Critical Duration of Intracellular Ca\textsuperscript{2+} Response Required for Continuous Translocation and Activation of Cytosolic Phospholipase A\textsubscript{2}\textsuperscript{*}

(Received for publication, September 23, 1998, and in revised form, November 20, 1998)

Tetsuya Hirabayashi\textsuperscript{§§}, Kazuhiko Kume\textsuperscript{††}, Kenzo Hirose\textsuperscript{‡}, Takehiko Yokomizo\textsuperscript{¶}, Masamitsu Iino\textsuperscript{‡}, Hiroshi Itoh\textsuperscript{‡}, and Takao Shimizu\textsuperscript{‡‡}

From the \textsuperscript{§§}Department of Biochemistry and Molecular Biology, \textsuperscript{††}Department of Pharmacology, Faculty of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033 and the \textsuperscript{¶}Department of Biological Sciences, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan

When cells are exposed to certain external stimuli, arachidonic acid (AA) is released from the membrane and serves as a precursor of various types of eicosanoids. A Ca\textsuperscript{2+}-regulated cytosolic phospholipase A\textsubscript{2} (cPLA\textsubscript{2}) plays a dominant role in the release of AA. To closely examine the relation between Ca\textsuperscript{2+} response and AA release by stimulation of G protein-coupled receptors, we established several lines of Chinese hamster ovary cells expressing platelet-activating factor receptor or leukotriene B\textsubscript{4} receptor. Measurement of intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) demonstrated that cell lines capable of releasing AA elicited a sustained [Ca\textsuperscript{2+}]\textsubscript{i} increase when stimulated by agonists. The prolonged [Ca\textsuperscript{2+}]\textsubscript{i} elevation is the result of Ca\textsuperscript{2+} entry, because this elevation was blocked by EGTA treatment or in the presence of Ca\textsuperscript{2+} channel blockers (SKF 96365 and methoxyverapamil). cPLA\textsubscript{2} fused with a green fluorescent protein (cPLA\textsubscript{2}-GFP) translocated from the cytosol to the perinuclear region in response to increases in [Ca\textsuperscript{2+}]\textsubscript{i}. When EGTA was added shortly after [Ca\textsuperscript{2+}]\textsubscript{i} increase, the cPLA\textsubscript{2}-GFP returned to the cytosol, without liberating AA. After a prolonged [Ca\textsuperscript{2+}]\textsubscript{i} increase, even by EGTA treatment, the enzyme was not readily redistributed to the cytosol. Thus, we propose that a critical time length of [Ca\textsuperscript{2+}]\textsubscript{i} elevation is required for continuous membrane localization and full activation of cPLA\textsubscript{2}.

Arachidonic acid (AA)\textsuperscript{3} is a precursor for biosynthesis of eicosanoids, including prostaglandins, thromboxanes, leukotrienes, and lipoxins. In the resting state, the bulk of the AA in mammalian cells is esterified in glycerophospholipids at the sn-2 position (1). Liberation of AA occurs mainly by activation of phospholipase A\textsubscript{2} (PLA\textsubscript{2}) in response to varieties of extracellular stimuli such as cytokines, hormones, neurotransmitters, mitogens, antigens, and endotoxins (2). Mammalian cells have structurally diverse forms of PLA\textsubscript{2} including secretory PLA\textsubscript{2}, Ca\textsuperscript{2+}-independent PLA\textsubscript{2}, and cytosolic PLA\textsubscript{2} (cPLA\textsubscript{2}) (3–5). Among these PLA\textsubscript{2}s, receptor-mediated AA release is primarily attributed to cPLA\textsubscript{2} because the enzyme preferentially hydrolyzes phospholipids containing AA and is regulated by physiological levels of intracellular calcium concentrations ([Ca\textsuperscript{2+}]\textsubscript{i}) and phosphorylation of Ser-505 by mitogen-activated protein kinase (MAPK) (6–9). Consistently, stimulated peritoneal macrophages derived from cPLA\textsubscript{2}-deficient mice fail to produce eicosanoids (10, 11). Sensitivity of cPLA\textsubscript{2} to Ca\textsuperscript{2+} is mediated through an amino-terminal CaLB domain which is homologous to the C2 domain of protein kinase C and several other Ca\textsuperscript{2+}-dependent phospholipid-binding proteins (6, 12). Immunofluorescent studies have shown that cPLA\textsubscript{2} translocates from the cytosol to the nuclear envelope and the endoplasmic reticulum in response to [Ca\textsuperscript{2+}]\textsubscript{i} increase (13, 14). Other proteins involved in eicosanoid production, such as cyclooxygenase isoforms (15–17), 5-lipoxygenase, 5-lipoxygenase-activating protein, and leukotriene C\textsubscript{4} synthase (18–20) are also localized to these sites constitutively or upon cell stimulation.

The mobilization of Ca\textsuperscript{2+} can be elicited by ligand binding to cell-surface receptors that activate phospholipase C\textsubscript{a} through heterotrimeric G protein-dependent or protein tyrosine kinase-dependent pathway. Calcium signaling patterns occur as single transients, repetitive oscillations, or sustained plateaux. Although several investigators have noted the importance of Ca\textsuperscript{2+} influx in receptor-mediated AA release (21–23), little is known about how these Ca\textsuperscript{2+} signaling patterns regulate cPLA\textsubscript{2} translocation and AA release.

To address this question, we monitored Ca\textsuperscript{2+} signaling pattern, cPLA\textsubscript{2} translocation, and AA release using Chinese hamster ovary (CHO) cell lines stably expressing platelet-activating factor (PAF) receptor (24) and/or leukotriene B\textsubscript{4} (LTB\textsubscript{4}) receptor (25). In CHO cells, cPLA\textsubscript{2} is expressed endogenously and coupled to receptor-mediated release of AA (26, 27). We found that a critical time length of [Ca\textsuperscript{2+}]\textsubscript{i} elevation is required for continuous translocation of cPLA\textsubscript{2} to the membrane and for the release of AA.

EXPERIMENTAL PROCEDURES

Materials—The sources of materials used in this work were as follows: PAF C-16 from Cayman Chemical (Ann Arbor, MI); cremophore EL from Sigma; \textsuperscript{1}H\textsuperscript{(H)arachidonic acid, \textsuperscript{1}H}WEB 2086, and \textsuperscript{1}Hleukotriene B\textsubscript{4} from NEN Life Science Products; fura-2 acetoxymethyl from Dojindo (Kumamoto, Japan); SKF 96365, ionomycin, and nifedipine from Calbiochem; methoxyverapamil from RBI (Natick, MA); anti-cPLA\textsubscript{2} monoclonal IgG (4–4B-3C) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and fatty acid-free bovine serum albumin fraction V from Bayer (Kankakee, IL).

WEB 2086 and leukotriene B\textsubscript{4} were generous gifts from Boehringer Ingelheim (Germany) and Drs. Y. Kobayashi and F. Sato (Tokyo Institute of Technology, respectively).
Plasmid Construction—For a chimeric protein containing GFP at the carboxyl terminus of cPLA2, cDNA encoding human cPLA2 was amplified by polymerase chain reaction with primers upstream (5'GGAA-GATCTATGTCATTTATAGATCCTT-3') and downstream (5'TGCGGTCGACTGCTTTGGGTTTACTTAGA-3') using pSV2-cPLA2 (provided by Drs. I. Kudo and M. Murakami, Showa University, Tokyo) as a template. The resulting polymerase chain reaction product was cloned into BglII–SalI sites of pEGFP-N3 vector (CLONTECH, Palo Alto, CA) to obtain a cPLA2-GFP fusion construct. The orientation of cPLA2 and the integrity of the reading frame was verified by restriction analysis and DNA sequencing.

Cell Culture and Transfection—CHO-K1 cells were maintained under 5% CO2 at 37°C in growth medium (Ham's F12 medium supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin). CHO cells stably expressing PAF receptor or LTB4 receptor were generated as described previously (25, 28). Briefly, cDNAs of guinea pig PAF receptor and human LTB4 receptor were subcloned into mammalian expression vectors, pcDNAI/Neo and pcDNA3 (Invitrogen, Carlsbad, CA), respectively, and transfected into CHO-K1 cells. Clones resistant to Geneticin (1 mg/ml) were isolated by limiting dilution, tested for receptor expressions by binding assay, and maintained in the presence of 0.3 mg/ml Geneticin. LTB4 receptor expressing cells were generated by co-transfection of PAF11 cells expressing PAF receptor (see below) with the expression vector for LTB4 receptor and pPUR encoding the puromycin resistance gene (CLONTECH), and by selection with puromycin (10 μg/ml, Sigma).

For cPLA2-GFP expression, cells were seeded at a density of 5 × 10⁵ cells/60-mm dishes and transiently transfected with 2 μg of the expression vector encoding a cPLA2-GFP fusion protein (pcPLA2-GFP) or a control vector pEGFP-N3 with LipofectAMINE PLUS (Life Technologies, Inc.), according to the manufacturer's protocol, and used for experiments 48–72 h after transfection. The amount of cPLA2-GFP was comparable to that of endogenous cPLA2, as determined by immunoblotting with anti-cPLA2 antibody (data not shown).

Binding Assay—Binding assays for the PAF receptor or the LTB4 receptor on CHO-K1 cells were performed as described previously (29). Briefly, cells on a 24-well culture plate were incubated for 1 h at 25°C with various concentrations of [3H]WEB 2086 or [3H]LTB4 in the presence or absence of unlabeled ligand (20 μM WEB 2086 or 4 μM LTB4, respectively). The cells were washed three times with Hepes/Tyrode’s/BSA buffer (140 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl2, 0.49 mM MgCl2, 5.6 mM D-glucose, 12 mM NaHCO3, 0.37 mM NaH2PO4, 10 mM Hepes (pH 7.4), and 0.1% (w/v) fatty acid-free BSA) and then lysed in 1% Triton X-100. The radioactivity of the cell lysate was determined by liquid scintillation counting. $K_d$ and $B_{max}$ values were calculated by Scatchard analysis.

Single Cell Calcium Imaging—4 × 10⁴ cells were seeded on coverslips (16-mm diameter) in the growth medium and incubated for 24 h.
Expression and downstream signals of PAF and LTB$_4$ receptor in CHO cells

$B_{\text{max}}$ and $K_i$ values for each line were determined by Scatchard analysis of the binding of [3H]WEB 2086 or [3H]LTB$_4$ to the cells. Ca$^{2+}$ response to 100 nM PAF or 1 μM LTB$_4$ was measured as described under "Experimental Procedures." $S$ represents a sustained [Ca$^{2+}$], increase that continued over 5 min. $T$ represents a transient [Ca$^{2+}$], increase that returned to the base line within 5 min. AA release 10 min after stimulation (100 nM PAF or 1 μM LTB$_4$) was measured, and the values were subtracted with background releases without stimulation and expressed as the means ± S.D. from triplicate determinations.

| Cell line | PAF12 | PAF14 | PAF11 | PAF-LT | LT13 | LTF2 |
|-----------|-------|-------|-------|--------|------|------|
| PAFR      | $B_{\text{max}}$ (fmol/10$^5$ cells) | 5     | 14    | 35     | 23   |      |
|           | $K_i$ (nM) |       |       |        |      |      |
| LTB$_4$   | $B_{\text{max}}$ (fmol/10$^5$ cells) |       |       |        |      |      |
|           | $K_i$ (nM) | 2.4   | 2.5   | 2.7    | 2.6  |      |
| Ca$^{2+}$ response | to PAF | to LTB$_4$ | | | | |
|           | $T$ | $S$ | $S$ | $T$ | $S$ | |
| AA release | by PAF (%) | 0.16 ± 0.17 | 7.24 ± 0.61 | 7.53 ± 0.28 | 6.57 ± 0.76 | 0.16 ± 0.02 | 0.13 ± 0.05 | 5.33 ± 0.46 |
|           | by LTB$_4$ (%) | | | | | | |

After another 24 h incubation in serum-free medium containing 0.1% (w/v) fatty acid-free BSA, the cells were washed twice with Hepes/Tyrode's/BSA buffer and incubated at 37°C with 10 μM fura-2 acetoxyethyl ester and 0.01% (w/v) cremophore EL for 1 h in the buffer. After loading, cells were washed with and maintained in the same buffer in the dark at room temperature. Fura-2 fluorescence intensity was measured by alternating excitation at 340 and 380 nm and detecting emission at 505 nm with a 40× objective (Nikon UV-fluor), an SIT camera, and an ARGUS-50/CA image processor (Hamamatsu Photonics, Hamamatsu, Japan). To determine intracellular free Ca$^{2+}$ concentrations, the 340/380 ratio of fluorescence intensities were compared with the ratio for Ca$^{2+}$ standard in solution (Molecular Probes, Eugene, OR).

AA Release Assay—Cells were seeded onto 12-well culture plates at a density of 8 × 10$^4$ cells/well in the growth medium. After 24 h incubation, the medium was removed, and the cells were labeled by incubation for 24 h in 0.75 ml of serum-free medium containing 3.7 kBq of [3H]arachidonic acid (3.7 TBq/mmol) and 0.1% (w/v) fatty acid-free BSA. After another 24 h incubation in serum-free medium containing 0.1% (w/v) fatty acid-free BSA, the cells were washed twice with Hepes/Tyrode's/BSA buffer, and fluorescence images were taken in a FLUOVIEW confocal laser scanning microscope system (Olympus, Tokyo) with a 100× oil immersion objective (NA 1.35), 488-nm laser line for excitation and a 520 ± 20-nm band pass filter for emission.

RESULTS

Agonist-induced Calcium Response and AA Release in CHO Cells Expressing PAF or LTB$_4$ Receptor—Guinea pig PAF receptor and human LTB$_4$ receptor were expressed in CHO-K1 cells that have no detectable endogenous receptors. Six cell lines PAF11, PAF12, PAF14, LT13, LTF2, and PAF-LT were examined for binding assay, agonist-induced Ca$^{2+}$ response (Fig. 1), and AA release. Results obtained using these cell lines are summarized in Table I. In cells with low levels of receptor expression (PAF12 and LT13 cells), stimulation with agonists caused a rapid and transient [Ca$^{2+}$], increase, which returned to the base line within 2–5 min (Fig. 1, B and F). These cell lines did not release AA in response to PAF or LTB$_4$. On the other hand, in cells with high levels of receptor expression (PAF11, PAF14 and LTF2 cells), stimulation with agonists induced a sustained [Ca$^{2+}$], increase which remained elevated at least for 5 min (Fig. 1, A, C, and E) and AA release. In PAF-LT cell line with a high level of PAF receptor and a low level of LTB$_4$ receptor expression, PAF induced a sustained [Ca$^{2+}$], increase (Fig. 1D) and AA release, whereas LTB$_4$ induced a transient [Ca$^{2+}$], increase (Fig. 1G) and little AA release. These results suggest that the sustained [Ca$^{2+}$], increase is necessary for receptor-mediated AA release from CHO cells.

Requirement of Calcium Influx for AA Release—PAF11 cells with a relatively higher receptor expression were used in the following studies. 100 nM PAF induced AA release in the time
course shown in Fig. 2A. It had a 2-min lag phase, followed by a linear increase up to 10 min. PAF induced AA release in a dose-dependent manner, and ED$_{50}$ was around 4 $\times$ 10$^{-10}$ M (Fig. 2B).

We next examined contributions of intracellular and extracellular Ca$^{2+}$ sources in the agonist-induced Ca$^{2+}$ response and AA release. PAF stimulation in the presence of either Ca$^{2+}$ chelator EGTA (10 mM) or Ca$^{2+}$ channel blockers (50 $\mu$M SKF 96365 or 100 $\mu$M (z)-methoxyverapamil) (30, 31) evoked a transient [Ca$^{2+}$]$_i$ increase, which returned to the base line within 2 min following the addition (Fig. 3A). Single cell Ca$^{2+}$ imaging confirmed that addition of EGTA after PAF stimulation created a transient [Ca$^{2+}$]$_i$ increase, which returned to the base line within 1 min following the addition (Fig. 5A). Fig. 5C shows that EGTA added 1 min after PAF stimulation substantially inhibited the PAF-induced AA release but did not alter the AA release when added 5 min after the stimulation.

Since stimulation of the PAF receptor results in various signals besides [Ca$^{2+}$]$_i$ elevation (32), similar experiments were carried out using a Ca$^{2+}$ ionophore ionomycin to study the role of Ca$^{2+}$ signaling in a more direct manner. The prolonged [Ca$^{2+}$]$_i$ increase was generated by 2 $\mu$M ionomycin and had about the same magnitude as the PAF-stimulated plateau (Fig. 5B). The transient [Ca$^{2+}$]$_i$ increase was created by ionomycin with the following addition of EGTA, and this increase returned to the base line within 1 min after the addition (Fig. 5B). The ionomycin-induced sustained [Ca$^{2+}$]$_i$ rise caused AA release, whereas the brief [Ca$^{2+}$]$_i$ rise did not (Fig. 5D), confirming that a prolonged [Ca$^{2+}$]$_i$ increase (over 2 min) is essential for AA release.

Translocation and Reversal of cPLA$_2$ by the Change in [Ca$^{2+}$]$_i$—Due to its inherent fluorescence and unique compact structure, green fluorescent protein (GFP) has been reported in many studies to serve as a valuable reporter in the localization
Duration of Calcium Influx Regulates Arachidonate Release

we constructed a chimeric protein cPLA2-GFP by fusing EGFP to the carboxyl terminus of cPLA2. To ensure that the fusion of GFP did not affect structure and enzyme activity of cPLA2, the cPLA2-GFP construct was analyzed for AA release. The expression of cPLA2-GFP enhanced PAF-induced AA release in PAF11 cells by about 1.5-fold at 10 min. The correlation of AA release with the duration of [Ca\(^{2+}\)], increase was essentially identical to that in untransfected or GFP-transfected cells (Fig. 5, C and E). In addition, gel shift of cPLA2-GFP in response to agonist stimulation was comparable with that of endogenous cPLA2 (data not shown). The gel shift has been established to result from phosphorylation at Ser-505 (7). These results suggest that the fusion of GFP to the carboxyl terminus of cPLA2 does not perturb cPLA2 function.

We then monitored the localization of the cPLA2-GFP in living cells by confocal laser fluorescence microscopy. In the resting state, cPLA2-GFP was almost homogeneously present in the cytosol (Fig. 6, A and B, 0 min). Stimulation of the cells with PAF or ionomycin triggered translocation of cPLA2-GFP to the perinuclear region within 1 min, and the cPLA2-GFP fluorescence was retained for over 10 min (Fig. 6, A and B). When PAF12 cells transiently expressing cPLA2-GFP were stimulated with PAF, the translocation of cPLA2-GFP to the perinuclear region was transient and reversed within 5 min (Fig. 6C). In contrast to cPLA2-GFP, unconjugated GFP was distributed homogeneously in the cytosol and nucleus, and its distribution was not affected by PAF or ionomycin (data not shown).

Next, the effect of termination of [Ca\(^{2+}\)] increase by EGTA on PAF-induced translocation of cPLA2-GFP was examined. cPLA2-GFP translocation was reversed rapidly when EGTA was added 1 min after PAF stimulation (Fig. 7A, upper panels).

In contrast, the reversal was much slower when EGTA was added 5 min after stimulation; a large portion of cPLA2-GFP was retained at the perinuclear region for another 5 min after the addition of EGTA (Fig. 7A, lower panels). Similar redistributions of cPLA2-GFP were obtained by application of ionomycin instead of PAF (Fig. 7B). These results agree with the finding that AA release was inhibited when EGTA was added 1 min after PAF application, while it was not inhibited after 5 min (Fig. 5C), which means that a prolonged [Ca\(^{2+}\)] increase for about 5 min induces stable localization of cPLA2 at the perinuclear region and continuous hydrolysis of AA from membrane phospholipids.

DISCUSSION

cPLA2 plays a major role in releasing AA upon cell stimuli and in production of eicosanoids (10, 11, 26). This enzyme is activated by [Ca\(^{2+}\)] increases in physiological ranges. Although previous reports have shown by immunohistochemical and biochemical methods that cPLA2 is translocated to membranes in response to [Ca\(^{2+}\)] increases, the dynamic change of the enzyme localization in the living cell has not been reported. The question we addressed here was how different Ca\(^{2+}\) signaling patterns control cPLA2 translocation and AA release. We found that a critical time length of [Ca\(^{2+}\)] elevation is required for continuous membrane localization and full activation of cPLA2.

Optimal concentrations of PAF and LTB\(_4\) triggered comparable maximal increases in [Ca\(^{2+}\)] in CHO cell lines expressing PAF and/or LTB\(_4\) receptor. However, duration of the [Ca\(^{2+}\)] increase varied with the cell line (Fig. 1). Correlations between Ca\(^{2+}\) signaling pattern (transient or sustained) and response in AA release (Table I) raised the possibility that the agonist-
induced Ca\(^{2+}\) signaling pattern is responsible for the release of AA. Calcium mobilization from internal stores and subsequent Ca\(^{2+}\) entry from the extracellular space are the two major components of Ca\(^{2+}\) signaling following activation of cell-surface receptors. The inability of PAF to induce AA release in Ca\(^{2+}\)-free medium (Fig. 4A) or in the presence of Ca\(^{2+}\) channel blockers (Fig. 4B) indicates the critical role of Ca\(^{2+}\) influx in AA release. This is consistent with the previous observations, in which AA release has been linked to Ca\(^{2+}\) influx (21). Ca\(^{2+}\) entry across the plasma membrane occurs via voltage-, store-, or receptor-operated channels. Although the primary route of Ca\(^{2+}\) influx in CHO cells remains to be elucidated, 100 \(\mu\)M nifedipine, an L-type voltage-dependent Ca\(^{2+}\) channel blocker, had no apparent effects on the Ca\(^{2+}\) influx induced by PAF or LTB\(_4\) (data not shown).

Stimulation of PAF receptor exerts various responses, including inhibition of adenyl cyclase, activations of MAPK, phosphatidylinositol 3-kinase, phospholipases C and D, and tyrosine kinases, as well as increase in \([\text{Ca}^{2+}]_i\), (24, 32, 34, 35). Although MAPK was activated by PAF and LTB\(_4\) to about the same extent in PAF-LT cells (data not shown), LTB\(_4\) did not induce AA release (Table I), suggesting that MAPK activation with a transient \([\text{Ca}^{2+}]_i\) increase is insufficient to induce AA release. The experiments using ionomycin and EGTA confirmed that a sustained \([\text{Ca}^{2+}]_i\) increase is essential for AA release (Fig. 5D). The difference in the extent of AA release between PAF and ionomycin (Fig. 5, C and D) is probably due to phosphorylation of cPLA\(_2\) by MAPK. Whereas PAF induced MAPK activation (34) and mobility shift of cPLA\(_2\) in SDS-polyacrylamide gel electrophoresis (36), ionomycin did not activate MAPK or promote the gel shift of cPLA\(_2\) (data not shown). These results are consistent with the previous reports that phosphorylation of cPLA\(_2\) increases its intrinsic enzymatic activity (7, 37, 38) and augments Ca\(^{2+}\)-induced AA release (26, 39).

It was recently reported that, in mouse peritoneal macrophages, the effects of phosphorylation of cPLA\(_2\) on AA release differ depending on whether calcium response is transient or sustained (40). Further studies are necessary to identify fully the individual and combined roles of calcium and phosphorylation for the activation of cPLA\(_2\) in various types of cells.

Calcium-mediated translocation of heterologously expressed cPLA\(_2\) to the nuclear envelope and endoplasmic reticulum was shown previously in CHO cells using an anti-cPLA\(_2\) antibody (14). Because immunohistochemical studies provide only a static picture and have a possible loss in signal linearity due to fixation, permeabilization, and staining of the cells, we took advantage of the GFP fusion protein and digital imaging technique to monitor cPLA\(_2\) translocation in real time in living cells. Although the endoplasmic reticulum forms a fine reticular network throughout the cytoplasm and around the nuclear envelope in CHO cells (41), cPLA\(_2\)-GFP was translocated upon stimulation with a calcium ionophore A23187 induced translocation of cPLA\(_2\) to the nuclear membrane fraction and preferential loss of AA from this membrane fraction (42). Therefore, functional activation of cPLA\(_2\) takes place after translocation to the perinuclear region. Mechanisms involved in the preferential targeting of the enzyme to this site are under investigation.

Another important finding of the present study is that a brief \([\text{Ca}^{2+}]_i\) rise caused a transient translocation of cPLA\(_2\) without AA release, whereas an increase in \([\text{Ca}^{2+}]_i\) of longer duration (~5 min) induced a prolonged translocation of cPLA\(_2\) and AA release even after \([\text{Ca}^{2+}]_i\) returned to the resting level (Figs. 5 and 7). To explain these observations, we propose a two-step...
mechanism for cPLA₂ activation. In the initial step, cPLA₂ is translocated to the perinuclear region upon increase in [Ca²⁺], and weakly binds to the membranes. The enzyme is not yet activated, and the translocation can be readily reversed upon decrease in [Ca²⁺]. In the second step, the binding of cPLA₂ to the membranes becomes stronger with a marked reduction in the dissociation rate. Concomitantly, the enzyme is activated. The transfer from the first step to the second requires a critical length of continuous [Ca²⁺], increase was −2 min in PAF11 cells but may differ depending on the cell type and the level of CPLA₂ expression. Possible mechanisms by which cPLA₂ is transferred from the weak-binding state to the strong-binding state include conformational change of cPLA₂ by phosphorylation (7), dissociation of inhibitory proteins such as p11 (43), and interaction with anchoring or activating factors (44). The requirement of critical duration of [Ca²⁺] increase for the activation of cPLA₂ may serve as a safety mechanism to discriminate appropriate signals from transient fluctuations of [Ca²⁺]. Once the [Ca²⁺] transient exceeds the critical duration, the strong-binding state of cPLA₂ allows the cells to produce AA for prolonged periods even after [Ca²⁺] returns to the resting value.

Acknowledgments—We thank Drs. H. Hanaka and T. Izumi for plas-mids; Dr. Y. Kaziro and other laboratory members (University of Tokyo and Tokyo Institute of Technology) for valuable discussions; and M. Ohara for comments.

REFERENCES
1. Irvine, R. F. (1982) Biochem. J. 204, 3–16
2. Clark, J. D., Schievella, A. R., Nalefski, E. A., and Lin, L.-L. (1995) J. Lipid Mediat. Cell Signal. 12, 83–117
3. Dennis, E. A. (1997) Trends Biochem. Sci. 22, 1–2
4. Tischfield, J. A. (1997) J. Biol. Chem. 272, 17247–17250
5. Balsinde, J., and Dennis, E. A. (1997) J. Biol. Chem. 272, 16069–16072
6. Clark, J. D., Lin, L.-L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N., and Knopf, J. L. (1991) Cell 65, 1943–1951
7. Lin, L.-L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A., and Davis, R. J. (1993) Cell 72, 269–278
8. Kramer, R. M., and Sharp, J. D. (1997) FEBS Lett. 410, 49–53
9. Leslie, C. C. (1997) J. Biol. Chem. 272, 16709–16712
10. Usui, N., Kume, K., Nagase, T., Nakatani, N., Ishii, S., Tashiro, F., Komagata, Y., Maki, K., Ikuta, K., Ouchi, Y., Miyazaki, J., and Shimizu, T. (1997) Nature 390, 618–622
11. Bonventre, J. V., Huang, Z., Taheri, M. R., O’Leary, E., Li, E., Moskowitz, M. A., and Sapienza, A. (1997) Nature 390, 622–625
12. Nalefski, E. A., and Falke, J. J. (1996) Protein Sci. 5, 2375–2390
13. Glover, S., Bayburt, T., Jonas, M., Chi, E., and Gelb, M. H. (1995) J. Biol. Chem. 270, 15359–15367
14. Schievella, A. R., Regier, M. K., Smith, W. L., and Lin, L.-L. (1995) J. Biol. Chem. 270, 30749–30754
15. Rollins, T. R., and Smith, W. L. (1980) J. Biol. Chem. 255, 4872–4875
16. Serhan, C. N., Haegstrom, J. Z., and Leslie, C. C. (1996) FASEB J. 10, 1147–1158
17. Spencer, A. G., Woods, J. W., Arakawa, T., Singer, I. I., and Smith, W. L. (1998) J. Biol. Chem. 273, 9886–9893
18. Woods, J. W., Evans, J. F., Ethier, D., Scott, S., Vickers, P. J., Hearn, L., Heibein, J. A., Charleson, S., and Singer, I. (1993) J. Exp. Med. 178, 1935–1946
19. Goetzl, E. J., An, S., and Smith, W. L. (1995) FASEB J. 9, 1051–1058
20. Penrose, J. F., Spektor, J., Lam, B. K., Friend, D. S., Xu, R., Jack, R. M., and Austen, K. F. (1995) Am. J. Respir. Crit. Care Med. 152, 283–289
21. Brooks, R. C., McCarthy, K. D., Lapetina, E. G., and Morell, P. (1989) J. Biol. Chem. 264, 20147–20153
22. Krump, E., Pouliot, M., Naccache, P. H., and Borgeat, P. (1995) Biochem. J. 310, 681–688
23. Miyakawa, T., Kojima, M., and Ui, M. (1998) Biochem. J. 329, 107–114
24. Honda, Z., Nakamura, M., Miki, I., Minami, M., Watanabe, T., Seyama, Y., Okado, H., Toh, H., Ito, K., Miyamoto, T., and Shimizu, T. (1991) Nature 349, 342–346
25. Yokomizo, T., Izumi, T., Chang, K., Takuwa, Y., and Shimizu, T. (1997) Nature 387, 620–624
26. Lin, L.-L., Lin, A. Y., and Knopf, J. L. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 6147–6151
27. Vial, D., and Piomelli, D. (1995) J. Neurochem. 64, 2675–2772
28. Takano, T., Honda, Z., Sakakana, C., Szeto, K., Kameyama, K., Haga, K., Haga, T., Kurokawa, K., and Shimizu, T. (1994) J. Biol. Chem. 269, 22453–22458
29. Ishii, I., Izumi, T., Tsukamoto, H., Uneyama, H., Ui, M., and Shimizu, T. (1997) J. Biol. Chem. 272, 7846–7854
30. Gussovsky, F., Luinders, J. E., Kohn, E. C., and Felder, C. C. (1993) J. Biol. Chem. 268, 7768–7772
31. Kiley, N., Leeffer, J. P., Pittius, C. W., and Hollt, V. (1987) J. Biol. Chem. 262, 4083–4089
32. Izumi, T., and Shimizu, T. (1995) Biochim. Biophys. Acta 1259, 317–333
33. Cubitt, B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A., and Tsien, R. Y. (1995) Trends Biochem. Sci. 20, 448–455
34. Honda, Z., Takane, T., Gotoh, Y., Nishida, E., Ito, K., and Shimizu, T. (1994) J. Biol. Chem. 269, 2307–2315
35. Stephenson, L., Jackson, T., and Hawkins, P. T. (1993) J. Biol. Chem. 268, 17162–17172
36. Sakakana, C., Ferby, I., Waga, I., Bito, H., and Shimizu, T. (1994) Biochem. Biophys. Res. Commun. 205, 18–23
37. Nemenoff, R. A., Winitz, S., Qian, N. X., Van Putten, V., Johnson, G. L., and Heasley, L. E. (1993) J. Biol. Chem. 268, 1960–1964
38. Kramer, R. M., Roberts, E. F., Manetta, J. V., Hyslop, P. A., and Jakubowski, J. A. (1993) J. Biol. Chem. 268, 26786–26804
39. Xu, X. X., Rock, C. O., Qu, Z.-H., Leslie, C. C., and Jackowski, S. (1994) J. Biol. Chem. 269, 31693–31700
40. Qu, Z.-H., Gijon, M. A., de Carvalho, M. S., Spence, D. M., and Leslie, C. C. (1998) J. Biol. Chem. 273, 8203–8211
41. Náray-Fejes-Toth, A., and Fejes-Toth, G. (1996) J. Biol. Chem. 271, 15436–15442
42. Peters-Golden, M., Song, K., Marshall, T., and Brock, T. (1996) Biochem. J. 318, 797–803
43. Wu, T., Angus, C. W., Yao, X. L., Logun, C., and Shelhamer, J. H. (1997) J. Biol. Chem. 272, 17145–17153
44. Mosior, M., Six, D. A., and Dennis, E. A. (1998) J. Biol. Chem. 273, 2184–2191
Critical Duration of Intracellular Ca^{2+} Response Required for Continuous
Translocation and Activation of Cytosolic Phospholipase A_{2}

Tetsuya Hirabayashi, Kazuhiko Kume, Kenzo Hirose, Takehiko Yokomizo, Masamitsu
Iino, Hiroshi Itoh and Takao Shimizu

J. Biol. Chem. 1999, 274:5163-5169.  
doi: 10.1074/jbc.274.8.5163

Access the most updated version of this article at http://www.jbc.org/content/274/8/5163

Alerts:  
• When this article is cited  
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 44 references, 27 of which can be accessed free at
http://www.jbc.org/content/274/8/5163.full.html#ref-list-1