Intracellular Ca^{2+}, inositol 1,4,5-trisphosphate and additional signalling in the stimulation by platelet-activating factor of prostaglandin E_2 formation in P388D_1 macrophage-like cells

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In the P388D_1 macrophage-like cell line, phospholipase A_2 activity and prostaglandin production are stimulated by platelet-activating factor (PAF) and bacterial lipopolysaccharide (LPS). We have investigated the role of Ins(1,4,5)P_3 and Ca^{2+} in signal transduction of PAF-induced prostaglandin E_2 (PGE_2) formation in these cells. The role of Ca^{2+} in the activation mechanism was studied by fluorescence imaging of intracellular Ca^{2+} in individual adherent cells and by determining the PGE_2 production in the same population of cells. This new approach enabled us to correlate directly events on the single-cell level with a physiologically relevant response of the cell population. Priming the cells with LPS was required for PAF to stimulate PGE_2 formation, yet LPS affected neither the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) nor the PAF-induced rise in $[Ca^{2+}]_i$. In addition, basal and PAF-stimulated Ins(1,4,5)P_3 levels were not affected by LPS priming. However, the Ca^{2+} transient, the release of Ins(1,4,5)P_3 and the formation of PGE_2 induced by PAF were inhibited in cells pretreated with pertussis toxin. Buffering the $[Ca^{2+}]_i$ with intracellular BAPTA [bis-(*o*-aminophenoxy)ethane-*NNN'N'*-tetra-acetic acid] blocked the PAF-stimulated rise in $[Ca^{2+}]_i$ and PGE_2 formation. Removal of extracellular Ca^{2+} during PAF stimulation prevented the influx of Ca^{2+}, but did not affect the initial $[Ca^{2+}]_i$ transient, nor did it inhibit PGE_2 formation. Under the same conditions, ionomycin stimulated an identical $[Ca^{2+}]_i$ transient, but, in contrast with PAF stimulation, no PGE_2 formation was observed. PGE_2 production could be rescued by prompt subsequent addition of PAF, which caused no further $[Ca^{2+}]_i$ change on its own. These results show that the transient initial rise in $[Ca^{2+}]_i$, produced either by PAF via the formation of Ins(1,4,5)P_3 or directly by ionomycin, is necessary, but not sufficient for the formation of PGE_2 in LPS-primed P388D_1 cells. Furthermore, we have demonstrated for the first time that PAF triggers a second signal that is not mediated by a change in $[Ca^{2+}]_i$. However, both signals are required to induce PGE_2 formation.

INTRODUCTION

Stimulation of macrophages by various factors such as platelet-activating factor (PAF) leads to the release of arachidonic acid and to the formation of eicosanoids, which play key roles in many aspects of acute and chronic inflammation [1]. The rate-limiting step in the biosynthesis of eicosanoids is thought to be the release of arachidonic acid from membrane phospholipids by phospholipase A_2 (PLA_2) [2]. In the macrophage-like cell line P388D_1, various PLA_2 activities have been identified and characterized [3–5]. Inhibitor studies on an isolated Ca^{2+}-dependent membrane-associated PLA_2 *in vitro* and in intact cells have correlated PLA_2 activity, arachidonic acid release and the formation of prostaglandin E_2 (PGE_2) in P388D_1 cells [5–7]. PLA_2 activities have also been demonstrated in many tissues and cells, including macrophages and other monocytic cell lines. Recently, a new type of Ca^{2+}-dependent cytosolic PLA_2 was described [8,9]; however, its role during physiological stimulation has yet to be demonstrated. The signalling pathways by which PLA_2 are activated or regulated and the function of these enzymes in intact cells remain to be elucidated.

The variety of PLA_2 activities in P388D_1 cells make this particular cell system a unique and attractive model in which to study these signalling events. We have demonstrated that bacterial lipopolysaccharides (LPS) prime P388D_1 cells for an enhanced [^{3}H]arachidonic acid release and subsequent PGE_2 formation upon stimulation with PAF [7]. Although LPS priming appears to involve transcriptional events, protein synthesis is required for primed PAF stimulation of PGE_2 secretion. Furthermore, PAF-stimulated PGE_2 formation is inhibited by genistein, an inhibitor of tyrosine-specific protein kinases (TPK), but it is insensitive to treatment with H-7, an inhibitor of protein kinase C. Also, P388D_1 cells do not release PGE_2 upon stimulation with oleoylacetylglycerol [7]. These results suggest a role for TPK in the PAF-induced activation of PLA_2, whereas protein kinase C appears not to be involved.

We have now focused our studies on the early events in PAF-induced cell stimulation leading to the activation of PLA_2. In the present study, we have investigated the release of Ins(1,4,5)P_3 and the role of intracellular Ca^{2+} in the PAF-induced activation of PLA_2.

Abbreviations used: LPS, bacterial lipopolysaccharide; PAF, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); PLA_2, phospholipase A_2; PLC, phospholipase C; BAPTA, bis-(o-aminophenoxy)ethane-*NNN'N'*-tetra-acetic acid; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; PGE_2, prostaglandin E_2; TPK, tyrosine-specific protein kinases; DPBS, Dulbecco's phosphate-buffered saline; HBS, Hanks' buffered saline.

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EXPERIMENTAL

Materials

P388D$_1$ cells (TIB 63) were obtained from the American Type Culture Collection (Bethesda, MD, U.S.A.). Iscove's modified Dulbecco's medium (endotoxin < 0.05 ng/ml) was from Whittaker Bioproducts (Walkersville, MD, U.S.A.). Fetal bovine serum (lot no. 1111-1096) was from Hyclone Laboratories (Logan, UT, U.S.A.). Non-essential amino acids were from Irvine Scientific (Santa Ana, CA, U.S.A.). PAF (prepared from bovine heart lecithin), the Ca^{2+} ionophore ionomycin and gentamycin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dulbecco's phosphate-buffered saline (DPBS), minimum essential medium, Hanks' buffered saline (HBS) and 2-mercaptoethanol were from Gibco BRL (Grand Island, NY, U.S.A.). Tyrphostin 25 was from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). BAPTA/AM [bis-(*o*-aminophenoxy)ethane-*NNN'N'*-tetra-acetic acid, acetoxymethyl ester] was from Calbiochem (La Jolla, CA, U.S.A.). Trioctylamine and 1,1,2-trichloro-1,2,2-trifluoroethane were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). LPS from *Salmonella* Re595 was kindly provided by Dr. R. J. Ulevitch, Scripps Clinic and Research Foundation (La Jolla, CA, U.S.A.). Fura-2/AM was from Molecular Probes (Eugene, OR, U.S.A.). Pluronic was from BASF Corp. (Wyandotte, MI, U.S.A.).

Cell culture

P388D$_1$ cells were maintained at 37 °C in a humidified atmosphere of air/CO_2 (9:1) in Iscove's modified Dulbecco's medium with 10% fetal-bovine serum (heat-inactivated), glutamine (4 mM), gentamycin (50 μg/ml) and supplemented with non-essential amino acids. Adherent cells were selected by passage of only adherent cells. Cells were maintained in 175 cm^2 flasks and passaged every 3–4 days (doubling time ~ 30 h) by scraping from the flask with a Nunc (Naperville, IL, U.S.A.) cell scraper. At least 10 passages were performed before the cells were plated for an experiment. Cells were not used after passage 30, after which their doubling time increases and their adherency decreases. For the standard experimental set-up, adherent cells were scraped and plated in six-well dishes (Falcon) at 1×10^6 cells/well. For Ca^{2+} determination, adherent cells were scraped and plated in 25 mm Petri dishes in which the centre portion of the bottom had been replaced by glass coverslips attached with vacuum grease. Cells were allowed to adhere overnight in Iscove's modified Dulbecco's medium with 10% fetal-bovine serum, penicillin G/streptomycin (100 units/ml and 100 μg/ml respectively) and supplemented with non-essential amino acids. All experiments were performed in serum-free Iscove's modified Dulbecco's medium with penicillin G (100 units/ml)/streptomycin (100 μg/ml), glutamine (4 mM) and supplemented with non-essential amino acids (Buffer A).

Experimental protocol

For standard conditions, P388D$_1$ cells at 1×10^6 cells/well were placed in buffer A for 30 min (wash no. 1) before treatment with LPS (100 ng/ml) for 1 h (priming step). After LPS priming, cells were washed with buffer A for 30 min (wash no. 2) and then stimulated with PAF (20 nM), unless stated otherwise, for 20 min at 37 °C (stimulation step). The PGE_2 levels in the media or cellular Ins(1,4,5)P_3 levels were then determined as described below. Inhibitors were present during wash no. 2 and the stimulation step unless stated otherwise. For all experiments that required a 4 h preincubation with pertussis toxin, this preincubation step was introduced after wash no. 1 and before the priming step. The volumes of all washes and incubations were 2 ml/well, unless stated otherwise. In all cases, the inhibitors and pertussis toxin were added in buffer A.

For $[Ca^{2+}]_i$ determinations, LPS-primed cells were loaded in HBS containing Pluronic (0.01%) and 0.5 μM fura-2/AM for 30 min at room temperature during wash no. 2. All experiments were performed in HBS, except for the Ca^{2+}-free experiments (Figure 3), where Ca^{2+}-free DPBS was used. Inhibitors were present during wash no. 2 and the stimulation step. Cells were stimulated with PAF (20 nM) or ionomycin (0.5 μM) for 10 min at 34 °C. Fluorescence Ca^{2+} images were obtained and calibrated as previously described [10–13]. The images were taken every 5 s. $[Ca^{2+}]_i$ traces are the means of 10–15 individual cells of the same dish. $[Ca^{2+}]_i$ traces shown in the Figures are from a typical dish of an experiment. The $[Ca^{2+}]_i$ increase reported is the difference between the basal level of $[Ca^{2+}]_i$ and the level of the transient peak.

PGE_2 levels in the media above these cells were determined immediately after the $[Ca^{2+}]_i$ data were collected. Even though the levels of PGE_2 in the Ca^{2+} experiments were usually lower than those obtained with the standard PAF activation, the stimulus–response pattern for PGE_2 formation was identical (results not shown). The lower PGE_2 levels cannot be due to the shorter stimulation time (10 versus 20 min), since PGE_2 formation has ceased 3 min after stimulation (R. Asmis and E. A. Dennis, unpublished work). All PGE_2 determinations were therefore performed by using both the standard PAF activation conditions and the $[Ca^{2+}]_i$ assay conditions to ensure comparability with our previous work [7], to allow for a detailed statistical analysis, and to allow a direct correlation between the $[Ca^{2+}]_i$ signal and PGE_2 formation.

PGE_2 assay

After stimulation, the medium was removed, centrifuged to remove cells and assayed for PGE_2 content by a specific radioimmunoassay (Advanced Magnetics, Cambridge, MA, U.S.A.). PGE_2 levels are expressed as percentages of control, and the value corresponding to 100% is given in ng/10^6 cells in the legends of the Figures for each experiment.

Assay for Ins(1,4,5)P_3

Cells were stimulated for 15 s in a final volume of 1 ml. LiCl (15 mM) was present during wash no. 2 and during the stimulation step. Stimulation was stopped and the cells were lysed by addition of 1 ml of cold trichloroacetic acid (40%, w/v) for 60 min at 4 °C. A 1 ml portion of the supernatant was removed and extracted once with 2 vol. of 1,1,2-trichloro-1,2,2-trifluoroethane/trioctylamine (3:1, v/v). The upper aqueous layer was removed, and the Ins(1,4,5)P_3 concentrations were determined with an Ins(1,4,5)P_3 radioreceptor assay kit (New England Nuclear, Boston, MA). Extraction yields were tested routinely and the results corrected accordingly.

Cytotoxicity assay

Cytotoxicity was measured after each standard experiment by fluorescein diacetate staining of cells as described previously [7]. Fluorescein diacetate (1 μg/ml) was added to each well and incubated at 37 °C, for 15 min. Fluorescence was observed by

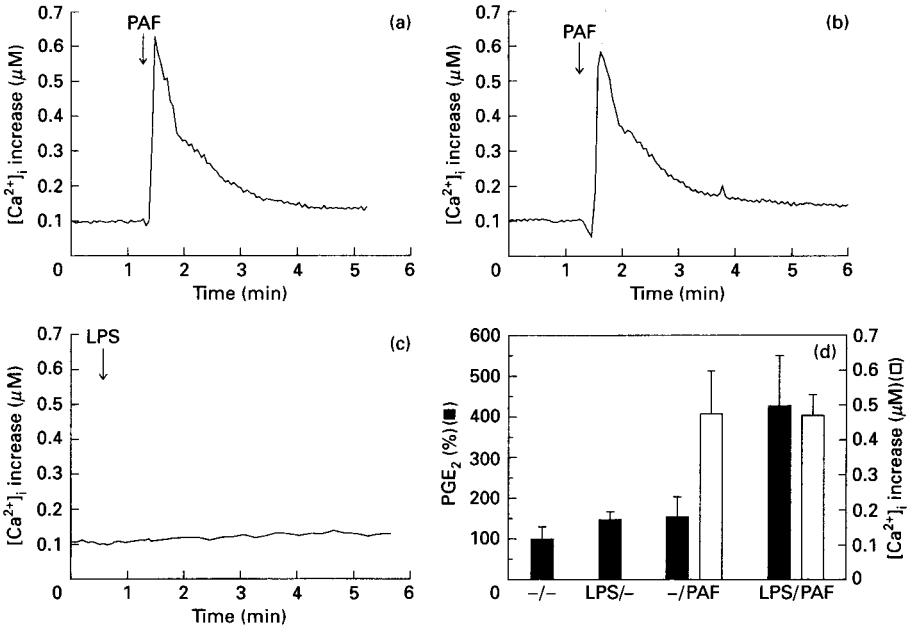

Figure 1 Effect of PAF and LPS on $[Ca^{2+}]_i$ and PGE_2 formation

$[Ca^{2+}]_i$ was measured as described in the Experimental section after PAF (20 nM) stimulation of unprimed (**a**) or LPS-primed (100 ng/ml) (**b**) $P388D_1$ cells, or after LPS (1 μg/ml) stimulation of cells for 1 h (**c**). (**d**) PGE_2 levels (■) were determined immediately after each $[Ca^{2+}]_i$ measurement as described in the Experimental section and are presented as percentages of the unprimed unstimulated control (100% = 0.62 ng of $PGE_2/10^6$ cells). The amplitude of the corresponding PAF-induced transient $[Ca^{2+}]_i$ peak (□) is shown for comparison.

using an inverted Olympus IMT-2 fluorescence microscope, and toxicity was determined as the ratio of non-fluorescent cells (dead) to fluorescent cells (living). Viability observed was > 98% under all conditions used in this paper.

Statistical analysis

For PGE_2, Ins(1,4,5)P_3 and Ca^{2+} determinations, all data points are the means ± S.E.M. of triplicate samples from a representative experiment, except in Figure 1(d), where four independent experiments were pooled. Statistical significance between treatment groups was determined by Duncan multiple-range tests ($P < 0.01$) unless stated otherwise. Standard-error bars are only shown when the S.E.M. is greater than the symbol size.

RESULTS

LPS priming does not affect $[Ca^{2+}]_i$ levels

$P388D_1$ cells show an enhanced formation of PGE_2 upon PAF stimulation only if they have been primed with LPS [7]. Because of the reported Ca^{2+}-dependence of PLA_2 [9,14], we investigated whether LPS priming enhanced the rise in $[Ca^{2+}]_i$ induced by PAF, thereby increasing PLA_2 activity and subsequently enhancing PGE_2 formation. PAF (20 nM) induced a large and rapid increase in $[Ca^{2+}]_i$ (Figure 1a). As observed previously in peritoneal macrophages [15], this increase consists of an initial transient peak which appears to result from the release of Ca^{2+} from intracellular stores, followed by a plateau due to entry of extracellular Ca^{2+} (see below). As shown in Figures 1(a) and 1(b), LPS priming of $P388D_1$ cells affected neither the amplitude nor the shape of the PAF-induced $[Ca^{2+}]_i$ response. To determine if LPS itself caused any changes in $[Ca^{2+}]_i$, cells were treated with LPS, and $[Ca^{2+}]_i$ was monitored for 1 h. As shown in Figure 1(c), LPS at concentrations as high as 1 μg/ml did not induce any changes in $[Ca^{2+}]_i$. This result differs from those obtained in peritoneal macrophages, in which LPS has been shown to induce the release of Ca^{2+} from intracellular stores in 47% of the cells [16].

The correlation between the amplitude of the transient Ca^{2+} signal (white bars) and the formation of PGE_2 (black bars) determined in the same cell dishes under various conditions is shown in Figure 1(d). LPS priming slightly increased PGE_2 levels in unstimulated $P388D_1$ cells. PAF did not stimulate PGE_2 formation unless the cells were primed with LPS before stimulation. However, no LPS priming was required for PAF to trigger the full Ca^{2+} signal. Indeed, the amplitude of the $[Ca^{2+}]_i$ increase induced by PAF in unprimed or LPS-primed cells is identical. These experiments clearly show that LPS priming is not due to $[Ca^{2+}]_i$ changes. Furthermore, these results indicate that the PAF-induced increase in $[Ca^{2+}]_i$ is not sufficient to trigger the formation of PGE_2 in $P388D_1$ cells.

$[Ca^{2+}]_i$ rise is necessary for PGE_2 formation

Since the PAF-induced $[Ca^{2+}]_i$ rise was not sufficient to trigger PGE_2 production, we tested whether a $[Ca^{2+}]_i$ rise was actually required for the formation of PGE_2 in LPS-primed $P388D_1$ cells.

Long-term treatment of cells with Ca^{2+}-free medium containing 2 mM EGTA should lead to the depletion of Ca^{2+} stores. Indeed, under these conditions, the PAF-induced rise in $[Ca^{2+}]_i$ was strongly decreased (Figure 2). Furthermore, no Ca^{2+} influx was observed, due to the lack of external Ca^{2+}. PGE_2 formation was inhibited to control levels in the $[Ca^{2+}]_i$-assay conditions.

Another way to prevent the rise of $[Ca^{2+}]_i$ is to load the cells with BAPTA/AM, the permeant ester of the Ca^{2+} chelator BAPTA [17]. Single-cell Ca^{2+} measurements showed that BAPTA/AM (30 μM) completely blocked the initial Ca^{2+} peak

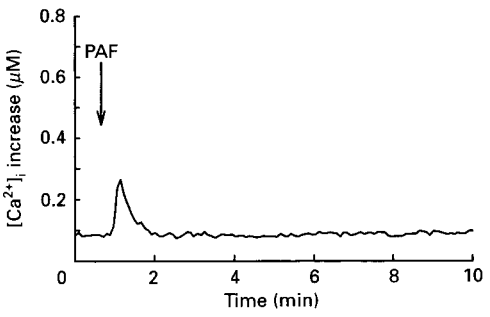

Figure 2 Effect of long-term EGTA preincubation on PAF-induced $[Ca^{2+}]_i$ rise in P388D$_1$ cells

LPS-primed P388D$_1$ cells were treated with Ca^{2+}-free medium with 2 mM EGTA for 30 min during wash no. 2, and stimulated with 20 nM PAF in the respective medium.

induced by PAF (Figure 3a). However, we observed a slight but delayed transient increase in $[Ca^{2+}]_i$. Subsequent PGE_2 measurements demonstrated that the PAF-induced PGE_2 formation is inhibited to basal levels, which were not affected by BAPTA. Under standard experimental conditions, BAPTA inhibited PGE_2 formation in a dose-dependent manner, completely abolishing PAF-stimulated PGE_2 production at 30 μM (Figure 3b).

All these results demonstrate that a Ca^{2+} rise is required for the PAF-stimulated formation of PGE_2. Interestingly, basal PGE_2 levels were not abolished by the long-term treatment with EGTA, nor did they appear to be affected by BAPTA. Basal PGE_2 production therefore appears not to require a rise in $[Ca^{2+}]_i$.

Role of Ins(1,4,5)P_3 formation

Stimulation of P388D$_1$ cells with physiological concentrations of PAF (20 nM) in the presence of Li^+ (15 mM) resulted in a 2–4-fold increase in Ins(1,4,5)P_3 levels, regardless of priming with LPS (Table 1). In the absence of Li^+, no significant levels of Ins(1,4,5)P_3 were detected under any condition tested (results not shown). Despite the presence of Li^+, PAF-induced formation of Ins(1,4,5)P_3 was very transient (Figure 4). An increase in Ins(1,4,5)P_3 levels was observed 5 s after stimulation. Ins(1,4,5)P_3 levels were maximal at 15 s, at which point its concentration was 2–4 times the basal value. It decreased thereafter and reached basal levels approx. 2 min after PAF stimulation.

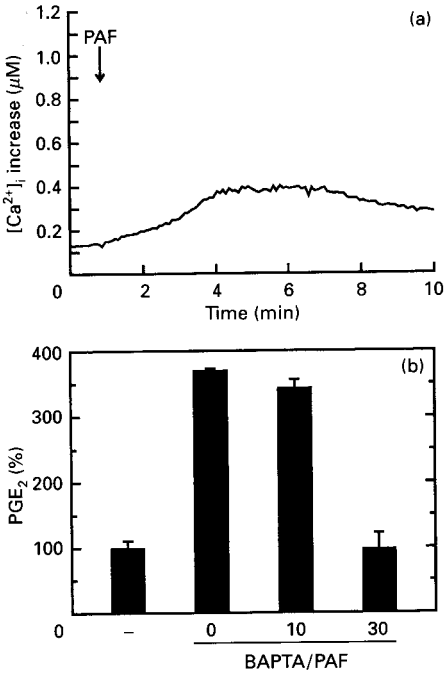

Figure 3 Effect of BAPTA on the PAF-induced $[Ca^{2+}]_i$ rise and PGE_2 formation in P388D$_1$ cells

LPS-primed P388D$_1$ cells were treated with BAPTA/AM (30 μM) for 30 min during wash no. 2 and were then stimulated with 20 nM PAF (**a**). The dose-dependent effect of BAPTA/AM (concentrations indicated in μM) on PAF-induced (20 nM) PGE_2 formation under standard experimental conditions is shown in (**b**). PGE_2 formation is given as percentage of the LPS-primed unstimulated control (100% = 0.90 ng of $PGE_2/10^6$ cells).

The Ca^{2+} ionophore ionomycin is a potent activator of PGE_2 formation in P388D$_1$ cells, but caused a slight decrease in basal Ins(1,4,5)P_3 levels (Table 1). LPS priming had no significant effect on basal Ins(1,4,5)P_3 levels, nor on PAF-stimulated Ins(1,4,5)P_3 levels. LPS priming is therefore not mediated through the formation of Ins(1,4,5)P_3 nor the release of $[Ca^{2+}]_i$, as shown above (Figure 1). Pertussis toxin (100 ng/ml) inhibited both PAF-induced Ins(1,4,5)P_3 and PGE_2 formation, but had no effect on the ionomycin-stimulated PGE_2 release (Table 1). Under the

Table 1 Effect of LPS priming and pertussis toxin on PAF-stimulated Ins(1,4,5)P_3 and PGE_2 formation in P388D$_1$ cells

P388D$_1$ cells were preincubated with pertussis toxin (PTX), primed with LPS and stimulated with PAF or ionomycin where indicated. Ins(1,4,5)P_3 and PGE_2 formation were determined under standard experimental conditions, and are given as percentages of unprimed and unstimulated control [100% = 0.48 ng of $PGE_2/10^6$ cells or 5.5 pmol of Ins(1,4,5)$P_3/10^6$ cells respectively].

PTX (100 ng/ml)	LPS (100 ng/ml)	PAF (20 nM)	Ionomycin (0.5 μM)	Ins(1,4,5)P_3 formation (% of control)	PGE_2 formation (% of control)
–	–	–	–	100±16	100±12
–	+	–	–	123±16	235±31
+	+	–	–	109±27	181±13
–	–	+	–	313±11	135±19
–	+	+	–	293±21	510±42
+	+	+	–	92±20	250±17
–	–	–	+	29±9	458±20
+	–	–	+	54±6	432±20

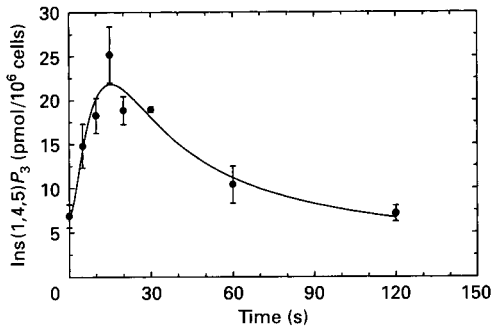

Figure 4 Time-dependence of Ins(1,4,5)P_3 formation in PAF-stimulated $P388D_1$ cells

Ins(1,4,5)P_3 levels were determined at various time points after PAF (20 nM) stimulation of unprimed $P388D_1$ cells as described in the Experimental section. LiCl (15 mM) was present during wash no. 2 and the stimulation step.

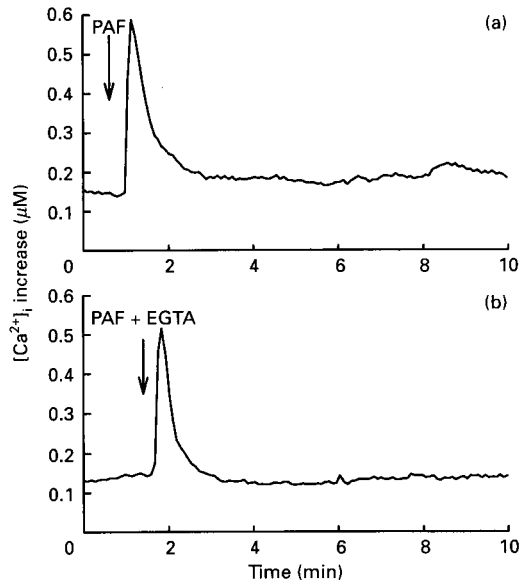

Figure 5 Role of Ca^{2+} influx in PAF-stimulated $P388D_1$ cells

LPS-primed $P388D_1$ cells were stimulated with PAF (20 nM) in the absence (**a**) or presence (**b**) of EGTA (2 mM).

conditions of the Ca^{2+}-assay protocol, the PAF-stimulated Ca^{2+} transient was inhibited by about 50 % in cells preincubated with pertussis toxin (100 ng/ml). These results indicate that the PAF-induced release of Ins(1,4,5)P_3, rise in $[Ca^{2+}]_i$ and formation of PGE_2 may be mediated by a pertussis-toxin-sensitive G-protein.

Role of Ca^{2+} influx

In order to investigate the role of the Ca^{2+} influx and the necessity for a sustained $[Ca^{2+}]_i$ rise in PAF signalling, we separated the transient $[Ca^{2+}]_i$ spike, due to release of intracellular stores, from the sustained $[Ca^{2+}]_i$ elevation caused by the influx of extracellular Ca^{2+}. EGTA (2 mM) was added during the PAF

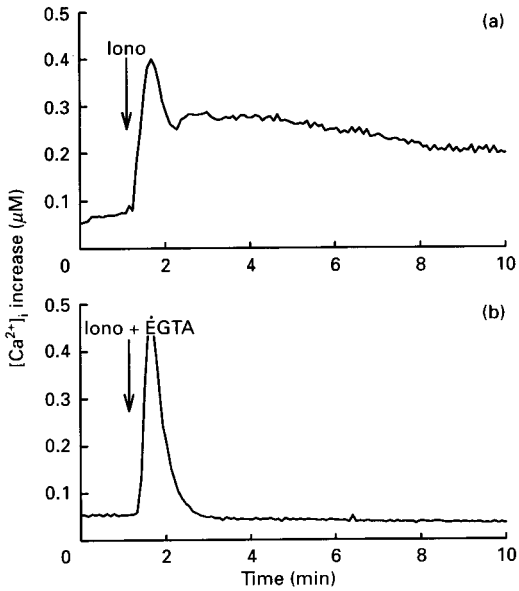

Figure 6 Role of Ca^{2+} influx in ionomycin-stimulated $P388D_1$ cells

LPS-primed $P388D_1$ cells were stimulated with ionomycin (0.5 μM) in the absence (**a**) or presence (**b**) of EGTA (2 mM).

stimulation step only. This removed the extracellular Ca^{2+}, yet the assays were done before the intracellular stores were affected. The PAF-induced Ca^{2+} signal decayed more rapidly in the presence of EGTA, as shown in Figure 5, demonstrating that the plateau of the Ca^{2+} signal was due to Ca^{2+} influx. However, the amplitude of the transient $[Ca^{2+}]_i$ peak resulting from the release of Ca^{2+} from intracellular stores remained unchanged. Under these conditions, the PAF-induced Ca^{2+} signal is therefore solely the result of the release of Ca^{2+} from intracellular stores. PAF-stimulated PGE_2 formation was not inhibited by EGTA under these short incubation conditions (results not shown); this shows that a sustained $[Ca^{2+}]_i$ rise provided by a Ca^{2+} influx is not required for PAF signalling.

We next investigated the role of the Ca^{2+} transient by activating the cells with the Ca^{2+} ionophore ionomycin in the presence of EGTA. In the presence of external Ca^{2+}, ionomycin stimulation resulted in a fast biphasic $[Ca^{2+}]_i$ rise (Figure 6a) similar to that found with PAF (Figure 1a). However, the plateau of the ionomycin-induced $[Ca^{2+}]_i$ rise was larger and sustained for a longer period than that induced by PAF (compare Figures 5a and 6a). In the presence of EGTA, the Ca^{2+} signal triggered by PAF and ionomycin appeared to be identical (compare Figures 5b and 6b); in contrast with the case when PAF was employed, the ionomycin-induced PGE_2 formation was decreased to control levels. Within the resolution of our imaging system, the $[Ca^{2+}]_i$ rise appeared uniform in the cells during PAF activation as well as during ionomycin stimulation. No regions of high local $[Ca^{2+}]_i$ were observed after PAF addition which could have explained the high PAF stimulation under these conditions. These results suggest that the transient rise in $[Ca^{2+}]_i$ is necessary, but not sufficient, to trigger the formation of PGE_2 in $P388D_1$ cells.

Table 2 shows the effect of EGTA, present during the stimulation step, on PAF- and ionomycin-induced PGE_2 formation under the standard PAF-activation conditions. The presence of EGTA decreased both the basal and the PAF-stimulated PGE_2 formation by 10–50 %, varying from experiment to ex-

Table 2 Role of Ca^{2+} influx determined by the standard PAF activation protocol in P388D$_1$ cells

LPS-primed P388D$_1$ cells were stimulated with buffer A, PAF (20 nM) or ionomycin (0.5 μM) in the absence or presence of EGTA (2 mM). PGE_2 formation is given as percentage of LPS-primed unstimulated control (100% = 1.9 ng of $PGE_2/10^6$ cells).

Stimulus	EGTA (2 mM)	PGE_2 formation (% of control)
Buffer A	−	100±16
	+	56±16
PAF (20 nM)	−	212±4
	+	118±15
Ionomycin (0.5 μM)	−	216±11
	+	66±15

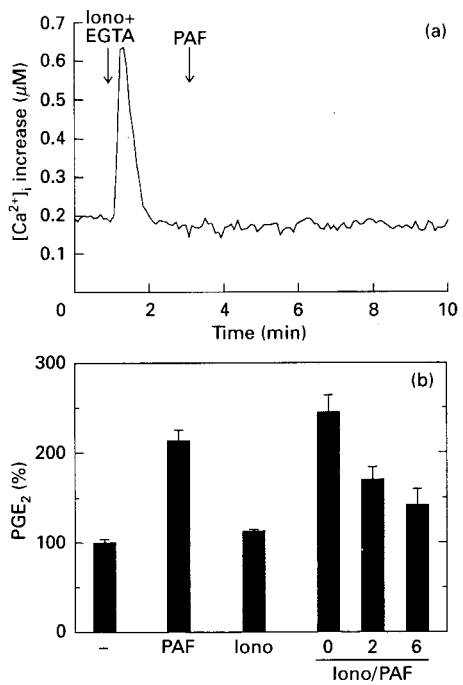

Figure 7 PAF triggers a second Ca^{2+}-independent signal in P388D$_1$ cells

LPS-primed P388D$_1$ cells were stimulated with ionomycin (0.5 μM) in the presence of EGTA (2 mM). PAF (20 nM) was added 2 min after stimulation. A typical $[Ca^{2+}]_i$ measurement is shown in (**a**). (**b**) shows the PGE_2 formation using the standard PAF-activation protocol. Cells were stimulated with buffer A (−), PAF (20 nM), or ionomycin (Iono; 0.5 μM). PAF (20 nM) was added to ionomycin-stimulated cells (Iono/PAF) at the times indicated in min (0, 2, 6). PGE_2 formation was determined under standard experimental conditions, and is given as percentage of the LPS-primed unstimulated control (**b**: 100% = 1.06 ng of $PGE_2/10^6$ cells).

periment. However, the potentiation of the PAF-stimulated PGE_2 formation compared with controls was not affected by EGTA in any experiment. It is unclear if this decrease in the PGE_2 signals is due to the lack of extracellular Ca^{2+} or is the result of a secondary effect of EGTA on the cells. The effect was not due to the loss of cells, as determined by protein assay (results not shown). In contrast with PAF, the ionomycin-stimulated PGE_2 formation was completely abolished when EGTA was present during the stimulation step, as observed in the single-cell Ca^{2+} measurements. We therefore conclude that no sustained Ca^{2+} increase is required for PAF-induced PGE_2 formation, but it is required for ionomycin to stimulate PGE_2 production.

PAF triggers two signals

Since in the presence of EGTA, the Ca^{2+} signal induced by PAF and ionomycin were identical, but only PAF stimulated PGE_2 formation, we conclude that PAF must trigger a second signal in addition to the rise in $[Ca^{2+}]_i$. In order to test this hypothesis and to separate the two signals, we performed the following experiment: The cells were first stimulated with ionomycin in the presence of EGTA, thereby inducing the required Ca^{2+} signal (Figure 7a) without initiating PGE_2 production. Then, after 2 min we added PAF. No further Ca^{2+} signal was observed, because the refilling of the Ca^{2+} stores was prevented by the absence of extracellular Ca^{2+}. However, PGE_2 formation was now stimulated (Figure 7b). These results confirm that PAF triggers a second signal independently of the rise in $[Ca^{2+}]_i$.

Next we investigated the time-dependence of the reconstituted PGE_2 signal under standard experimental conditions. In the presence of EGTA, we again observed PAF-induced PGE_2 formation, whereas the ionomycin-stimulated production of PGE_2 was decreased to nearly control levels, as shown in Figure 7(b). When PAF was added with ionomycin (0 min), the PGE_2 signal was not significantly increased compared with the PGE_2 formation induced by PAF alone. PGE_2 formation was decreased when PAF was added 2 min after ionomycin stimulation, but was significantly higher than in cells stimulated only with ionomycin. Addition of PAF 6 min after ionomycin stimulation showed no significant increase in PGE_2 formation compared with ionomycin alone. Since the increase in PGE_2 production induced by PAF in ionomycin-stimulated cells is not the result of an increase in $[Ca^{2+}]_i$, as we have demonstrated in the single-cell measurements, we conclude that PAF triggers two signals, one of which is the Ca^{2+} signal that PAF and ionomycin appear to have in common. The second response can be triggered independently of the Ca^{2+} signal. However, the second signal has to be triggered within a few minutes after the $[Ca^{2+}]_i$ rise, suggesting that the activation induced by the Ca^{2+} transient is down-regulated rapidly.

Effect of inhibition of tyrosine-specific protein kinases (TPK) on $[Ca^{2+}]_i$, Ins(1,4,5)P_3 and PGE_2

We have demonstrated that genistein, an inhibitor of TPK [18], is a potent inhibitor of PAF-stimulated PGE_2 formation [7]. For the present studies, we used the more specific TPK inhibitor tyrphostin 25 [19]. Under standard experimental conditions tyrphostin 25 inhibited PAF-induced PGE_2 formation in a dose-dependent manner, with an apparent IC_{50} of 8 μM (results not shown), which is very similar to the value that we have determined previously for genistein (IC_{50} = 7 μM) [7]. At 30 μM, tyrphostin 25 inhibited PAF-stimulated PGE_2 production by 98±0.3%, and in the presence of EGTA it blocked by 97±6%. PAF-induced Ins(1,4,5)P_3 formation, however, was only inhibited by 29±3%. Although tyrosine phosphorylation is essential for PAF-induced PGE_2 formation, it is not required for Ins(1,4,5)P_3 release.

The phospholipase C (PLC) involved in the PAF-stimulated release of Ins(1,4,5)P_3 in P388D$_1$ cells appears not to be activated by TPK. When the Ca^{2+}-assay protocol was used to activate

Table 3 Effect of tyrphostin 25 on $[Ca^{2+}]_i$ and PGE_2 formation in $P388D_1$ cells

LPS-primed $P388D_1$ cells were stimulated with either PAF (20 nM) or ionomycin (0.5 μM) for 10 min, and PGE_2 levels and $[Ca^{2+}]_i$ were determined. Tyrphostin 25 (30 μM) was present during wash no. 2 and the stimulation step. PGE_2 formation is given as percentage of LPS-primed unstimulated control (100% = 0.40 ng of $PGE_2/10^6$ cells).

Stimulus	Tyrphostin 25 (30 μM)	PGE_2 formation (% of control)	$[Ca^{2+}]_i$ increase (μM)
Buffer A	−	100±15	0
PAF (20 nM)	−	353±38	0.41±0.02
	+	149±10	0.46±0.01
Ionomycin (0.5 μM)	−	376±62	0.29±0.04
	+	145±10	0.30±0.04

cells, preincubation with tyrphostin 25 (30 μM) blocked PGE_2 formation stimulated by either PAF or ionomycin, but did not affect $[Ca^{2+}]_i$ induced by either PAF or ionomycin (Table 3). Tyrphostin 25 (30 μM) also inhibited ionomycin-stimulated PGE_2 production (92±3%) under standard experimental conditions. All these results suggest that tyrosine phosphorylation is not the second signal triggered by PAF. It is more likely an additional event common to both PAF and ionomycin stimulation. Since tyrphostin 25 was added 30 min before the stimulation step in the experiments sited above, inhibition of tyrosine phosphorylation could occur before PAF or ionomycin stimulation. An interesting possibility is that LPS priming involves tyrosine phosphorylation.

DISCUSSION

In $P388D_1$ cells, the formation of PGE_2 correlates very well with the release of [^{3}H]arachidonic acid and with the response of the membrane-associated Ca^{2+}-dependent PLA_2 activity to various inhibitors, but not with the cyclo-oxygenase activity [6]. Whereas LPS priming of $P388D_1$ cells increases [^{3}H]arachidonic acid release and PGE_2 formation, cyclo-oxygenase activity remains unaffected [7]. The most likely candidate for the rate-limiting step in the formation of prostanoids in $P388D_2$ cells is therefore the PLA_2-mediated release of arachidonic acid. Although several different types of PLA_2 activities have been identified and characterized in $P388D_1$ cells [3–5], little is known about the regulation of PLA_2 activity in these cells.

PAF at micromolar concentrations stimulates the formation of PGE_2 in $P388D_1$ cells [6]. However, the cells require LPS priming before stimulation for physiological doses of PAF to trigger PGE_2 production. Although transcriptional events appear to be required for LPS priming, PAF stimulation involves protein synthesis [7]. LPS priming and PAF stimulation are clearly separate events in $P388D_1$ cells, which make these cells a unique model in which to study the different stages of activation in macrophages. These findings also suggest that LPS and PAF trigger different signalling pathways, resulting in the stimulation/regulation of PLA_2 activity responsible for the formation of PGE_2 in $P388D_1$ cells. The results reported herein further support this hypothesis. In contrast with PAF, LPS does not trigger a rise in $[Ca^{2+}]_i$ in $P388D_1$ cells, nor does it affect the PAF-induced Ca^{2+} signal. LPS priming is therefore not mediated by a rise in $[Ca^{2+}]_i$. These results are in good agreement with our observations on Ins(1,4,5)P_3 formation in these cells. LPS does not induce Ins(1,4,5)P_3 formation, nor does it affect PAF-stimulated Ins(1,4,5)P_3 release. The role of LPS priming, however, remains unclear and is under further investigation.

At physiologically relevant concentrations, PAF triggers a biphasic rise of $[Ca^{2+}]_i$ in both unprimed and LPS-primed cells. The first phase, a Ca^{2+} spike, is due to the release of intracellular Ca^{2+}, whereas the second phase is a long, slower, influx of extracellular Ca^{2+}. However, LPS priming was required for PAF to induce PGE_2 formation, demonstrating that the PAF-induced $[Ca^{2+}]_i$ rise alone is not sufficient to trigger PGE_2 production. Yet, the PAF-induced $[Ca^{2+}]_i$ rise is required for PGE_2 formation, as we have demonstrated by either depleting the intracellular stores through long-term exposure to EGTA or by blocking the $[Ca^{2+}]_i$ rise with the Ca^{2+} chelator BAPTA. Again, these findings correlate well with our results on Ins(1,4,5)P_3 formation.

We have found that in LPS-primed $P388D_1$ cells stimulated with PAF (stimulatory conditions under which $[Ca^{2+}]_i$ is increased and both [^{3}H]arachidonic acid release and PGE_2 formation are strongly enhanced [7]), no significant increase in Ins(1,4,5)P_3 could be detected unless the cells were pretreated with Li^+. Several explanations are possible for the Li^+ requirement. One possibility is that the amount of Ins(1,4,5)P_3 actually required to trigger the necessary Ca^{2+} signal in $P388D_1$ cells is very small and is below the detection limit of the assay (1 pmol/10^6 cells). It has been suggested that the action of Ins(1,4,5)P_3 is highly co-operative, and that as few as three molecules may be sufficient to open a Ca^{2+} channel [20]. Another possibility is that the increase in InsP_3 is due to an increase in Ins(1,3,4)P_3 rather than Ins(1,4,5)P_3. It has been reported that Li^+ treatment of pancreatic acinar cells resulted in 6-fold higher levels of Ins(1,3,4)P_3 compared with Ins(1,4,5)P_3 [21]. However, the affinity of the InsP_3-binding protein used in the assay for Ins(1,4,5)P_3 is 3000 times that for Ins(1,3,4)P_3, making this possibility highly unlikely. Further investigations are necessary to elucidate just how Li^+ affects Ins(1,4,5)P_3 metabolism in $P388D_1$ cells.

Pretreatment of $P388D_1$ cells with pertussis toxin inhibited the PAF-stimulated Ins(1,4,5)P_3 formation, Ca^{2+} signalling and PGE_2 formation in these cells, suggesting that one of the earliest events in PAF stimulation of $P388D_1$ cells is the receptor-mediated activation of PLC via a pertussis-toxin-sensitive G-protein. In normal bone-marrow-derived macrophages, the release of [^{3}H]InsP_3 is also pertussis-toxin-sensitive [22], whereas in the leukaemic U-937 cells both the release of [^{3}H]InsP_3 and the mobilization of intracellular Ca^{2+} were insensitive to pertussis-toxin treatment [23].

All the above results suggest a cause-and-effect relationship between PAF-induced Ins(1,4,5)P_3 formation, mobilization of intracellular Ca^{2+} and PGE_2 formation in $P388D_1$ cells. Even though the sensitivity to pertussis toxin or cholera toxin of the various G-proteins appears to vary in other macrophages and monocytic cell lines [24], the general scheme [PAF→PAF receptor→G-protein→PLC→Ins(1,4,5)P_3→Ca^{2+} signal] appears to be a common mechanism of PAF activation in cells.

Of the known forms of PLC, only the PLC-β_1 isotype has been shown to be responsive to G-proteins [25,26]. PLC-γ_1 and PLC-γ_2 require tyrosine phosphorylation for activity [27], whereas the mechanism for regulation of PLC-δ remains unknown. Our pertussis-toxin experiments suggest that the PAF-induced Ins(1,4,5)P_3 formation in $P388D_1$ cells is G-protein-mediated and that the PLC involved, therefore, is not of the PLC-γ subform. This finding is supported by the lack of effect of tyrphostin-25, a specific TPK inhibitor, on PAF-induced Ins(1,4,5)P_3 formation in these cells. On the other hand, the G-proteins which have been

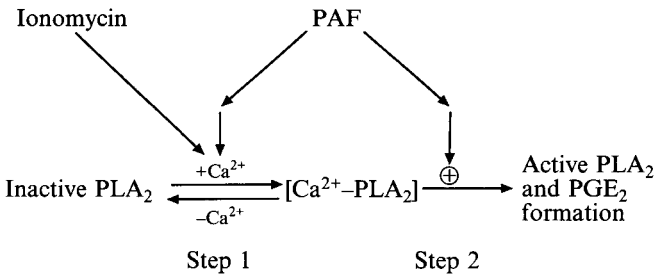

Figure 8 Model for PLA_2 activation

A $[Ca^{2+}]_i$ rise induced by PAF or by ionomycin can convert the inactive form of PLA_2 into a labile, inactive, $[Ca^{2+}–PLA_2]$ form through Step 1. This step is reversible. Step 2, which converts PLA_2 into its active form, is slow and poorly reversible. This step can, however, be accelerated by a second signal provided by PAF.

identified to regulate PLC-β_1 are of the pertussis-toxin-insensitive G_q family (for review see [28]). However, recent evidence suggests the PLC-β_2 can be stimulated by $\beta\gamma$-subunits of G-proteins [28]. Should such a stimulatory role be demonstrated for the $\beta\gamma$-subunits of the pertussis-toxin-sensitive G-protein families G_o and G_i, then PLC-β_2 would become a likely candidate for the PLC subtype responsible for the release of Ins(1,4,5)P_3 in $P388D_1$ cells. Clearly, further investigations are necessary to demonstrate convincingly the G-protein(s) and the PLC subtypes involved in the stimulus–response coupling of PAF-activated PGE_2 formation in $P388D_1$ cells.

Although the formation of Ins(1,4,5)P_3 and the subsequent release of Ca^{2+} from intracellular stores are necessary for the PAF-stimulated formation of PGE_2, a sustained $[Ca^{2+}]_i$ rise provided by Ca^{2+} influx is not required. Indeed, we have shown that, in the presence of EGTA, PAF stimulated PGE_2 formation 2–4-fold over controls, which is similar to the results observed in the presence of external Ca^{2+}. These results are in contrast with observations made in PAF- or zymosan-stimulated peritoneal macrophages, where arachidonic acid release was strongly dependent on external Ca^{2+} [29]. Also, in C62B glioma cells, PLA_2 activation appears to require Ca^{2+} influx [30]. On the other hand, we have shown that EGTA inhibited ionomycin-induced PGE_2 formation, even though the sharp intracellular Ca^{2+} spike was identical with that triggered by PAF under these conditions. However, PGE_2 secretion can be restored by subsequent addition of PAF, even though no further $[Ca^{2+}]_i$ increase is observed. These results suggest that PAF triggers two signals: the rise in $[Ca^{2+}]_i$ and a second signal that is not Ca^{2+}-mediated.

Previous studies on PAF-activated neutrophils suggested that a rise in cytosolic Ca^{2+} is necessary, but not sufficient, to promote the activation of PLA_2 [31,32]. Our results with ionomycin and normal extracellular Ca^{2+} show that a large and sustained rise in $[Ca^{2+}]_i$ fully activated PLA_2. With a more physiological stimulant, PAF, the $[Ca^{2+}]_i$ elevation is accompanied by a second signal to activate PLA_2 fully. A simple model to explain our results is presented in Figure 8. This model postulates that PLA_2 reversibly binds with Ca^{2+} to form a labile complex $[Ca^{2+}–PLA_2]$, which is still unable to release arachidonic acid, but which can be further converted into the final, active, phospholipase. The latter step is slow unless accelerated by a second Ca^{2+}-independent signal provided by PAF. Once $[Ca^{2+}–PLA_2]$ is formed, it is no longer dependent on elevated Ca^{2+}. According to this model, PAF provides the transient rise in $[Ca^{2+}]_i$ required to convert PLA_2 into the labile $[Ca^{2+}–PLA_2]$ complex, as well as the additional signal to accelerate the final activation step. If the $[Ca^{2+}]_i$ rise is buffered or blunted (Figures 2a and 3a), activation is prevented (Figures 2b and 3b). If ionomycin is used without external Ca^{2+} to generate a matching brief $[Ca^{2+}]_i$ transient (Figures 6b and 7a) without the second signal, presumably the PLA_2 temporarily forms the $[Ca^{2+}–PLA_2]$ complex, but reverts to the initial state as $[Ca^{2+}]_i$ falls, so activation is prevented (Figure 6c). Ionomycin with external Ca^{2+} prolongs the $[Ca^{2+}]_i$ increase (Figure 6a) and the formation of the labile $[Ca^{2+}–PLA_2]$ complex sufficiently so that slow conversion into the active state can gradually occur (Figure 6c).

Further evidence for this model is shown in Figure 7. These experiments temporally separate the two sequential steps and begin to characterize the kinetics of Step 1. A brief $[Ca^{2+}]_i$ transient induced by ionomycin in the absence of extracellular Ca^{2+} triggers Step 1. Subsequent addition of PAF does not affect $[Ca^{2+}]_i$, but gives rise to the second signal to promote Step 2. Enzyme activation requires this PAF-induced signal to be delivered within 6 min of the $[Ca^{2+}]_i$ transient and suggests that the transiently formed $[Ca^{2+}–PLA_2]$ intermediate reverts to the starting form within that period unless trapped by the final activation step.

One attractive possibility is that the $[Ca^{2+}–PLA_2]$ form is bound at the membrane surface. Indeed, recently described intracellular forms of PLA_2 have been shown to possess Ca^{2+}-sensitive translocation domains [8,33]. This step could be required for positioning the enzyme properly at the lipid–water interface. It is possible that the enzyme moves to the membrane as soon as it binds Ca^{2+} and that the labile $[Ca^{2+}–PLA_2]$ form in our model would be the translocated form of the enzyme. However, translocation could be a slow process, and an interesting possibility is that the role of the second signal is to accelerate this phenomenon. Then Step 2 would correspond to the translocation event. Of course, another possibility is that the second signal relates to secretion of an extracellular PLA_2 responsible for these events. These possibilities are under investigation.

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