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Direct Relationship between Intracellular Calcium Mobilization and Phospholipase D Activation in Prostaglandin E-Stimulated Human Erythroleukemia Cells†

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ABSTRACT: The relationship between calcium mobilization and phospholipase D (PLD) activation in response to E-series prostaglandins (PGEs) was investigated in human erythroleukemia cells. Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was increased by PGE_1 and PGE_2 over the same concentration range at which PLD activation was seen. Pretreatment of cells with pertussis toxin greatly inhibited the PGE-stimulated increase in $[Ca^{2+}]_i$, implying that a G protein participates in the PGE receptor signaling process. The peak level and also the plateau level of Ca^{2+} mobilization stimulated by these prostaglandins were markedly decreased in Ca^{2+}-depleted medium, indicating that both extracellular and intracellular Ca^{2+} stores contribute to the changes in $[Ca^{2+}]_i$. Likewise, activation of PLD by PGE_1 and PGE_2 was abolished by pertussis toxin pretreatment or incubation in Ca^{2+}-depleted medium. U73122, a putative phospholipase C inhibitor, blocked both Ca^{2+} mobilization and PLD activation in PGE-stimulated cells. Furthermore, the intracellular loading of BAPTA, a Ca^{2+} chelator, inhibited both Ca^{2+} mobilization and PLD activation by PGE_1 and PGE_2 in a similar dose-dependent manner. Simultaneous measurement of $[Ca^{2+}]_i$ and PLD activity in the same cell samples indicated that PLD activity increases as a function of $[Ca^{2+}]_i$ in a similar fashion in cells stimulated either by PGEs or by the calcium ionophore ionomycin. Taken together, these findings suggest that a rise in $[Ca^{2+}]_i$ is necessary for PGE-stimulated PLD activity in human erythroleukemia cells.

Hydrolysis of phospholipids by phospholipase D (PLD)[1] yields phosphatidic acid (PA) and polar head group "bases" such as choline and ethanolamine (Heller, 1978). PA may itself act as a second messenger to elicit cellular effects (Ohsako & Deguchi, 1981; Moolenaar et al., 1986; Murayama & Ui, 1987) or be further metabolized by PA phosphohydrolase to diacylglycerol (Billah et al., 1989), an activator

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[1] Abbreviations: HEL cells, human erythroleukemia cells; IP_3, inositol 1,4,5-trisphosphate; PA, phosphatidic acid; PEt, phosphatidylethanol; PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2; PLC, phosphoinositide-specific phospholipase C; PLD, phospholipase D; PMA, 4β-phorbol 12-myristate 13-acetate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; MTH medium, modified Tyrode-Hepes medium.

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of protein kinase C. Within the past few years, increasing attention has been focused on the possible role of PLD in cellular signal transduction pathways and functional responses.

Although PLD activation has been observed in response to a variety of agonists acting through membrane receptors (Billah & Anthes, 1990; Shukla & Halenda, 1991), the mechanisms which couple receptor occupation to PLD activation have not been determined. Several studies imply a relationship between PLD and phosphoinositide-specific phospholipase C (PLC) since most of the agonists, if not all, which stimulate PLC also activate PLD (Billah & Anthes, 1990). In some cell types, the PLD activity stimulated by receptor-mediated agonists is dependent on the extracellular Ca^{2+} concentration (Pai et al., 1988; Mullmann et al., 1990; Anthes et al., 1991; Wu et al., 1991) or an intact protein kinase C pathway (Huang & Cabot, 1990; Martinson et al., 1990; Cook & Wakelam, 1991; van Blitterswijk et al., 1991). Furthermore, in general, calcium ionophores and phorbol esters are able to activate PLD (Billah & Anthes, 1990; Shukla & Halenda, 1991). These findings have led to the suggestion that PLD activation is secondary to the receptor-coupled activation of PLC, although no conclusive evidence for such a pathway has so far been reported.

In human erythroleukemia (HEL) cells, PLD is stimulated by PGE_1 and PGE_2 in a Ca^{2+}-dependent manner (Wu et al., 1991). Furthermore, a G protein seems to be an essential component in the PGE signal transduction pathway in HEL cells because pertussis toxin, an ADP-ribosyltransferase which uncouples certain types of G proteins from cell-surface receptors (Gilman, 1987), abolishes PGE-stimulated PLD activation (Wu et al., 1991). These data suggest that PLD may be activated through the following pathway: PGE receptor → pertussis toxin-sensitive G protein → Ca^{2+} mobilization → PLD. In this study, we further explored the mechanism responsible for PGE-stimulated PLD activation with a focus on the role of $[Ca^{2+}]_i$. On the basis of results from several different experimental approaches, we conclude that PLD is activated primarily via an increase in $[Ca^{2+}]_i$ in HEL cells stimulated by E-series prostaglandins.

Experimental Procedures

Materials. The following reagents were purchased from the sources indicated: [^{3}H]myristic acid (39.3 Ci/mmol), New England Nuclear (Boston, MA); [^{3}H]adenine (10–25 Ci/mmol), American Radiolabeled Chemicals (St. Louis, MO); phosphatidylethanol standard, Avanti (Pelham, AL); fetal bovine serum, GIBCO (Grand Island, NY); prostaglandins, Cayman Chemical (Ann Arbor, MI); pertussis toxin, List Biological Laboratories (Campbell, CA); fura-2/AM and BAPTA/AM, Molecular Probes (Eugene, OR); ionomycin, Calbiochem (La Jolla, CA). All other reagents were purchased from Sigma (St. Louis, MO). U73122 was a generous gift from Dr. John E. Bleasdale, The Upjohn Co. (Kalamazoo, MI). UK14304 was kindly provided by Pfizer Central Research (Sandwich, Kent, England).

Cell Culture. HEL cells were grown in RPMI 1640 medium supplemented with 5% (v/v) fetal bovine serum and 1 mM glutamine under an atmosphere of 5% CO_2/95% air at 37 °C. The cell density was maintained at (1–2) × 10^6 cells/mL in suspension culture by dilution with fresh medium.

Assay of PLD Activity. PLD activity was determined as previously described (Wu et al., 1991), by measuring the formation of [^{3}H]phosphatidylethanol (PEt), an unequivocal indicator of PLD activity which is produced via PLD-catalyzed transphosphatidylation (Yang et al., 1967; Heller, 1978). Briefly, HEL cell phospholipids were metabolically prelabeled with [^{3}H]myristic acid, and cells were then incubated in buffer containing 0.5% (v/v) ethanol at 37 °C for 5 min, followed by drug treatments for various times. PEt was separated from other phospholipids by thin-layer chromatography, and radioactivity was measured by liquid scintillation counting. PEt formation is expressed as the percent of total cell-associated radioactivity (Wu et al., 1991).

Cyclic AMP Production. Conversion of [^{3}H]ATP to cyclic AMP was assayed in [^{3}H]adenine-prelabeled HEL cells in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (0.2 mM), as described previously (Wu et al., 1991).

$[Ca^{2+}]_i$ Measurements. HEL cells were harvested by centrifugation and resuspended in a modified Tyrode–Hepes (MTH) medium (134 mM NaCl, 2.9 mM KCl, 4 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 5 mM dextrose, 0.2 mg/mL fatty acid-free bovine serum albumin, and 10 mM Hepes, pH 7.4 at 37 °C) with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (cell density 1 × 10^6 cells/mL). Cells were then loaded with 2 μM fura-2/AM for 60 min at 37 °C. After cells were loaded, they were pelleted, washed, and incubated for a further 30 min to allow the fura-2/AM to deesterify maximally to fura-2. Cells were then washed once and resuspended at 1 × 10^6 cells/mL in fresh medium. Just prior to assay, cell aliquots (2 mL) were spun in a microcentrifuge, and cells were resuspended in 2 mL of MTH with 0.1 mM $MgCl_2$ and 1 mM $CaCl_2$ in most of the experiments, or with various calcium concentrations in the ionomycin experiments (Figures 5 and 6), and transferred to a magnetically-stirred quartz cuvette maintained at 37 °C. Fluorescence was then measured with 1-s resolution in a SPEX CM1T11I spectrofluorometer (SPEX, Edison, NJ) with excitation at 340 and 380 nm and emission at 505 nm. The Ca^{2+}-saturated and Ca^{2+}-free fluorescence levels of the fura-2 were determined by addition of 50 μM digitonin in the presence of 1 mM $CaCl_2$ and of 20 mM EGTA/0.25 M Tris buffer (pH 8.7), respectively (Tsien et al., 1982). Autofluorescence was measured in cells which were not loaded with fura-2/AM. After subtraction of the autofluorescence, $[Ca^{2+}]_i$ was calculated according to the ratio method as follows:

$$[Ca^{2+}]_i = K_d S_{f380/b380}(R - R_{min})/(R_{max} - R)$$

where K_d is 224 nM, $S_{f380/b380}$ is the ratio of the intensities of the free and bound dye forms at 380 nm, R is the fluorescence ratio (340 nm/380 nm) of the intracellular fura-2, and R_{min} and R_{max} are the minimal and maximal fluorescence ratios, respectively (Grynkiewicz et al., 1985). The R_{max} was about 30, indicating complete hydrolysis of fura-2/AM to fura-2.

Pretreatment of Cells with U73122 or BAPTA/AM. Base-line fluorescence was usually recorded for 1 min before agonists were added. For those samples requiring pretreatment with U73122 (1 μM), the drug was added after the 1-min base-line tracing, and the fluorescence was monitored for 5 min before cells were stimulated with agonists. In Figure 4, BAPTA/AM (1, 5, or 10 μM) or vehicle (0.1% DMSO) as a control was added to cells during the final 30 min of loading with fura-2/AM. After being loaded, cells were processed, and $[Ca^{2+}]_i$ was monitored as described above.

Simultaneous Measurement of $[Ca^{2+}]_i$ and PLD Activity. $[Ca^{2+}]_i$ and [^{3}H]PEt formation were measured simultaneously in some experiments (Figures 4 and 6) to assess the relationship between $[Ca^{2+}]_i$ and PLD activation under identical conditions in the same cell samples. For these experiments, cells were treated essentially as described above for $[Ca^{2+}]_i$ measurement except that 5 μCi/mL [^{3}H]myristic acid was added during the loading period and 0.5% ethanol was present during stimulation. Basal $[Ca^{2+}]_i$ was then followed for 1 min, and cells were

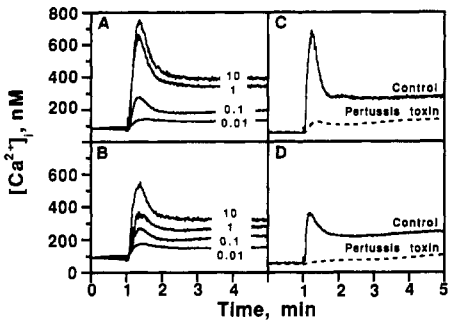

FIGURE 1: Stimulation of Ca^{2+} mobilization by PGE_1 and PGE_2 in HEL cells and the effect of pertussis toxin. (Panels A and B) Changes of $[Ca^{2+}]_i$ in response to the indicated concentrations (μM) of PGE_1 (panel A) or PGE_2 (panel B) are shown. Data are representative of seven (PGE_1) or four (PGE_2) separate experiments. (Panels C and D) After pretreatment with or without 100 ng/mL pertussis toxin for 24 h, HEL cells were loaded with fura-2/AM, and changes in $[Ca^{2+}]_i$ stimulated by 10 μM PGE_1 (panel C) or PGE_2 (panel D) were determined. Control, solid line; pertussis toxin, dashed line. Tracings are representative of three (PGE_1) or two (PGE_2) experiments.

incubated with or without agonists for a further 5 min. At the end of the 5-min stimulation period, duplicate 0.5-mL cell aliquots, out of a total sample volume of 3 mL, were quickly withdrawn from the cuvette and quenched in chloroform/methanol solvent in order to determine [^{3}H]PEt formation. The remaining 2-mL cell sample was used to calibrate the fura-2 signal for conversion to $[Ca^{2+}]_i$. We observed no difference in basal or agonist-stimulated $[Ca^{2+}]_i$ in medium with or without 0.5% ethanol (data not shown).

RESULTS

Ca^{2+} Mobilization in Response to PGEs. Since our major aim in this study was to assess the role of Ca^{2+} in PLD activation, we first examined the effects of PGE_1 and PGE_2 on Ca^{2+} mobilization in fura-2-loaded HEL cells. Basal $[Ca^{2+}]_i$ was 66 ± 5 nM (n = 7), consistent with previous reports in HEL cells (Motulsky & Michel, 1988; Michel et al., 1989). The time course of changes in $[Ca^{2+}]_i$ following addition of prostaglandins at various concentrations from 0.01 to 10 μM is depicted in Figure 1 (panels A and B). Both PGE_1 and PGE_2 elicited a rapid rise of $[Ca^{2+}]_i$ in a dose-dependent manner; Ca^{2+} mobilization displayed a similar prostaglandin concentration dependence to that previously reported for PLD activation (Wu et al., 1991). A rise in $[Ca^{2+}]_i$ could be detected at prostaglandin concentrations as low as 0.01 μM. The peak $[Ca^{2+}]_i$ occurred within 10–20 s after agonist stimulation. The average values of the peak $[Ca^{2+}]_i$ in response to various concentrations of PGE_1 and PGE_2 are shown in Table I. It is interesting to note that the PGE-initiated rise in $[Ca^{2+}]_i$ reached a sustained plateau after 90–120 s. The plateau level averaged 328 ± 26 nM (n = 7) and 257 ± 33 nM (n = 4) after stimulation by 10 μM PGE_1 and PGE_2, respectively.

Effect of Pertussis Toxin Treatment on Ca^{2+} Mobilization. Previously, we demonstrated that pertussis toxin pretreatment abolished PGE-stimulated PLD activity, implying that a G protein is involved in the PGE receptor signal transduction pathway (Wu et al., 1991). It was then of interest to test the effect of this toxin on Ca^{2+} mobilization in response to PGEs. As shown in Figure 1 (panels C and D), the peak and plateau levels of $[Ca^{2+}]_i$ stimulated by PGE_1 and PGE_2 were dramatically decreased by treatment of cells with 100 ng/mL pertussis toxin. This toxin concentration has been shown to produce maximal ADP-ribosylation of G proteins (Brass et al., 1991) and to abolish PGE-stimulated PLD activity (Wu et al., 1991). These results indicate that the changes in $[Ca^{2+}]_i$

Table I: Effects of PGE_1 and PGE_2 on $[Ca^{2+}]_i$ in HEL Cells[a]

treatment	peak $[Ca^{2+}]_i$ (nM) (mean ± SE)
basal	66 ± 5
PGE_1 (μM)	
0.01	134 ± 22
0.1	289 ± 35
1	627 ± 48
10	721 ± 55
PGE_2 (μM)	
0.01	130 ± 26
0.1	226 ± 36
1	327 ± 58
10	416 ± 71

[a] Changes of $[Ca^{2+}]_i$ in response to the indicated concentrations of PGEs were measured in fura-2-loaded cells as described under Experimental Procedures. Data are a summary of seven (PGE_1) or four (PGE_2) experiments. Representative tracings are shown in Figure 1.

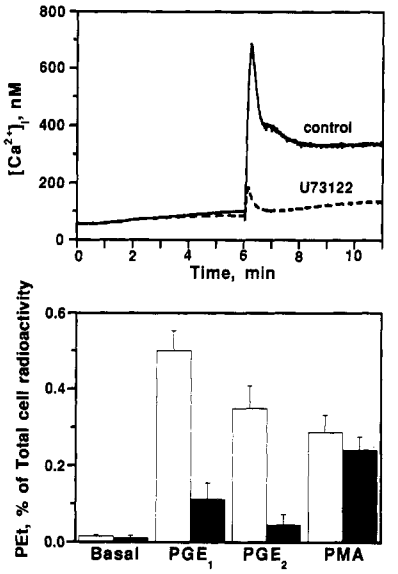

FIGURE 2: Effect of U73122 on PGE-stimulated Ca^{2+} mobilization and PLD activity in HEL cells. (Upper panel) $[Ca^{2+}]_i$ was recorded for 1 min, followed by the addition of 1 μM U73122 (dashed line) or 0.1% DMSO (control, solid line). After a 5-min pretreatment, 10 μM PGE_1 was added. Tracings are representative of four experiments. (Lower panel) [^{3}H]PEt formation was measured in HEL cells pretreated with (filled bars) or without (control, open bars) 1 μM U73122 for 5 min and then stimulated with PGEs (10 μM) or PMA (100 nM) for an additional 15 min. Data are means ± SE from four experiments, each conducted in duplicate. PMA itself at 100 nM caused no change in $[Ca^{2+}]_i$ in HEL cells (data not shown).

initiated by PGEs are regulated through a pertussis toxin-sensitive G protein (G_i or G_o) and are in keeping with the notion that PLD activation is secondary to Ca^{2+} mobilization. However, alternate possibilities are that PLD and $[Ca^{2+}]_i$ are controlled by distinct G proteins, both of which serve as substrates for pertussis toxin, or that the same G protein regulates both responses independently of one another.

Effect of U73122 on PGE-Stimulated Ca^{2+} Mobilization and PLD Activity. If PLD activation is in fact secondary to a rise of $[Ca^{2+}]_i$ in HEL cells, then it would be expected that any approach which diminishes PGE-stimulated Ca^{2+} mobilization by an action at the post-G-protein level will also block PLD activation. U73122, a putative PLC inhibitor, has been shown to block agonist-induced Ca^{2+} mobilization in neutrophils and platelets (Bleasdale et al., 1990; Smith et al., 1990), possibly through inhibition of PLC and the resultant formation of inositol 1,4,5-trisphosphate (IP_3). As shown in Figure 2 (upper panel), the addition of 1 μM U73122 to HEL cells did not result in any change in basal $[Ca^{2+}]_i$ through the 5-min

Table II: Effect of U73122 on Cyclic AMP Production[a]

treatment	cyclic AMP (% conversion) control	U73122	n
basal	0.07 ± 0.02	0.05 ± 0.01	4
PGE_1	1.44 ± 0.27	1.39 ± 0.43	4
FSK	0.41 ± 0.14	0.44 ± 0.15	3
UK14304	0.06 ± 0.02	0.05 ± 0.02	3
FSK + UK14304	0.18 ± 0.06	0.17 ± 0.06	3

[a] HEL cells were loaded with [^{3}H]adenine, pretreated with or without 1 μM U73122 for 5 min, and then incubated for 5 min with PGE_1 (10 μM), UK14304 (10 μM), or forskolin (FSK, 20 μM) in the presence of 0.2 mM isobutylmethylxanthine. ATP and cyclic AMP were separated by Dowex and alumina column chromatography after reactions were terminated with trichloroacetic acid. Radioactivity in these nucleotides was measured by scintillation counting, and data are presented as the percent conversion of radioactive ATP to cyclic AMP. Values are means ± SE from the indicated number of experiments.

pretreatment, but greatly diminished the PGE_1-stimulated $[Ca^{2+}]_i$ rise compared to control. Similar results were obtained with PGE_2 as the agonist (data not shown). Under the same conditions, PLD activity stimulated by PGE_1 or PGE_2 was inhibited by 79% and 90%, respectively (Figure 2, lower panel). Phorbol myristate acetate (PMA), which has previously been shown to stimulate PLD in HEL cells and many other cell types (Shukla & Halenda, 1991), was employed as a positive control. PLD activation in response to PMA remained intact in the presence of 1 μM U73122 (Figure 2, lower panel), suggesting that U73122 does not directly inhibit PLD under these conditions. Likewise, ionomycin-stimulated PEt formation was not inhibited by 1 μM U73122 (data not shown).

Effects of U73122 on other cellular activities were also studied. U73122 did not influence PGE_1-stimulated cyclic AMP production (Table II), suggesting there was no effect of this inhibitor either at the receptor level or on G_s or adenylyl cyclase. Data from Figure 1 and the previous report by Wu et al. (1991) suggest the involvement of a pertussis toxin-sensitive G protein (G_i or G_o) in the PGE receptor signal transduction pathway. Since G_i but not G_o is expressed in HEL cells (Michel et al., 1989), we were especially concerned with the possibility that U73122 might inhibit G_i. To address this potential nonspecific action, the effect of U73122 on α_2-adrenergic receptor-mediated inhibition of adenylyl cyclase activity, a pathway regulated by G_i (Gilman, 1987), was tested. As shown in Table II, no change in the inhibition of forskolin-stimulated cyclic AMP production by UK14304 (an α_2-adrenergic agonist) was found in the presence of 1 μM U73122. This result tends to rule out a direct effect of U73122 on G_i. Taken together, these data further support a role for the agonist-induced $[Ca^{2+}]_i$ increase in regulation of PLD activity, since U73122 inhibited both Ca^{2+} mobilization and PLD activation and the mechanism of this inhibition was unlikely to be a nonspecific effect at the receptor or G-protein level.

Extracellular [Ca^{2+}] Dependence of PGE-Mediated Effects in HEL Cells. Following the initial peak in $[Ca^{2+}]_i$, the presence of a sustained elevated plateau level of $[Ca^{2+}]_i$ suggested that Ca^{2+} influx from the extracellular space might contribute to Ca^{2+} mobilization in response to PGEs (Figure 1). Since our previous study showed an extracellular Ca^{2+} requirement for PGE-stimulated PLD activation (Wu et al., 1991), we wished to determine the extent to which the PGE-elicited rise in $[Ca^{2+}]_i$ depends on extracellular Ca^{2+}. Ca^{2+} mobilization and PLD activation were compared in cells incubated in normal MTH medium with 1 mM Ca^{2+}, or in cells suspended just before the $[Ca^{2+}]_i$ measurement in Ca^{2+}-omitted medium containing 0.1 mM EGTA ("Ca^{2+}-depleted medium"). The free Ca^{2+} concentration, as measured by a Ca^{2+} electrode (Orion), in Ca^{2+}-depleted medium was 73 ± 15 nM (n = 3). Removal of extracellular Ca^{2+} only slightly decreased basal $[Ca^{2+}]_i$ in HEL cells (66 ± 10 and 55 ± 7 nM in normal Ca^{2+} and Ca^{2+}-depleted medium, respectively, n = 5). Figure 3 (upper panel) shows that in Ca^{2+}-depleted medium, PGE_1 was still able to evoke a rapid but relatively small increase of $[Ca^{2+}]_i$ which quickly diminished to base line. Similar results were seen with PGE_2 as the agonist (data not shown). This indicated that the changes of $[Ca^{2+}]_i$ in response to PGEs in normal MTH medium (containing 1 mM Ca^{2+}) were from extracellular as well as intracellular stores. In normal Ca^{2+} vs Ca^{2+}-depleted medium, respectively, the peak $[Ca^{2+}]_i$ levels were 697 ± 27 nM compared to 207 ± 22 nM in cells stimulated with 10 μM PGE_1 (n = 3) and were 344 ± 87 nM vs 166 ± 14 nM with 10 μM PGE_2 (n = 2). PGE-stimulated but not PMA-stimulated PLD activity was abolished by incubation of cells in the Ca^{2+}-depleted medium (Figure 3, lower panel). These data suggest that the small and transient rise of $[Ca^{2+}]_i$ in Ca^{2+}-depleted medium may be insufficient to activate PLD via the receptor-coupled pathway.

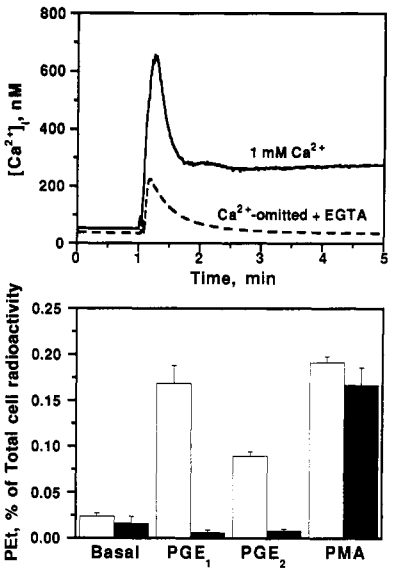

FIGURE 3: Extracellular [Ca^{2+}] dependence of Ca^{2+} mobilization and PLD activation. Changes of $[Ca^{2+}]_i$ in response to PGE_1 (upper panel) and PLD activity in response to various stimuli (lower panel) were determined in fura-2-loaded and [^{3}H]myristic acid-labeled HEL cells, respectively. Experiments were performed in normal MTH medium containing 1 mM $CaCl_2$ (control, solid line in Ca^{2+} tracings, and open bars in lower panel) or in Ca^{2+}-omitted medium with 0.1 mM EGTA (dashed line, filled bars). Concentrations of PGEs and PMA were 10 μM and 100 nM, respectively. $[Ca^{2+}]_i$ tracings are representative of three experiments. Data in the lower panel are means ± SE from three experiments performed in duplicate.

Inhibition of the $[Ca^{2+}]_i$ Rise and PLD Activity by BAPTA. BAPTA/AM is a cell permeant precursor for the Ca^{2+}-selective chelator BAPTA (Lew et al., 1982). This compound has been used to buffer agonist-induced increases in $[Ca^{2+}]_i$ and thereby gain insight into the role of $[Ca^{2+}]_i$ in cell responses (Negulescu et al., 1989). To test the effect of BAPTA on PGE-initiated responses, HEL cells were loaded with both fura-2 and [^{3}H]myristic acid along with various amounts of BAPTA. $[Ca^{2+}]_i$ and PEt formation in response to PGEs was then measured in the same samples. Loading of BAPTA into the cells led to a buffering of the rise in $[Ca^{2+}]_i$ initiated by PGE_1 (Figure 4, inset) and PGE_2 (data not shown) in a dose-dependent manner. When cells were preincubated in the presence of 1 μM BAPTA/AM, there was a somewhat slower

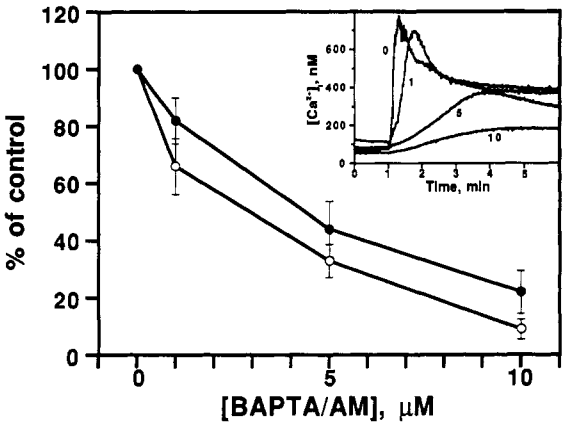

FIGURE 4: Comparison of the effect of BAPTA on the $[Ca^{2+}]_i$ rise and PLD activation in response to PGE_1. Cells were simultaneously labeled with [^{3}H]myristic acid and loaded with fura-2/AM in the presence or absence of the indicated concentrations of BAPTA/AM as described under Experimental Procedures. After base-line $[Ca^{2+}]_i$ was determined for 1 min, cells were stimulated for 5 min with 10 μM PGE_1. Open symbols, peak $[Ca^{2+}]_i$ levels; closed symbols, [^{3}H]PEt formation. Data are means ± SE of three experiments. The peak $[Ca^{2+}]_i$ level in response to PGE_1, determined in cells without BAPTA/AM treatment, was 938 ± 51 nM; PEt formation under these conditions was 0.255 ± 0.046% of total cell radioactivity. Inset: Data shown are representative fura-2 tracings from cells loaded with the indicated concentrations (μM) of BAPTA/AM and then stimulated with 10 μM PGE_1 at the 1-min point on the x axis.

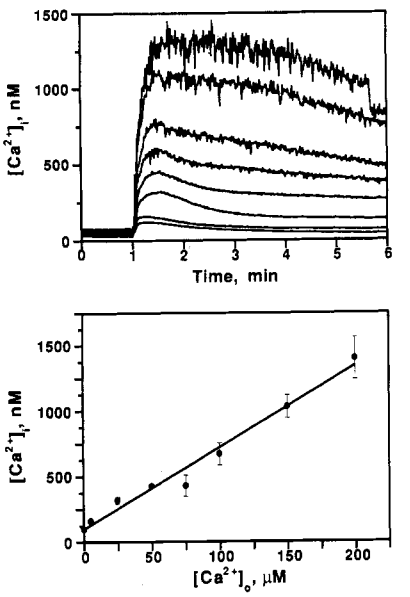

FIGURE 5: Changes of $[Ca^{2+}]_i$ in ionomycin-stimulated HEL cells as a function of $[Ca^{2+}]_o$. (Upper panel) The incubation media for each tracing (from bottom to top) were as follows: Ca^{2+}-omitted plus 1 mM EGTA, Ca^{2+}-omitted without EGTA, or containing 25, 50, 75, 100, 150, or 200 μM added Ca^{2+}, respectively. Ionomycin (1 μM) was added at the 1-min point. (Lower panel) The peak $[Ca^{2+}]_i$ levels obtained from three such experiments are depicted as means ± SE.

rise in $[Ca^{2+}]_i$ but little effect on the magnitudes of the peak and plateau levels of $[Ca^{2+}]_i$. At 5 μM, BAPTA/AM caused both a delayed rate of rise and a great reduction in the peak level of $[Ca^{2+}]_i$ in PGE-stimulated HEL cells. When cells were loaded with 10 μM BAPTA/AM, changes of $[Ca^{2+}]_i$ after addition of PGE_1 and PGE_2 were reduced to a slight and very gradual increase over basal $[Ca^{2+}]_i$. Under identical conditions, BAPTA/AM pretreatment similarly inhibited the $[Ca^{2+}]_i$ rise and PLD activation in response to PGE_1 (Figure 4) and PGE_2 (not shown). On the other hand, this Ca^{2+} chelator had no effect on PMA-stimulated PEt formation; the response to PMA in control cells was 0.14 ± 0.03% of total cell radioactivity and 0.15 ± 0.02% in cells pretreated with 10 μM BAPTA/AM (n = 3). Therefore, a rise in $[Ca^{2+}]_i$ appears to be a requirement for PGE-induced activation of PLD.

Relationship between $[Ca^{2+}]_i$ and PLD Activation. If the PGE-induced increase of $[Ca^{2+}]_i$ is indeed responsible for activation of PLD, it is expected that maneuvers which directly elevate $[Ca^{2+}]_i$ within the physiological range should mimic the effect of receptor activation. Thus, experiments were designed to vary $[Ca^{2+}]_i$ within the range of 100 nM–2 μM in a controlled manner. This was accomplished by incubating HEL cells with a fixed amount of the calcium ionophore ionomycin (1 μM) in medium containing various amounts of added Ca^{2+}. Figure 5 (upper panel) shows that ionomycin evoked a rapid and sustained rise of $[Ca^{2+}]_i$ in a manner dependent on the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). There was a direct and linear relationship (correlation coefficient = 0.979) between $[Ca^{2+}]_o$ and peak $[Ca^{2+}]_i$ in ionomycin-stimulated HEL cells (Figure 5, lower panel); note, however, that 1 μM ionomycin did not *equilibrate* $[Ca^{2+}]_i$ with $[Ca^{2+}]_o$. Under these conditions, PLD activation was not detectable below a peak $[Ca^{2+}]_i$ of about 300–400 nM (Figure 6, inset). Above this level, PLD activity increased linearly as a function of $[Ca^{2+}]_i$. We then assessed the relationship between $[Ca^{2+}]_i$ and PLD activation in HEL cells stimulated by various PGE concentrations. As shown in Figure 6, both PGE_1 and PGE_2 (0.01–10 μM) activated PLD over a range of $[Ca^{2+}]_i$ similar to that evoked by ionomycin. These data, together with the results presented above, indicate that an increase of $[Ca^{2+}]_i$ is a critical step in the PGE receptor-coupled pathway for activation of PLD.

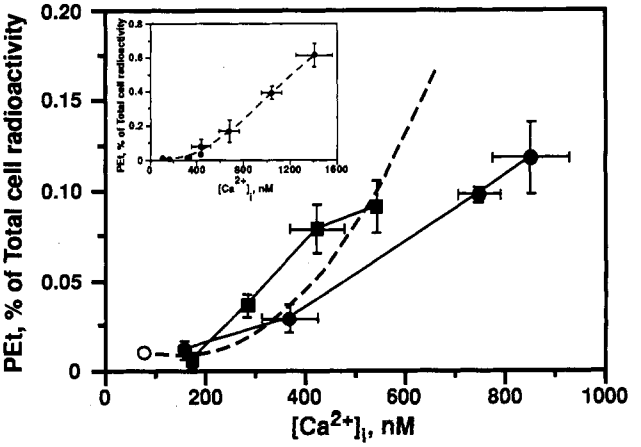

FIGURE 6: Relationship between activation of PLD and Ca^{2+} mobilization induced by ionomycin and PGEs. Assays of $[Ca^{2+}]_i$ and PEt formation were performed in the same samples incubated in media containing 0.5% ethanol and 1 mM Ca^{2+}. Cells were treated with 0.01, 0.1, 1, or 10 μM PGE_1 (filled circles) or with PGE_2 (filled squares) or were not treated (open circle). The dashed line represents the ionomycin curve from the inset with the exception of the two highest points on the curve. Data are means ± SE of three (PGE_1) or means ± range of two (PGE_2) experiments. Inset: $[Ca^{2+}]_i$ and PEt formation in HEL cells stimulated with 1 μM ionomycin for 5 min were determined in the same samples in media containing 0.5% ethanol and the various amounts of added Ca^{2+} as described in Figure 5. Data are means ± SE from three experiments.

Discussion

No clear picture has yet emerged as to the cellular mechanism for regulation of PLD. In several types of cells, including HL-60 granulocytes (Pai et al., 1988), neutrophils

(Mullmann et al., 1990), and monocyte-like U937 cells (Anthes et al., 1991), activation of PLD in response to receptor-mediated agonists requires extracellular Ca^{2+}, suggesting that agonists exert their effect via an increase of $[Ca^{2+}]_i$. Studies in human platelets also imply that thrombin stimulates PLD through Ca^{2+} mobilization (Huang et al., 1991). However, the relationship between $[Ca^{2+}]_i$ and PLD in agonist-stimulated cells has not been investigated in detail. Other reports regarding 1321N1 astrocytoma cells (Martinson et al., 1990) and several fibroblast cell lines (Huang & Cabot, 1990; Cook & Wakelam, 1991; van Blitterswijk et al., 1991) show that protein kinase C may participate in PLD activation, since protein kinase C inhibitors and/or down-regulation of the kinase by long-term treatment with phorbol esters blocked agonist-stimulated PLD activity. The Ca^{2+} dependence, or lack thereof, of PLD in these cell types has not yet been established. It is also possible that this enzyme is directly regulated by one or more G proteins, since GTPγS stimulates PLD activity in permeabilized cells (Martin & Michaelis, 1989), cell homogenates (Anthes et al., 1989), and membrane preparations (Bocckino et al., 1987).

In HEL cells, PGE_1 and PGE_2 stimulate PLD in a pertussis toxin-sensitive and Ca^{2+}-dependent manner, and the activation of this phospholipase is not mediated through the G_s/cyclic AMP pathway (Wu et al., 1991). On the basis of this information, we hypothesized that an agonist-induced rise in $[Ca^{2+}]_i$ might be responsible for the subsequent activation of PLD. In the present study, we further explored the mechanism responsible for PLD activation by PGE_1 and PGE_2 in HEL cells. We conclude that PGE receptor-coupled PLD in this cell line appears to be regulated primarily through an increase of $[Ca^{2+}]_i$, as supported by the following findings. First, PGEs stimulate both Ca^{2+} mobilization and PLD over a similar range of concentrations [Figure 1 and Wu et al. (1991)]. Second, pertussis toxin inhibits both PLD activity and changes of $[Ca^{2+}]_i$ stimulated by PGEs [Figure 1 and Wu et al. (1991)]. Third, U73122 blocks PEt formation and Ca^{2+} mobilization in response to PGEs without affecting other cellular activities mediated through G_s and G_i (Figure 2 and Table II). Fourth, buffering of extracellular Ca^{2+} with EGTA or of intracellular Ca^{2+} with BAPTA results in a reduction of the PGE-initiated rise in $[Ca^{2+}]_i$ as well as PLD activation (Figures 3 and 4). Lastly, PLD can be activated by directly raising $[Ca^{2+}]_i$ with ionomycin, within the same range of $[Ca^{2+}]_i$ that is observed following addition of PGEs (Figure 5 and 6). In this context, it has been generally observed that agonist-induced activation of PLD in cells of hemopoietic origin, e.g., neutrophils and the HL-60, U937, and HEL cell lines, is Ca^{2+}-dependent (Billah & Anthes, 1990; Anthes et al., 1991; Shukla & Halenda, 1991). Thus, it is possible that PLD in hemopoietic cells is coupled to receptors through elevation of $[Ca^{2+}]_i$.

The cellular mechanism responsible for Ca^{2+} mobilization in PGE-stimulated HEL cells has not been identified. A small and transient increase of $[Ca^{2+}]_i$ occurred in Ca^{2+}-depleted medium (Figure 3), suggesting that this response is mediated by a second messenger via action on intracellular Ca^{2+} stores. The likely candidate is IP_3, a second messenger produced upon hydrolysis of phosphatidylinositol 4,5-bisphosphate by PLC (Rana & Hokin, 1990). After binding of IP_3 to its receptors, Ca^{2+} is released from IP_3-responsive pools, and this may in turn trigger an influx of Ca^{2+} through plasma membrane Ca^{2+} channels (Berridge & Irvine, 1989). If the increase of $[Ca^{2+}]_i$ in response to PGEs is dependent upon IP_3 production, then it would appear that activation of PLD is secondary to PLC. In fact, many agonists are known to activate both PLC and PLD, but a cause/effect relationship between these two phospholipases has not yet been established [reviewed in Billah and Anthes (1990) and Shukla and Halenda (1991)].

Another question is how Ca^{2+} exerts its stimulatory effect on PLD. Ca^{2+} may bind directly to PLD, resulting in dissociation of a regulatory subunit(s) or exposure of the catalytic site, or the enzyme might translocate from cytosol to membrane in response to a rise in $[Ca^{2+}]_i$. Alternatively, Ca^{2+}-induced PLD activation may occur secondarily through activation of Ca^{2+}-dependent protein kinases, such as protein kinase C or a calmodulin-regulated protein kinase, whose substrates are PLD itself or a separate regulatory protein. However, preliminary experiments have shown that the protein kinase inhibitor staurosporine (100 nM) inhibits PMA-stimulated PEt formation by more than 80% ($n = 4$) but does not inhibit the response to prostaglandins or ionomycin (data not shown). This finding suggests that protein kinase C is not directly involved in PGE- or Ca^{2+}-stimulated PLD activation in HEL cells, raising the possibility that fundamentally different mechanisms exist for receptor-coupled regulation of PLD among different cell types (see above).

PGEs evoke Ca^{2+} mobilization in a variety of cell types, including mesangial cells (Mene et al., 1987), chromaffin cells (Yokohama et al., 1988), Swiss 3T3 cells (Yamashita & Takai, 1987), MDCK cells (Aboolian et al., 1989), and HEL cells (present study). The different cell types vary considerably in their dependence on extracellular $[Ca^{2+}]$ for this response. We found that the rise of $[Ca^{2+}]_i$ induced by PGEs is regulated by a pertussis toxin-sensitive G protein (Figure 1). PGE receptors are also coupled to a pertussis toxin-sensitive signaling pathway (G_i-mediated inhibition of adenylyl cyclase) in chromaffin cells (Negishi et al., 1989) and cortical collecting tubule cells (Sonnenburg &Smith, 1988). One point also worth noting is that the rise in $[Ca^{2+}]_i$, after reaching the initial peak, is maintained at a level higher than base line in PGE-stimulated HEL cells (Figure 1). By comparison, other agonists such as thrombin (Brass et al., 1991), epinephrine (Michel et al., 1989), and neuropeptide Y (Motulsky & Michel, 1988) also initiate a rapid and transient increase of $[Ca^{2+}]_i$ in HEL cells, but a sustained phase of elevated $[Ca^{2+}]_i$ is not apparent with these agonists. The PGE-induced Ca^{2+} mobilization is apparently more sustained and of higher magnitude in HEL cells than in the other types of cells discussed above. These results suggest that Ca^{2+} mobilization evoked by PGEs may be mediated through different mechanisms among these different cell types, and also suggest that receptor-specific pathways for control of $[Ca^{2+}]_i$ may exist in HEL cells.

The G-protein complement of HEL cells has been well characterized. Michel et al. (1989) found that G_o is not detectable in these cells. Williams et al. (1990) reported that at least three subtypes of G_i (G_{i1}, G_{i2}, and G_{i3}) are found in HEL cells and their relative abundance is $G_{i2} \gg G_{i3} > G_{i1}$, as determined with isotype-specific antibodies. All three types of G_i were substrates for pertussis toxin (Williams et al., 1990). Pertussis toxin-sensitive G proteins appear to regulate some cellular responses in HEL cells, including thrombin-stimulated IP_3 production (Brass et al., 1991) and α_2-adrenergic receptor-mediated (Michel et al., 1989) and neuropeptide Y receptor-mediated (Motulsky & Michel, 1988) Ca^{2+} mobilization and inhibition of adenylyl cyclase activity. Our results with pertussis toxin suggest that PLD activation (Wu et al., 1991) and the increase of $[Ca^{2+}]_i$ (Figure 1) in response to PGE_1 and PGE_2 are also G_i-mediated. At this stage, it is not known which isotypes of G_i are responsible for these cellular

effects. The availability of subtype-specific antibodies may help to address this issue.

Although several studies suggest that PLD may be involved in neutrophil function (Rossi et al., 1990; Kessels et al., 1991), insulin release (Metz & Dunlop, 1990), steroidogenesis (Liscovitch & Amsterdam, 1989), and acetylcholine synthesis (Kanfer, 1980), the physiological significance of PLD activation is still not clear mainly because the role of PA has not yet been established. Exogenously added PA has been shown to inhibit cyclic AMP production (Murayama & Ui, 1987) and to stimulate cyclic GMP production (Ohsako & Deguchi, 1981), phosphoinositide turnover (Moolenaar et al., 1986; Murayama & Ui, 1987), Ca^{2+} mobilization (Moolenaar et al., 1986), and cell proliferation (Moolenaar et al., 1986; Siegmann, 1987; Yu et al., 1988). This lipid can interfere with the activity of $p21^{ras}$ GTPase activating protein (Tsai et al., 1989). In some cases, PA derived from the PLD pathway may be an important source of diacylglycerol through the action of PA phosphohydrolase (Billah et al., 1989). Alternatively, PA may be converted to lysophosphatidic acid (Billah et al., 1982), which itself is able to activate PLC, to inhibit cyclic AMP production, and to stimulate DNA synthesis (van Corven et al., 1989). At present, it is too early to speculate as to the functional response(s) which is (are) mediated by PLD in HEL cells. However, it is likely that PLD has an important cellular role since stimulation of this phospholipase can be observed at physiologically relevant $[Ca^{2+}]_i$.

In summary, the present study demonstrates that E-series prostaglandins evoke a rise in $[Ca^{2+}]_i$, derived from both intracellular and extracellular stores, through a pathway regulated by a pertussis toxin-sensitive G protein in HEL cells. Our results further imply that Ca^{2+} mobilization is a key intermediate in the receptor-coupled pathway leading to PLD activation.

Registry No. PGE_1, 745-65-3; PGE_2, 363-24-6; PLD, 9001-87-0; Ca^{2+}, 7440-70-2; PLC, 9001-86-9.

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Molecular Motions and Dynamics of a Diunsaturated Acyl Chain in a Lipid Bilayer: Implications for the Role of Polyunsaturation in Biological Membranes

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ABSTRACT: The nature and dynamics of the motions of a diunsaturated fatty acyl chain in a lipid bilayer were examined using a comprehensive simulation program for ^2H NMR line shapes developed by Wittebort et al. [Wittebort, R. J., Olejniczak, E. T., & Griffin, R. G. (1987) *J. Chem. Phys. 36*, 5411–5420]. A motional model in which the isolinoleoyl chain (18:2$^{\Delta 6,9}$) adopts two conformations consistent with the low energy structures proposed for 1,4-pentadiene [Applegate, K. R., & Glomset, J. A. (1986) *J. Lipid Res. 27*, 658–680], but undergoes a rapid jump between these states, is sufficient to account for the experimentally observed quadrupolar couplings, the ^2H–^2H and ^1H–^2H dipolar couplings, the longitudinal relaxation times, and the changes in the *average* conformation of the chain that occur with a variation in temperature. The jump motion originates via rotations about the C7–C8 and the C8–C9 carbon bonds and leads to the low order parameters assigned to the C8 methylene segment (0.18) and the C9–C10 double bond (0.11). In contrast, the C6–C7 double bond, which is not involved in the two-site jump, characterized by a relatively large order parameter (0.56). Fatty acyl chains containing three or more double bonds likely cannot undergo the same jump motion and consequently will be highly ordered structures. Correlation times for diffusion of the molecular long axis of the diunsaturated acyl chain about the bilayer normal ($\sim 10^{-10}$ s) and for the local jump motion ($\sim 10^{-10}$ s) were calculated. Relative to their rates of diffusion about their molecular long axes, the rate of the local jump motion in the diunsaturated bilayer is much slower than the rates of the local motions (trans/gauche isomerization) occurring in saturated bilayers. The presence of large amounts of highly unsaturated fatty acyl chains in biological membranes should create a dynamic state that allows considerable intermolecular motion but still maintain a high degree of local order within the hydrophobic region of the bilayer.

ω-3 Polyunsaturated fatty acyl chains perform an important structural role in biological membranes. They are found in large quantities in the membranes of the rod outer segment, postsynaptic neurons, and other excitable cells [see Tinoco et al. (1978)], and the high levels, particularly of docosahexaenoic acid (22:6$^{\Delta 4,7,10,13,16,19}$), are well conserved throughout nature (Crawford et al., 1977). A restriction of the dietary intake of ω-3 polyunsaturated fatty acids has also been correlated with a loss of visual acuity in the rhesus monkey (Neuringer et al., 1984), a decreased electrical response in rat photoreceptor cell membranes (Wheeler et al., 1975), and a poor discrimination-learning response of rats to a Y-maze test (Lamptey & Walker, 1976). Furthermore, the proper photochemical function of rhodopsin is dependent upon the presence of docosahexaenoyl chains (Wiedman et al., 1988).

It is often assumed that the role of polyunsaturated lipids is to lower the gel-to-liquid crystal phase transition temperature and thus to increase and perhaps modulate the "fluidity" of biological membranes. However, recent DSC and NMR[1] studies show that the role of polyunsaturated fatty acyl chains in membrane "fluidity" requires considerable clarification [see Baenziger et al. (1991)]. An alternative hypothesis is that the overlapping 1,4-pentadiene structure imparts unique conformational properties to the polyunsaturated acyl chains (Applegate & Glomset, 1986; Dratz & Deese, 1986). Computer modeling reveals that docosahexaenoyl chains may adopt two low energy conformations in which all six double bond axes are parallel to the bilayer normal, and the consecutive double bond planes form 90° angles with respect to each other (Applegate & Glomset, 1986). It was suggested that these structures pack relatively well in lipid bilayers and may be responsible for the important role of polyunsaturated lipids in biological membranes. Unfortunately, no experimental insight into the structural properties of polyunsaturated fatty acids has yet been obtained.

As a first step to gaining a more detailed understanding of the biological function of polyunsaturated lipids, we synthesized

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[1] Abbreviations: FID, free induction decay; NMR, nuclear magnetic resonance; iLPPC, 1-isolinoleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; PC, *sn*-glycero-3-phosphocholine; PiLPC, 1-palmitoyl-2-isolinoleoyl-*sn*-glycero-3-phosphocholine.

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