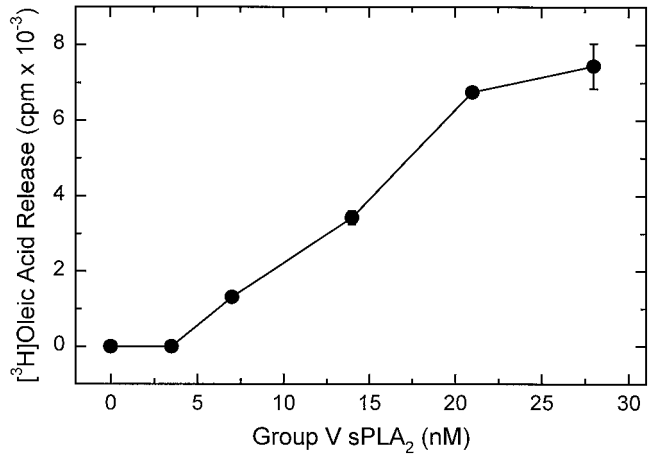

FIG. 5. **Effect of exogenous group V sPLA$_2$ on OA release.** The cells were treated with the indicated concentrations of recombinant group V sPLA$_2$ for 1 h. Afterward, supernatants were assayed for [^{3}H]OA release.

that the addition of exogenous group V sPLA$_2$ to the macrophages should result in an enhanced release of OA to the extracellular medium. This is exactly what happened in the experiment shown in Fig. 5.

DISCUSSION

Recent work by several laboratories has highlighted the importance of sPLA$_2$ (either group V or group IIA) in AA mobilization and attendant prostaglandin formation (26, 27). The sPLA$_2$ is thought to amplify the AA release signal initiated by the cPLA$_2$ to generate large amounts of free AA, part of which will eventually be converted into eicosanoids (26, 27).

That the sPLA$_2$ plays merely a hydrolytic role in the process of AA release has been argued against recently on the basis that AA mobilization in some cell types appears to be highly specific for AA (*i.e.* no release of other fatty acids is detected) (19–21). The latter finding would be inconsistent with the hydrolytic action of an enzyme such as the sPLA$_2$, which shows no fatty acid preference (22). Thus, an alternative explanation has been proposed that involves the sPLA$_2$ acting as a ligand-like molecule independent of enzyme activity. According to this hypothesis, the sPLA$_2$ acts as a a receptor-directed agonist that stimulates the selective release of AA via cPLA$_2$ activation. In addition to the lack of release of fatty acids other than AA (19–21), this hypothesis is also supported by data showing that sPLA$_2$s from different sources that have been rendered catalytically inactive by inhibitors are still able to elicit the AA release (20, 21).

In contrast, a large number of studies have shown that sPLA$_2$ inhibitors markedly diminish the release of AA (2, 5, 8, 10, 14, 28–31), thus supporting a hydrolytic role for the sPLA$_2$ in the process. Moreover, we (6) have recently found that the addition of exogenous group V sPLA$_2$ to the cells induces an AA release response that is not observed if chemically inactivated enzyme is used. In agreement with our data, Tada *et al.* (8) have found that catalytically inactive group IIA sPLA$_2$ mutants are incapable of promoting AA release from cytokine-primed cells.

The current results clearly show that the LPS-activated P388D$_1$ macrophages do release OA and that the regulatory features of the OA release are strikingly similar to those found previously for the AA release (2). Simultaneous measurement of the OA release *versus* AA release revealed that the activated

cells appear to release AA in preference over OA, which is fully consistent with recent data of Murakami and colleagues (10, 17). However, it is important to note that OA release was not detected in these previous studies, or it was detected at a very low level (10, 17). In contrast, we show in this study that OA release in the LPS-activated cells is actually quite significant (2-fold over basal).

LPS-activated OA release can be blocked by sPLA$_2$ inhibitors, which implies that a catalytically active sPLA$_2$ is needed for the OA release to occur. Hence, in the LPS-activated cells, AA-containing phospholipids are not the only substrates for the sPLA$_2$. Thus, it is tempting to speculate that the fatty release observed in cells may reflect the fatty acid composition of the specific phospholipid pools that come in contact with the sPLA$_2$. sPLA$_2$ docking in a membrane domain highly enriched in AA-containing phospholipids could explain why this enzyme appears to release AA in preference to other fatty acids *in vivo*.

Importantly, at least one of the sPLA$_2$ inhibitors utilized in this study, CMPE, is cell-impermeable. CMPE anchors to the extracellular leaflet of the plasma membrane by its phospholipid moiety, thereby protecting the membrane from the hydrolytic action of the sPLA$_2$ (23). From the results obtained with this compound, it can be concluded that the sPLA$_2$ pool involved in the OA release is the one on the cellular surface, because if the sPLA$_2$ were acting inside the cell, CMPE would not have had any effect on the release. Similar to OA release, we have observed that CMPE also strongly blunts AA release in the LPS-activated P388D$_1$ cells,[2] which also points at the cell surface as the site for sPLA$_2$-dependent AA mobilization. In agreement with these observations, we show that exogenous sPLA$_2$ is able to induce both AA (6) and OA (this study) release from the P388D$_1$ cells.

Our inhibitor studies indicate that, in addition to the sPLA$_2$, OA release in the LPS-activated P388D$_1$ cells also involves the cPLA$_2$. Since the cPLA$_2$ is highly AA-specific, it appears very unlikely that this enzyme contributes to the OA release by directly cleaving OA-containing phospholipids. We (5) have previously demonstrated that a catalytically active cPLA$_2$ is required for the cells to show enhanced expression of sPLA$_2$ in response to LPS. Thus, inhibition of OA release by cPLA$_2$ inhibitors most likely reflects the diminished capacity of the cells to synthesize sPLA$_2$, in an analogous manner to what we (5) and others (13) have previously described for the AA release.

P388D$_1$ macrophages contain a third PLA$_2$ type, the group VI iPLA$_2$. Given that iPLA$_2$ is not fatty acid-selective, a possible role for this enzyme in OA mobilization could in principle be envisioned. However, in analogy again with the AA release, we have failed to detect any role for this enzyme in the LPS-induced OA release. Under conditions wherein cellular iPLA$_2$ is completely inhibited by BEL, no effect on LPS-stimulated OA release is observed. In agreement with our data, a recent study by Ito *et al.* (32) also failed to detect any effect of BEL on OA release. Thus, these data lend further support to the idea that the group VI iPLA$_2$ may not be involved in cellular signaling in P388D$_1$ macrophages (33). In support of this view, elegant studies by Murakami *et al.* (10) have shown that overexpression of iPLA$_2$ in 293 fibroblasts does not modify the AA release response triggered by interleukin-1, but overexpression of either cPLA$_2$, group IIA sPLA$_2$, or group V sPLA$_2$ does result in all cases in an increased AA release in response to interleukin-1 (10).

In summary, we have found that LPS-stimulated P388D$_1$ macrophages release OA by a mechanism that involves the hydrolytic actions of both group V sPLA$_2$ and cPLA$_2$ and appears to be strikingly similar to the one previously described for AA release. The one other PLA$_2$ present in these cells (*i.e.* the group VI iPLA$_2$) appears not to be required for stimulated release.

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[2] J. Balsinde and E. A. Dennis, unpublished observation.

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